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Ecología del comportamiento sexual de los machos de la mariposa *Callophrys xami*, con algunas consideraciones acerca de la evolución del semen de insectos

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Tesis doctoral de Carlos Rafael Cordero Macedo

Abstract
In many insect species, males invest significant amounts of time and resources in the production of spermatophores and other ejaculate elements. Such reproductive investment may constrain the evolution of male mating strategies because it may be costly, *i. e.*, it may produce an increase in mortality or a decrease in future reproductive success. The production of ejaculates may increase male fitness because the transfer of an ejaculate may induce a decrease in female sexual receptivity and an increase in the rates of ovogenesis and oviposition, and because ejaculate constituents could be used by females to increase the quantity and quality of the male's offspring, and for their cryptic choice of males. In this thesis I present studies about these costs and benefits of ejaculate production. In the first part I present field and laboratory studies of the mating behaviour of the polygynous territorial butterfly *Callophrys xami* (Lycaenidae). In chapter 1, the typical courtship behavior, as well as behavioral variants, is described and discussed in terms of mate choice and variation in female receptivity. In chapter 2, field estimates indicate that mated females have an average of 1.37 spermatophores and that 21.4% had more than one spermatophore; no relation was found between the number of spermatophores and female wing wear and length. Laboratory observations and an experiment indicate that female sexual receptivity decreases after mating and that the probability of female remating is low. In chapter 3, the distribution of copulations between males and territories in the butterfly *Callophrys xami* (Lycaenidae) was evaluated in the field. Copulation rate was very low (.0014 copulations/male/h) and there was high individual variation in the number of copulations. Data suggests a sexual selection advantage for big, long lived males, and variation in territory quality. In appendix 3.1, I report that at least 13% of the males defended two or more territories sequentially, due to aggressive displacement from their territories by other males, and spontaneous shift to a different territory; however, in most territory shifts the causes were not determined. In chapter 4, I present the results of experiments designed to measure the effect of mating on male lifespan. In experiment 1, the lifespan of virgin males was not significantly different from that of multiply-mated males. To approach more natural, resource-limited conditions, in experiment 2, I used small males resulting from the experimental food limitation of last instar larvae; small virgin males lived significantly more days than small multiply-mated males. To evaluate the effect of the rates of mating and of spermatophore production on male survival, in experiment 3, I compared the longevity of pairs of males that produced a similar amount of spermatophore, but that mated different numbers of times, mated at different rates, and that produced spermatophores at different rates; the lifespan of "low mating rate" males was not different from that of "high mating rate" males. My results suggest that the cost of ejaculate production is probably one important cause of lifespan reduction, however, the role of other possible physiological costs in lifespan reduction is still unknown. In chapter 5, I tested three predictions from the hypothesis that spermatophore production is physiologically costly. In agreement with prediction 1, there was a direct correlation between the amount of "non-renewable" resources (resources available only for larvae) stored by the male and spermatophore weight. In agreement with prediction 2, recently mated males produced smaller spermatophores than males that had longer intermating intervals. In agreement with prediction 3, there was a time cost for males mating twice in one day, since second copulations of the day lasted several hours, whereas first copulations of the day lasted half an hour. The inability of small males and recently mated males to produce big ejaculates, and the increased investment of time in a copulation made by recently mated males, support the hypothesis that spermatophore production is physiologically costly for males. In the second part of the thesis, I first (chapter 6) present hypotheses for the evolutionary origin of substances, transfered by male insects within the ejaculate, that inhibit female receptivity (RIS) and stimulate ovulation and oviposition (OSS), and discuss how selection may have modified them subsequently. According to the handicap hypothesis, RIS and OSS are used by females to evaluate the quality of the ejaculate received. This hypothesis predicts that RIS and OSS must be reliable indicators of ejaculate quality, reliability being the result of the high RIS/OSS production costs and specific chemical composition. The second hypothesis proposes that RIS/OSS are selected only in regard to their effectiveness as stimulators through Fisher's sexual selection runaway process. According to this hypothesis, RIS/OSS not necessarily must be reliable indicators of sperm or ejaculate chemical constituents quality (other than ability to stimulate), and must show a high species-specificity (rapid evolutionary divergence). Based in these hypotheses, in chapter 7, I presenta new hypothesis on the evolutionary origin of nuptial seminal gifts. Empirical evidence is reviewed, and the kind of information necessary to evaluate the relative importance of each hypothesis is indicated.

Resumen

En muchos insectos, los machos invierten recursos en la producción de espermatóforos y otras secreciones durante el apareamiento. Esta inversión puede ser costosa, ya que puede reducir el éxito reproductivo futuro al disminuir la longevidad de los machos o la cantidad de recursos que pueden invertir en otros apareamientos. Además de la fertilización de huevos, los machos obtienen otros beneficios de los componentes del semen, tales como la reducción de la competencia espermática al provocar una disminución de la receptividad sexual de las hembras y un incremento en las tasas de ovogénesis y oviposición. En esta tesis presento un conjunto de estudios acerca de los costos y beneficios antes mencionados. En la primera parte presento un estudio observacional de campo de la conducta sexual de la mariposa poligínica *Callophrys xami* (Lycaenidae), así como un estudio experimental de laboratorio de los costos que pagan los machos que se aparean con varias hembras. Los principales resultados son: (a) Una descripción del cortejo, donde documento variación en dicha conducta, así como la ocurrencia de cópulas interrumpidas antes de la transferencia de espermatóforos. (b) Un estudio de la frecuencia de apareamiento de las hembras, donde muestro que existe una tendencia hacia la monandria, así como un experimento de laboratorio donde muestro que el apareamiento reduce la receptividad sexual de las hembras. (c) Un estudio de la distribución de los apareamientos entre los machos y los territorios observados en dos años consecutivos, donde muestro que la tasa de apareamientos de los machos es muy baja y la varianza en el número de apareamientos es muy alta; las características de los machos que fueron observados apareandose en varias ocasiones sugieren que los machos grandes y longevos tienden a aparearse un mayor número de veces; la variación en los patrones de ocupación y en el número de apareamientos de los territorios sugiere que varían en calidad. (d) Evidencia de que al menos el 13% de los machos defendieron dos o más territorios en secuencia, debido a que algunos machos fueron desplazados agresivamente de sus territorios y tuvieron que moverse a uno diferente, y algunos otros se cambiaron de territorio "voluntariamente". (e) Tres experimentos donde pongo a prueba la hipótesis de que el costo de la inversión hecha por los machos durante un apareamiento puede producir una reducción en la longevidad de los machos que se aparean a una tasa más elevada. En el experimento 1, la longevidad de machos vírgenes no fue diferente de la de machos que se aparearon varias veces. En el experimento 2, realizado con machos que fueron sometidos a una limitación de recursos durante su etapa larvaria, con el fin de simular condiciones de campo más realistas, encontre que los machos que se aparearon varias veces tuvieron una longevidad 40% menor a la de los machos vírgenes. En el experimento 3, la longevidad de los machos que se aparearon a una tasa relativamente elevada no fue distinta de la de los machos que se aparearon a una tasa relativamente baja. Dado que en mis experimentos elimine la existencia de costos ecológicos del apareamiento, el efecto negativo observado en el segundo experimento debe ser resultado de un costo fisiológico de las interacciones sexuales. (f) Un estudio experimental de tres predicciones derivadas de la hipótesis de que la producción de espermatóforos es fisiológicamente costosa para los machos. La primera predicción es que debe de haber una correlación directa entre el peso del espermatóforo y la cantidad de recursos "no renovables" almacenados por los machos; la segunda es que los machos que se aparearon recientemente deben de producir espermatóforos de menor tamaño, en comparación con machos que tuvieron períodos más largos de tiempo entre apareamientos; la tercera es que los machos que se aparearon recientemente deben de tardar más tiempo en transferir un espermatóforo, en comparación con machos que tuvieron períodos más largos de tiempo entre apareamientos. Las tres predicciones fueron corroboradas en mis experimentos. También muestro que algunas hembras incurren en un costo cuando se aparean con un macho que se apareó previamente el mismo día, ya que estas cópulas duran varias horas. En la segunda parte del trabajo presento una serie de hipótesis acerca de la evolución de las sustancias, presentes en el semen de muchos insectos, que producen un inhibición de la receptividad sexual (RIS) y una estimulan de la ovogénesis y la oviposición (OSS), así como una hipótesis acerca del origen evolutivo de los "regalos nupciales seminales". Las hipótesis se basan en la interpretación de que las RIS y las OSS son señales que el macho le envía a las hembras, y que éstas utilizan en su elección de pareja, las cuales han evolucionado de acuerdo al proceso desbocado o al principio de la desventaja. Una revisión de la literatura nos muestra que, aunque las suposiciones y predicciones de las hipótesis son razonables, los datos críticos para poner a prueba las hipótesis no existen todavía.

I. PRESENTACIÓN

La selección sexual es una consecuencia de las diferencias sexuales en inversión en los descendientes, las cuales tienen como resultado que las tasas reproductivas potenciales de los machos sean mucho mayores que las de las hembras y, por lo tanto, que éstas constituyan un recurso limitante para aquellos (1, 2, 3). Sin embargo, las diferencias sexuales se han reducido en muchas especies debido a que la selección favorece, en algunas circunstancias, el incremento en la inversión masculina. En muchas especies de insectos, los machos invierten cantidades considerables de recursos en la producción de espermatozoides, espermatóforos y otras secreciones que son transferidas a las hembras (1, 3). Dicha inversión puede ser costosa para los machos, ya que puede reducir su éxito reproductivo al disminuir su longevidad o la cantidad de recursos que podrían invertir en otros apareamientos. Estos costos, aunados a la competencia espermática y la elección críptica de pareja (3, 4), indican que la función que relaciona la adecuación de los machos con el número de parejas sexuales no necesariamente se incrementa de manera constante y, de hecho, podría decrecer cuando el número de apareamientos es muy elevado. Por otra parte, además de la ventaja de fertilizar huevos, los machos obtienen una serie de beneficios de los diferentes componentes del semen. Algunas de las sustancias reducen la competencia espermática al provocar una disminución de la receptividad sexual de las hembras y un incremento de la tasa de ovogénesis y oviposición, la remoción de los espermatozoides de otros machos, y un incremento en el número y la calidad de los descendientes (2, 3, 4).

En esta tesis presento una serie de estudios relacionados con los costos y beneficios arriba mencionados. La tesis se divide en dos partes relativamente independientes. En la primera presento los resultados de una serie de estudios observacionales y experimentales, realizados tanto en el campo como en el laboratorio, donde evalúo los patrones de apareamiento de machos y hembras, así como los posibles costos de apareamiento de los machos de la mariposa *Callophrys xami* Reakirt (Lepidoptera: Lycaenidae). En la segunda parte presento una serie de hipótesis acerca de la evolución de dos clases de sustancias presentes en el semen de insectos que efectan la fisiología y comportamiento reproductivo de las hembras: las sustancias inhibitorias de la receptividad sexual y las sustancias que estimulan la ovogénesis y la oviposición. Estas hipótesis son evaluadas por medio de una comparación de sus suposiciones y predicciones con los patrones encontrados en una revisión de la literatura. Además, presento una hipótesis acerca del origen evolutivo de los "regalos nupciales seminales" (= componentes del semen que tienen un valor nutricional o de protección para las hembras que los reciben). A continuación se presenta un resumen de los principales resultados de este trabajo.

RESUMEN Y CONCLUSIONES GENERALES

ECOLOGÍA DEL COMPORTAMIENTO SEXUAL DE LOS MACHOS DE LA MARIPOSA *CALLOPHRYS XAMI*

Los objetivos de este trabajo fueron evaluar los beneficios y posibles costos del apareamiento en los machos territoriales de la mariposa *C. xami*. Los machos de esta especie son adecuados para este tipo de estudio, ya que, como en muchas otras especies de lepidópteros, invierten una cantidad sustancial de recursos en la producción de espermatóforos y otras secreciones seminales (5, 6), y su comportamiento sugiere fuertemente una tendencia hacia la poliginia (7).

En el ***capítulo 1*** describo el cortejo. En el 26% de los cortejos observados, una de las fases (que probablemente involucra la liberación de feromonas masculinas y un costo energético elevado) fue muy breve o estuvo ausente. En el 7% de los apareamientos observados, la pareja se separó cuando habían transcurrido menos de 3 min desde el inicio de la cópula (la duración promedio de los apareamientos es de alrededor de media hora, si es la primera cópula del día del macho, y de varias horas —hasta la madrugada— si es la segunda cópula del día), seguramente antes de que se hubiera transferido un espermatóforo y espermatozoides a la hembra. Estas cópulas interrumpidas sugieren la existencia de elección poscopulatoria de pareja (4). Sin embargo, aunque las hembras son el sexo que se muestra activo durante las interrupciones, no es posible saber cual sexo está eligiendo interrumpir la cópula.

En el ***capítulo 2*** describo el comportamiento sexual de las hembras. Estimaciones de campo del número de esprmatóforos en las hembras muestran una tendencia a la monandria (promedio de 1.37 espermatóforos por hembra apareada), aunque el 21% tuvieron más de un espermatóforo. No encontré relación entre el número de espermatóforos y el tamaño y grado de desgaste de las alas de las hembras. Algunas observaciones casuales de individuos en cautiverio, así como un experimento de laboratorio, indican que la receptividad sexual de las hembras disminuye después de una cópula y que su probabilidad de reapareamiento es muy baja. La tendencia a la monandria y la disminución en la receptividad sexual de las hembras después de una cópula, aunadas al hecho de que las hembras se pueden aparear desde el primer día de su vida adulta y al patrón de oviposición que muestra un decrecimiento continuo en el número de huevos puestos con el tiempo (8), generan presiones de selección en favor de los machos que son más eficientes para copular con hembras vírgenes.

En el ***capítulo 3*** describo la distribución de los apareamientos entre los machos y entre los territorios. La tasa de apareamientos fue muy baja (.0014 apareamientos / macho / h) y hubo mucha variación entre los machos en el número de apareamientos. Unos pocos machos (n = 3) fueron observados apareandose en varias ocasiones (hasta un máximo de cuatro veces), y sus características

sugieren que los machos grandes y longevos tienden a aparearse un mayor número de veces. La variación entre territorios en el número de machos que los defendieron, la proporción de días en que fueron ocupados y en el número de apareamientos que ocurrieron dentro de ellos, sugiere que varían en calidad. La frecuencia de ocupación y el número de apareamientos que ocurrieron dentro de ellos, no estuvieron relacionados con el tamaño de los territorios. No hubieron correlaciones significativas entre años en la frecuencia con que los territorios fueron ocupados y en el número de apareamientos que ocurrieron dentro de ellos. Es posible que los territorios deban su calidad a su ubicación en zonas donde existe un mayor tránsito de hembras receptivas, y que su calidad varía debido a que el patrón de distribución espacial de las zonas de emergencia de hembras adultas, que a su vez determina la ubicación de las zonas de mayor tránsito, varía entre años.

El 13% de los machos observados defendieron dos o más territorios en secuencia. En el ***apéndice 3.1*** describo estos cambios de territorio. Se observaron dos causas de cambio de territorio: (i) algunos machos fueron desplazados agresivamente de sus territorios y tuvieron que moverse a uno diferente, mientras que (ii) otros se cambiaron de territorio "voluntariamente" (es decir, en ausencia de interacciones con otros individuos). Sin embargo, la importancia relativa de (i) y (ii) no pudo ser medida debido a que en el 84% de los casos no sabemos a qué se debió el cambio. La causa (i) sugiere que, al menos en algunas circunstancias, los territorios son un recurso limitante para los machos; mientras que (ii) sugiere que los machos pueden evaluar la calidad de sus territorios y, cuando se encuentran en uno que no les satisface, decidir moverse en busca de uno mejor.

En especies de insectos en las que los machos invierten cantidades sustanciales de recursos durante los apareamientos, la inversión reproductiva de los machos puede ser costosa, *i.e.* puede incrementar su mortalidad o disminuir los recursos disponibles para apareamientos futuros. En el ***capítulo 4*** puse a prueba experimentalmente la hipótesis de que el aparearse con varias hembras incrementa la mortalidad de los machos. En el experimento 1, la longevidad de los machos mantenidos vírgenes no fue diferente de la de los machos que se aparearon varias veces. En el experimento 2 los machos fueron sometidos a una limitación de recursos durante su etapa larvaria, con el fin de simular las condiciones de campo de una manera más realista. En este experimento, los machos que se aparearon varias veces tuvieron una longevidad 40% menor a la de los machos que se mantuvieron vírgenes. En el experimento 3, la longevidad de los machos que se aparearon a una tasa relativamente elevada no fue menor de la de los que se aparearon a una tasa relativamente baja. Como en mis experimentos elimine los "costos ecológicos" del apareamiento (*sensu* 9; p. ej. riesgo de depredación), el efecto negativo

observado en el segundo experimento debe ser resultado de los costos fisiológicos de las interacciones sexuales (estos incluyen los costos del cortejo precopulatorio, del acto de copular, del cortejo copulatorio, y de la producción del espermatóforos y otras secreciones). Aunque no puedo distinguir la importancia relativa de cada uno de los costos fisiológicos, sugiero que el costo fisiológico de producir un espermatóforo fue una causa importante (aunque, tal vez, no la más importante) de la reducción de la longevidad observada en el segundo experimento. El que no se haya encontrado una diferencia en la longevidad de los machos que se aparearon a diferentes tasas en el tercer experimento podría entenderse si la "tasa baja" de apareamientos fue lo suficientemente alta como para haber producido el efecto negativo en la longevidad (esto implica que la relación negativa entre la longevidad y la tasa de apareamientos no es lineal).

En el ***capítulo 5*** puse a prueba tres predicciones de la hipótesis, propuesta en el capítulo 4, de que la producción de espermatóforos es fisiológicamente costosa para los machos (10, 11, 12). La predicción 1 es que debe de haber una correlación directa entre el peso del espermatóforo y la cantidad de recursos "no renovables" (aquellos que sólo están disponibles para las larvas) almacenada por el macho adulto (el peso de los machos fue usado como una medida de dichos recursos); mis experimentos muestran la existencia de esta relación. La predicción 2 es que los machos que se aparearon recientemente deben de producir espermatóforos de menor tamaño, en comparación con machos que tuvieron períodos más largos entre apareamientos; esta relación fue observada en mis experimentos. La predicción 3 es que los machos que se aparearon recientemente deben de tardar más tiempo en transferir un espermatóforo, en comparación con machos que tuvieron períodos más largos entre apareamientos; esta relación fue observada en mis experimentos cuando comparamos la duración de las segundas cópulas del día de los machos (las cuales duran varias horas), con la duración de los apareamientos que ocurrieron uno o más días después del apareamiento previo (las cuales duran alrededor de media hora). Los resultados de este estudio también indican que algunas hembras incurren al menos en un costo de tiempo (para ellas el tiempo es un recurso limitante, ya que tienen una longevidad corta y sólo pueden poner huevos, durante unas pocas horas, en días cálidos, soleados y con poco viento) cuando se aparean con un macho que se apareó previamente el mismo día, ya que estas cópulas duran varias horas.

Conclusiones. Este estudio muestra que en *C. xami* las hembras tienden a ser monándricas, mientras que los machos son polígínicos. El número de apareamientos es un estimador razonable de la adecuación de los machos, ya que la cópula provoca una disminución la receptividad sexual de las hembras, al menos durante algún tiempo. Existe una gran varianza en el éxito de apareamiento de los

machos (1, 2), lo que indica que existe una elevada oportunidad para la selección sexual. La datos sugieren que la selección sexual podría actuar en favor de los machos más grandes (tanto porque estos se podrían aparear un mayor número de veces, como porque producen espermatóforos más grandes) y más longevos, aunque esto tiene que demostrarse. La producción de espermatóforos es fisiológicamente costosa, a juzgar por la incapacidad de producir espermatóforos "grandes" de los machos pequeños y de los machos apareados recientemente, así como el gran incremento en el tiempo invertido en un segundo apareamiento del día. El aparearse varias veces también es costoso para los machos, al menos si sufrieron una limitación de recursos durante su etapa larvaria (lo que parece ocurrir en el campo, a juzgar por el tamaño que presentan algunas mariposas; obs. pers.), debido a que disminuye su longevidad. Este es probablemente el primer estudio en el que se demuestra de manera directa este último tipo de costo en una especie de mariposa.

ALGUNAS CONSIDERACIONES ACERCA DE LA EVOLUCIÓN DEL SEMEN DE INSECTOS

En esta parte presento una serie de hipótesis acerca de la evolución de dos clases de sustancias presentes en el semen de los insectos que efectan la fisiología y el comportamiento reproductivo de las hembras: las sustancias inhibitorias de la receptividad sexual (RIS) y las sustancias que inducen y estimulan la ovogénesis y la oviposición (OSS). Además, presento una hipótesis, derivada de las ideas anteriores, acerca del origen evolutivo de los regalos nupciales seminales.

En el ***capítulo 6*** propongo dos hipótesis acerca del origen de las RIS/OSS. Dado que en muchas especies de insectos algunas cópulas no resultan en la transferencia de semen (4), aquellas hembras con la capacidad para detectar y discriminar en contra de estas cópulas se verían favorecidas por la selección. Una forma de detectar estas cópulas es utilizando alguno de los compuestos químicos que se transfieren con el semen como señales de una cópula exitosa. Alternativamente, en especies con regalos nupciales seminales (sustancias nutritivas o protectoras transferidas en el semen), el uso de las RIS/OSS pudo haber evolucionado en las hembras para sincronizar su fisiología y conducta reproductiva con la obtención de un regalo nupcial.

Posteriormente, propongo dos hipótesis de acuerdo a las cuales las RIS/OSS "primitivas", por selección tanto en las hembras como en los machos, pudieron evolucionar en señales de los machos hacia las hembras. En la primera propongo que las RIS/OSS son seleccionadas simplemente en consideración a su efectividad como estimuladoras por medio del proceso desbocado (*runaway*) de selección sexual. De acuerdo con esta hipótesis, las RIS/OSS no son indicadoras confiables de la calidad (otra que la mera

habilidad estimuladora) de los espermatozoides o de los constituyentes químicos del semen. Otra predicción de esta hipótesis es que las RIS/OSS deben de evolucionar rápida y divergentemente. En concordancia con esta predicción, las RIS/OSS son específicas de la especie en varios dípteros, que comprenden, incluso, especies hermanas (*sibling species*) del complejo *Drosophila melanogaster*.

En las especies en las que los machos varían en la calidad de su semen y el éxito reproductivo de las hembras es afectado por dicha variación, esperamos que evolucionen hembras que utilicen las RIS/OSS para evaluar la calidad del semen (y, posiblemente, la de los machos que las producen). En mi segunda hipótesis propongo que las RIS/OSS pudieron haber evolucionado de acuerdo al principio de la desventaja (13), y que son utilizadas por las hembras para evaluar la calidad del semen recibido (la cual es una función de la calidad genética o fenotípica de los espermatozoides y de la calidad nutricional o protectora, para la hembra o sus descendientes, de sus constituyentes químicos). Esta hipótesis predice que las RIS/OSS son indicadores confiables de la calidad del semen; siendo dicha confiabilidad el resultado de los elevados costos de producción y de la composición química específica de las RIS/OSS. En concordancia con las predicciones de esta hipótesis, existe evidencia empírica de que las RIS/OSS son costosas y de que están elaboradas con recursos que generalmente son limitantes para los insectos (*v. gr.* nitrógeno). El efecto dependiente de la dosis que generalmente presentan las RIS/OSS, así como datos de algunas especies que indican que la cantidad de RIS está directamente correlacionada con la cantidad total de semen, sugieren que las RIS/OSS son utilizadas por las hembras para evaluar la calidad del semen.

Los regalos nupciales seminales son sustancias nutritivas o defensivas, para las hembras o sus descendientes, que son transferidas por los machos en el semen. A partir de las ideas del capítulo 6, en el ***capítulo 7*** desarrollo una hipótesis acerca del origen evolutivo de estos regalos, de acuerdo con la cual los regalos nupciales seminales surgieron como resultado de: (i) un incremento en la cantidad y calidad de las RIS/OSS, a consecuencia de presiones de selección intersexual (elección femenina) en favor de sustancias que actúan como indicadoras de la calidad del semen o como simples "estimuladoras"; seguida de, (ii) la evolución en las hembras de mecanismos para metabolizar el "exceso" de RIS/OSS; y (iii) la subsecuente elaboración evolutiva de los regalos nupciales como resultado de presiones selectivas derivadas de la elección femenina o de la inversión paterna. Esta hipótesis explica el hecho de que en la mayoría de los casos conocidos, los regalos seminales no consisten de un "exceso" de espermatozoides, sino que son secreciones de las glándulas accesorias y de otras partes glandulares del aparato reproductivo del macho.

Conclusiones. Las hipótesis planteadas son alternativas a las interpretaciones "tradicionales" de manipulación por parte de los machos. Estas interpretaciones tradicionales se basan en la idea de que la selección actúa de manera más intensa sobre los machos que sobre las hembras en situaciones de conflicto. Sin embargo, esto no toma en cuenta el hecho de que las hembras cuentan con una mayor cantidad de posibilidades de respuesta evolutiva (p. ej., debido a que cuentan con toda una serie de "exaptaciones" para el control del destino de los espermatozoides dentro de su tracto reproductivo). Es prematuro concluir si las hipótesis aquí propuestas son más adecuadas para explicar la evolución de las RIS/OSS que las ideas tradicionales, sin embargo nos proveen de una serie de predicciones que es factible poner a prueba empíricamente. Lo mismo se puede decir de la hipótesis propuesta en el capítulo 7, aunque en este caso la evidencia necesaria para distinguir entre las hipótesis sobre el origen de los regalos nupciales seminales no será fácil de obtener.

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II. ECOLOGÍA DEL COMPORTAMIENTO SEXUAL DE LA MARIPOSA CALLOPHRYS XAMI (LYCAENIDAE)

CAPÍTULO 1

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The courtship behavior of *Callophrys xami* (Lycaenidae)

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Abstract. The courtship behavior of the butterfly *Callophrys xami* (Lycaenidae) was studied in the field. The typical courtship pattern as well as behavioral variants are described and discussed in terms of mate choice and variation in female receptivity. Copulatory behavior leading to separation of the pair well before the time necessary for successful mating (less than three minutes vs. 32 minutes) is described. The possible role of this last behavior in mate choice is discussed.

KEY WORDS: Courtship, copulation, behavioral variation, mate choice

INTRODUCTION

Intraspecific variation in courtship behavior in butterflies is well documented and it has been explained as a result of mate choice by males and/or by females (e. g. Rutowski, 1979, 1981-83, 1984; Wiklund and Forsberg, 1985; Krebs, 1988). Furthermore, Eberhard (1985, 1994, in press; Eberhard and Cordero, 1995) proposes that in animals courtship may continue during copulation and that females can also use this *copulatory courtship* for their choice of mate. In this paper, variation in precopulatory courtship and behavior during copulation in *Callophrys xami* is reported, and some of its possible causes discussed.

METHODS

The study was conducted in an ecological preserve of 146.8 ha, located on the campus of the Universidad Nacional Autónoma de México, south of Mexico City. This area is part of the Pedregal de San Angel, a zone characterized by volcanic soil, rough topography, markedly seasonal rainfall regime, and xerophytic shrubby vegetation (Rojo, 1994).

Callophrys xami is a multivoltine butterfly that can be found throughout the year. The Pedregal de San Angel population never reaches a very high density of individuals and it is more abundant from October to January (Soberón *et al.*, 1988). The main food plant of the larvae is the perennial *Echeveria gibbiflora* (Crassulaceae), an abundant species in the area (Soberón *et al.*, 1988). Males are territorial, and a male can occupy the same territory for as long as four weeks (Cordero, 1996). Territories are areas with well defined topographical limits, located beside or on natural or man-made trails; these areas lack concentrations of receptive females and larval and adult food resources. Males actively defend their territories by means of different types of aggressive flights, for an average of five hours per day (approximately between 1000 and 1500 h), and spend the rest of the time feeding and resting outside territories (Cordero and Soberón, 1990). Territories function as mating stations (Cordero and Soberón, 1990; Cordero, 1996).

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Observations were made in 1989, between November 1 and December 20, and in 1990, between November 10 and December 6. The number of territories observed was 19 in 1989 and 13 in 1990; the number of days a territory was visited varied between 29 and 38 in 1989 and between 14 and 24 in 1990 (details in Cordero, 1996). Most territorial males observed were captured and individually marked on the wings with indelible felt-tip pens (Sharpie®) that does not seem to have effects on males (Cordero and Soberón, 1990). Observations of courtships and copulations were made on groups of territories located on transects; we walked these transects at least two times per day during 31 days in 1989 and 11 days in 1990, making focal observations in each territory for at least three minutes after locating the resident male. Courtships and copulations were also observed during continuous observations of selected territories, made for 9 days in 1989 and for 13 days in 1990. Twelve courtships observed in 1983-1985 (during the course of another study; Cordero and Soberón, 1990) are included in the description of typical courtship.

Results

Typical successful courtship

The following description is based in 20 complete successful courtships observed in the field. This typical pattern was observed in the 12 successful courtships observed in 1983-1985, in five out of the 10 observed in 1989, and in three of the five observed in 1990. The temporal sequence of courtship and mating can be divided in seven phases (Fig. 1):

I. A female flies near (< 1 m) a flying or, more frequently, a perching male.

II. The male flies following the female and a courtship flight along a route parallel to the ground begins; during the courtship flight the male flies near (< 10 cm), slightly behind and a few cm above the female. This flight lasts about 30 seconds, unless a perturbation, such as a strong wind, momentarily interrupts the courtship, in which case the flight lasts longer.

III. Female and male alight on vegetation close to each other; there were a few cases in which one or more alighting attempts preceded final settling of the pair.

IV. Immediately after the couple alight on vegetation, the male walks in front of the female until reaching a head to head position, while fluttering vigorously; meanwhile, the female stays motionless with her wings closed. It is possible that during this phase (and, probably, since phase II) the male emits pheromones from androconia located near the forewing costal border.

V. After a few seconds, and still fluttering, the male walks beside the female until reaching a parallel, head to head and tail to tail position.

VI. The male moves the tip of his abdomen toward that of the female and, after making genital contact, stops fluttering; immediately after beginning copulation the male moves until reaching the "tail to tail" copula position typical of Lepidoptera. During copulation the couple stays motionless, unless some perturbation, such as strong wind or people coming too close to the mating pair, makes them fly *in copulo* to a different place on vegetation. The approximated time elapsed between alighting and beginning of copulation (phases IV to VI) is between 10 and 20 seconds.

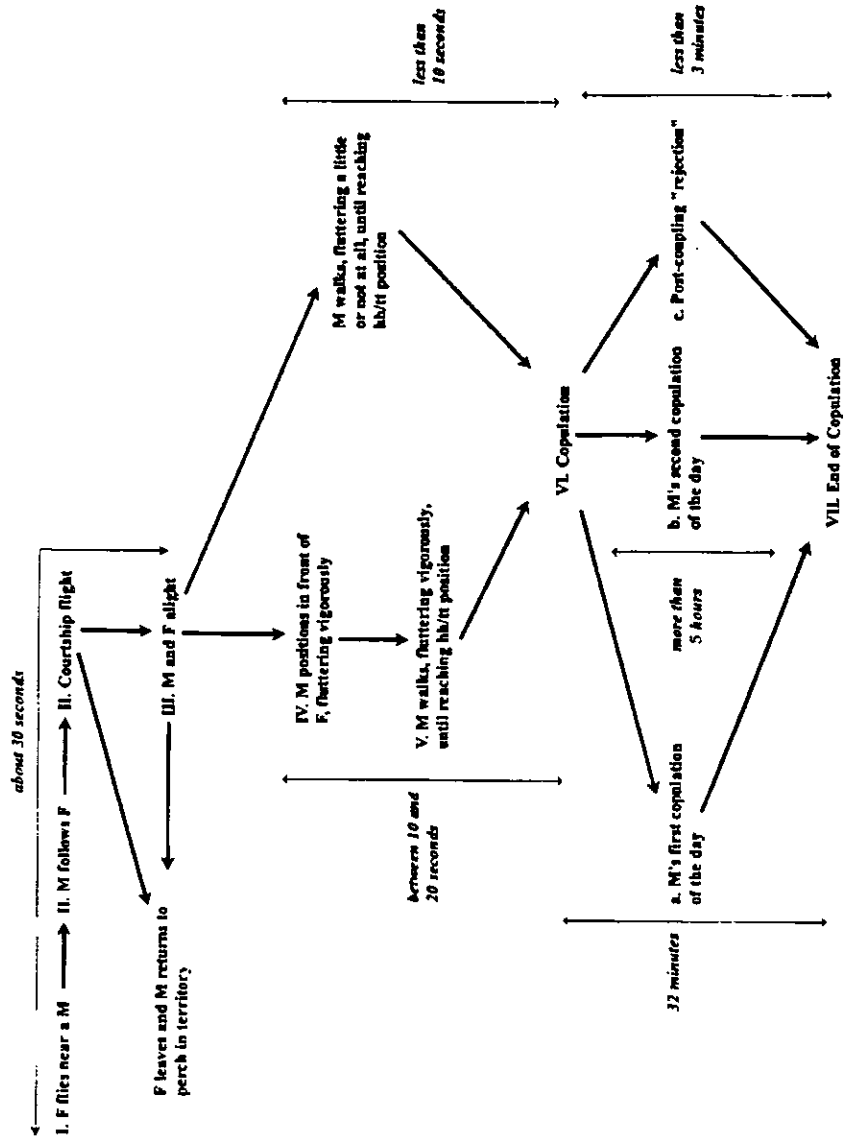

Fig. 1. Diagram of the mating behavior of *Callophrys xami* (Lycaenidae), showing variations in courtship and post-coupling behavior. Courtship description is based in 27 observations. M: male. F: female. hh/tt: M parallel to the F, head to head and tail to tail.

VII. The end of copulation begins with the female starting to move, turning and occasionally walking short distances (less than 20 cm); these movements are intercalated with female turnings around her longer body axis, until the pair ends genital contact. After separation the female and/or the male may remain in the mating place for a few minutes or they may fly away almost immediately. In few occasions the male found the female again, after seconds or a couple of minutes, and a short pursuit flight followed, which ended when the male returned to perch and the female left the area.

Courtship is mostly initiated inside territories, although the possibility exists that a female is found by a male when he is returning to his territory after, for example, an aggressive interaction. Copulations can take place inside territories, and they also occur outside, if the courtship flight takes the pair out of the territory. Average copulation duration in the field is 32.3 min (Standard Error = 4.9; Cordero, 1996); duration is much longer (range: 1h42 min to >14h; 23/24 copulations lasted > 5h) if the copulation is the second of the day for the male (Cordero, 1996). In captivity, courtship begins when a male detects a female at short distance, usually when the male and/or the female are walking on the cloth of the cylindrical, 58 cm high by 26 cm diameter, mating cages (Jiménez and Soberón, 1988-89); afterwards, courtship and mating proceeds from phase IV on, as in the field.

Courtship Variants

In five out of 10 successful courtships observed in 1989 and in two of the five observed in 1990, the time allocated to the vigorous fluttering (phases IV, V and part of VI) was considerably less than in the typical courtship described above, and in some cases it was almost absent (Fig. 1). This difference also occurred in some of the courtships observed in captivity.

We have observed several courtships in the field that did not result in mating. Several were observed in 1983-1985, 13 in 1989 and 29 in 1990. The duration of these unsuccessful courtships was variable, but observations lacked the detail necessary to look for differences between successful and unsuccessful courtships. These courtships lasted from a few seconds to rarely more than one minute. Almost all complete observations of unsuccessful courtships (some couples were lost from sight) ended before phase III, although at least in one case a male lost a female during alighting attempts (in this specific case, the strong wind blowing during observation, rather than mate choice [see discussion], may be responsible for the unsuccessful courtship).

Copula Interruption

In one of 17 copulations observed in 1989 and one of 10 observed in 1990, the female began walking after genital coupling began, dragging the male behind her. After a brief time, the female began to intercalate body twists during walking, behaving in the same way as when they are about to finish normal copulation (see phase VII of typical courtship), suggesting attempts to end copulation. Both cases resulted in the separation of the pair less than

three minutes after mating began (probably before ejaculate transfer and long before the end of normal copulations; Cordero, 1996).

In the case observed in 1989 the male encountered the female again, less than three minutes after separation, and courted her for a second time without success; afterwards the female left the area and the male returned to perch in the territory. The male had been defending the territory for at least five days before, and defended it three more days after; his minimum longevity was nine days (mean minimum longevity of males observed in 1989 more than one day = 6.9 days, s.e. = 0.8, range: 2-20, n= 51; Cordero, 1996); the forewing length (a measure of body size; Parlange, 1991; Cordero, 1996) of this male was 1.73 cm (mean forewing length of males observed in 1989 = 1.64 cm, s.e. = 0.01, range: 1.36-1.89 cm, n = 77; Cordero, 1996) and whether the male or the female had mated earlier the day of the interrupted copulation is unknown, given that we began observations in that territory at 1130. In the 1990 case the male had defended the territory at least one day before the "rejection" (his minimum longevity was four days); we did not measure the forewing length of this male. We do not know if the male or the female had mated earlier the day of the "rejection", given that we began observations after 1100. These two males were not observed mating for a second time.

We observed similar behavior in another two cases, one in the field and one in captivity, but these occurred after a perturbation. In the first case, the female exhibited the behavior after we attempted to put the field mating pair inside a cage; the time at which this mating began is unknown. The second case occurred when the mating cage accidentally fell to the ground more than five minutes after the mating had begun. We never observed this behavior in any of the 18 matings observed in 1983-1985.

Discussion

Variation in successful courtships

Typical courtship behavior of *Callophrys xami* is similar to the courtship of related species (e. g. Powell, 1968; Robbins, 1978); however, to my knowledge, variation in this behavior has not been reported in other *Callophrys* species. Courtship variants may be the result of differences in female receptivity. Males finding highly receptive females may save time, energy and pheromones with the shortening of courtship phases IV to VI. Female receptivity may be affected by time since last mating, time since emergence from the pupa (this applies to virgin females of species able to mate and lay eggs almost immediately after emergence, such as *C. xami*), number of sperm remaining in her spermatheca, size and quality of the last ejaculate received (e. g. Oberhauser, 1992; Kaitala and Wiklund, 1994), remaining quantity of receptivity inhibition substances transferred by her last partner (Cordero, 1995), number of mature eggs stored, feeding condition (e. g. Boggs,1990) and courting male quality (Thornhill and Alcock, 1983; Eberhard, in press; Cordero, 1995), among other factors.

In *C. xami* time since last mating is positively correlated with female receptivity (Cordero, 1996), but at this moment we do not know if time *per se* or other correlated factors (such as the quantity of some of the different ejaculate components remaining stored in the female) are responsible for differences in female receptivity. Males probably emit pheromones during phases IV to VI, and this also suggests that females involved in "short" courtships could have been highly receptive (and, therefore, in need of less stimulation). (A few observations suggest that at least some females come into actual or potential territories, flying within them in a way that could possibly make them very conspicuous to territorial males. If the function of this behavior is to make females easily detected by males, we expect to observe it only in receptive females.)

An alternative explanation for the "shortening" of courtships may be physical exhaustion of the male forcing him to reduce the possibly costly courtship phases IV to VI. However, our observations of the conditions of males, as well as the low frequency of male-male and male-female interactions reported in *C. xami* (Cordero and Soberón, 1990; Cordero, 1996), suggest that this second hypothesis is not a general explanation for these behavioral variants; besides, if this hypothesis is correct, we need to explain why females accepted these exhausted males. If we accept the idea that females gain information about male quality during courtship, a third hypothesis is that males involved in the "short" courtships could have been males of very high quality, quickly identified as such and accepted by females.

Mate choice and copula interruption

Although mate choice has not been studied in *C. xami*, differences in mate quality —and, therefore, selective pressures in favor of mate choice— are probably common. In females, the number of eggs remaining to be laid (and therefore, their value for males) can vary widely depending on the number of eggs already laid and on female size (Parlange, 1991; Cordero, 1996). In the laboratory, females mate a second time until they have laid a substantial proportion of their eggs (Cordero, 1996). In males, the quantity of nutritious ejaculate transferred to females varies with the size and mating history of the male (Cordero, 1996); besides, for a female to be the second male's mate of the day means not only a small ejaculate, but also a lengthy copulation (> 5 h *vs.* 30.3 minutes).

Given the common occurrence of unsuccessful courtships (56.5 % of 23 courtships observed in 1989, and 85.3 % of 34 observed in 1990), mate choice prior to copulation probably exists in *C. xami*. However, a detailed study remains to be conducted. "Copula interruption" suggests mate choice after mating began. However, it is not possible to tell with the available information whether female or male choice is responsible for this behavior. Females or males may be able to evaluate their mating partners after copulation begins and decide to interrupt it within a few minutes (Eberhard, 1985, 1994, in press; Eberhard and Cordero, 1995; Cordero, 1995). The fact that females are the behaviorally "active" sex during the process of separation cannot be

used as evidence of female choice, given that for females it may be convenient to interrupt mating with a male that, although desirable, is unwilling (because of mate choice reasons) to transfer an ejaculate. It is also conceivable that the male manipulates (chemically or mechanically) the female, inducing her to finish copulation against her own interests. However, the fact that in the case of 1989 the male found the female again, less than three minutes after separation, and unsuccessfully courted her for a second time, suggests that female choice is involved in at least some of the post-coupling "rejections". The fact that copula interruption occurs a few minutes after genital coupling begin may be due to rapid assessment of mate quality or to the fact that as time advances it may be physically difficult to interrupt copulation (Wickman, 1985).

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CAPÍTULO 2

The number of copulations of female *Callophrys xami* (Lycaenidae)

Abstract. Some aspects of the sexual behavior of females of the lycaenid butterfly *Callophrys xami* were studied. Field estimates of the frequency of successful copulations (number of spermatophore remains in the *corpus bursae*) indicate that mated females have an average of 1.37 spermatophores and that 21.4% had more than one spermatophore. No relation was found between the number of spermatophores and female wing wear and length. Laboratory observations and an experiment indicate that female sexual receptivity decreases after mating and that the probability of female remating is low. Discussion focuses in the role of female mating patterns in the evolution of several aspects of the mating system of this species.

KEY WORDS: Copulation, female remating, mating system

INTRODUCTION

Recent developments in sexual selection theory (Eberhard, 1996) suggest that the role of females in shaping the evolution of mating behavior has been underestimated. In butterflies most of the research on mating strategies has emphasized the role of male behaviour (e. g. Rutowski, 1991; Alcock, 1994; Deinert *et al.*, 1994). However, some studies indicate that female behavior has a very important role in the evolution of butterfly mating systems (Rutowski, 1980; Wiklund, 1982; Wickman, 1986, 1992, 1997; Dennis & Shreeve, 1988; Kaitala & Wiklund, 1994).

The mating behavior of males of the territorial butterfly *Callophrys xami* Reakirt (Lycaenidae) has been described in some detail (Cordero & Soberón, 1990; Cordero, 1993; Cordero, 1997); however, information on the sexual behavior of females of this species has not been published. In this paper we present field data on the frequency of successful copulations (*i. e.* those resulting in spermatophore transfer) of female *C. xami*. We also report several laboratory observations and an experiment on the effect of copulation and oviposition on the probability of female remating. We also describe two new observations of interrupted copulations.

METHODS

C. xami is a multivoltine butterfly that can be found throughout the year at relatively low numbers but reaches its highest density from October to January (Soberón *et al.*, 1988). The main larval food plant in the study area is the perennial *Echeveria gibbiflora* (Crassulaceae), an abundant species (Soberón *et al.*, 1988). Males defend territories that function as mating stations (Cordero & Soberón, 1990; Cordero, 1997). The courtship behavior is described in detail in Cordero (1993).

Field studies: female mating frequency

Field studies were conducted in a 146.8 ha ecological reserve maintained by the Universidad Nacional Autónoma de México, in the south of Mexico City. This reserve is in the Pedregal de San Angel, a zone characterized by volcanic soil, rough topography, markedly seasonal rainfall regime, and xerophytic shrubby vegetation.

The frequency of successful copulations by females (*i. e.* copulations resulting in spermatophore transfer) was evaluated by counting the number of spermatophores and spermatophore remains (mainly the sclerotized *collum*) in the *corpus bursae* of females (Burns, 1968; Drummond, 1984; Eberhard, 1985; Lederhouse *et al.*, 1989). Females were sampled during eigth sunny days between December 28, 1989 and January 23, 1990. All females observed during these days were collected and frozen until dissection; total sample size was 28 females. The length of the right forewing (a measure of body size; correlation between wing length and body weight: $r = 0.8$, $p < .001$, $n = 27$ [Cordero, unpublished data]) was measured with a calliper and each female was assigned to one of three wing wear categories: (1) similar to a recently emerged adult (wings mostly green with intact margins), (3) very worn female (wings mostly brown with worn margins), and (2) all individuals intermediate between (1) and (3). The effect of female wing length on the number of spermatophores was investigated with Spearman correlation because wing length had not a normal distribution. Since all females collected were in wing wear conditions 1 or 2, the effect of this characteristic on spermatophore number was assessed with a Mann-Whitney test.

Laboratory studies: female remating

During the course of other studies with *C. xami* we made a number of observations suggesting that mating normally induces a period of female sexual unreceptivity; some of these observations were recorded and are briefly summarized in the results.

We performed an experiment to investigate some of the conditions determining female remating. Since previous observations suggested that oviposition was necessary to induce remating in females, we investigated this factor. In this experiment, the probability of female remating in captivity was measured. 32 laboratory reared virgin females were introduced (mostly in pairs) in mating cages (cylindrical cages made of mesh cloth, 58 cm of height and 26 cm of diameter) with 1-3 virgin males, depending on male availability. Six females not copulating the first day were excluded from the experiment. After their first copulation, females were kept individually with fresh leaves (entire or in pieces) of *E. gibbiflora* as oviposition substrate, in white, translucid, one liter plastic containers; the cap of the containers had a circular "window" of six cm of diameter, covered with plastic mesh. Every morning, females were moved from the containers to mesh cloth cages and fed

ad libitum a 10% sugar solution. After feeding, females were returned to a container with fresh leaves without *C. xami* eggs. The females in the containers were placed during 2 h (beginning between 1000-1100 h) in an insectary under an incandescent ligth (100 W) to induce oviposition, and the rest of the time they were kept in the dark to avoid oviposition (personal observation). The daily number of eggs laid was counted and the eggs were kept on pieces of leaves of *E. gibbiflora* until they eclosioned or until we were sure they were not viable (we do not know if these eggs were fertilized or not), which happened when eggs become deflated, turned dark (instead of light green or white) or when more than 10 days had elapsed since the eggs were laid (eclosion occurs approximately 7 days after the egg is laid; Jiménez & Soberón, 1988-89; Cordero, unpublished data). We tried to induce females to remate after varying numbers of days ovipositing. Individual mated females were introduced in mating cages with three virgin males during 3-4 h (in a few occasions the males were not virgin; also in a few cases females were introduced with two, four or five males); the number of occasions mated females were presented to further males varied from one to five. Females that did not mate a second time were compared with females that remated; the males copulating with both types of females were also compared.

RESULTS

Female mating frequency in the field

The distribution of spermatophore numbers in the *corpus bursae* of females is presented in Fig. 1. The percentage of females with at least one spermatophore was 67.9%; 21.4% had more than one spermatophore. The mean ± SD number of spermatophores found in females with at least one spermatophore was 1.37 ± 0.6. Number of spermatophores was not associated with differences in either wing wear (Mann-Whitney, $U = 54$, $p > 0.05$; Fig. 1) or wing length ($r_s = -0.07$, $p > 0.05$, $n = 27$; Fig. 2).

Female remating in captivity: observations

A number of observations, suggesting that mating induces a period of female unreceptivity, were made in captivity during the course of other investigations and are summarized in Table 1.

Female remating in captivity: experimental results

Twenty six females mated the first day they were exposed to males. Twenty of these females were exposed to groups of 2-5 males a number of times after their first copulation (hereafter, FC); females were allowed to oviposit between remating attempts. Thirteen females did not remate (hereafter, FNR) and seven females copulated a second time (hereafter, FR) (Fig. 3). The FNR were

exposed to males between one and four times after their FC; the time interval between FC and the last exposition to males of these females varied between five and 13 days. The FR were exposed to males between one and three times after their FC: four females remated the first time they were exposed to males (four days after FC [n = 1], and five days after FC [n = 3]), two the second time (seven days after their FC), and one the third time (eight days after her FC).

The wing length of FR (n = 6) was similar to the wing length of FNR (n = 12) (Mann-Whitney, U = 18.5, p = 0.1); however, there was a non-significant trend for FR to be larger than FNR (FNR: median = 1.68 cm, range: 1.6 - 1.77 cm; FR: median = 1.74 cm, range: 1.65 - 1.78 cm). FR laid 108 ± 38 (range: 73 - 162) eggs before their SC and 209 ± 39 (range: 169 - 276) after their SC, for a total of 317 ± 38 (range: 242 - 352). FR mated for a second time after laying 33.8 ± 10.2 % (range: 21.6 - 48.9 %) of their lifetime egg production. There were not significant differences in the number of eggs laid previous to their second copulation between FR and FNR, considering equivalent periods of time (Table 2; Mann-Whitney tests for $X = 3$, $X = 4$ and $X = 6$, where $X =$ number of days after FC; all $p > 0.1$).

The wing length of males mated to FNR (n =7) was not different from that of the first mates of the FR (n = 5) (Mann-Whitney test, U = 17, p > 0.93); when we compared the relative size of males (male wing length / female wing length) we also did not find differences between males mated to FNR and FR (Mann-Whitney test, U = 11, p > 0.83). For five of the FR we have wing length data for the first and the second mate; in three cases the second mate was bigger than the first (1.67 cm *vs.* 1.66 cm, 1.64 *vs.* 1.55, and 1.65 *vs.* 1.63), and in two cases it was smaller (1.64 cm *vs.* 1.67 cm, and 1.53 *vs.* 1.67). In two of the FR the relative size of the first male was bigger (0.97 *vs.* 0.95, and 0.96 *vs.* 0.88) and in two cases it was smaller (0.95 *vs.* 0.96, and 0.94 *vs.* 0.99) than the relative size of the second male.

DISCUSSION

Frequency of copulation in the field

The average copulation frequency estimated for mated females (1.37 ± 0.6) indicates a relatively low degree of polyandry in comparison with other species of butterflies (Burns, 1968; Drummond, 1984; Gwynne, 1984; Eberhard, 1985; Svard & Wiklund, 1989; Wiklund & Forsberg, 1991; Braby, 1996). Furthermore, 78.6 % of the females (Fig. 1) had only one or no spermatophore in their *corpus bursae*. Since female remating intervals are probably long (see next section) in relation to female longevity in the field, we were expecting a low maximum number of spermatophores in females. The observed maximum of three spermatophores is intermediate in comparison with other butterfly species (Burns, 1968; Drummond, 1984; Gwynne, 1984; Eberhard,

1985; Svard & Wiklund, 1989; Wiklund & Forsberg, 1991; Braby, 1996). However, both the average number and the maximum number of spermatophores could be underestimates, since no females in the very worn ("old") wing wear condition were collected (Fig. 1). Therefore, although the data obtained in this study indicate a trend towards monandry in *C. xami*, it is possible that this is in part result of a sampling bias if "old" females were more difficult to detect or to capture. However, our experience of several years of field studies with this species does not provide evidence of a greater difficulty to catch "old" females. It is possible that females do not live long enough to become very worn, and, therefore, they are rare; in this case our estimates of copulation frequency would be unbiased. It is also possible that female longevity, and, therefore, the abundance of "old" females, varies in time as a result of, for example, varying predation pressure or weather conditions. Under these conditions, average and maximum number of spermatophores would vary with time depending on the age structure of females.

The method used to evaluate female copulation frequency is based on three assumptions: (a) Copulation always results in spermatophore transfer. This is not true, because there are some copulations of very short duration that do not result in the transfer of an spermatophore (Cordero, 1993; Table 1); however, these "interrupted" copulations are not common in the field (0/18 copulations observed in 1983-1985 and 2/27 copulations observed in 1989-1990; Cordero, 1993). Although the existence of interrupted copulations prevented the estimation of the total number of copulations performed by females, the figures obtained are probably good estimates (but see below) of the number of copulations resulting in spermatophore transfer.

(b) Males transfer only one spermatophore per copulation. This is not true, since in laboratory experiments we observed three copulations in which different males transfered two spermatophores in one copulation (Cordero *et al.*, unpublished MS). Violation of this assumption results in an overestimation of copulation frequency; however, if the frequency of copulations resulting in the transfer of two spermatophores in the laboratory is a good estimate of their frequency in the field (3/199 or 1.51%), its quantitative effect should be small.

(c) Spermatophores always leave recognizable remains within the *corpus bursae* of the female. This is not true, since in the laboratory it was not always possible to observe clear spermatophore remains in old females that had laid most of their eggs; however, judging from wing wear, few, if any, females in this condition were sampled. The possible violation of assumptions (a) and (c), and the fact that some of the females may have mated again had they not been collected, results in an underestimation of the frequency of copulations in females.

Female remating in captivity

The observations and experimental results suggest that some aspect of copulation delays (and, probably in some cases, inhibits for life) remating in female *C. xami*. The observed proportion of virgin females mating the first day they were exposed to males was very high: in the experiment this proportion was 81%, whereas in some other unpublished experiments reached 100% (Cordero *et al.*, unpublished MS). In contrast, the proportion of remated females varied from 0 % in observation (c) (Table 1), to 35 % in the experiment (Fig. 3). We only recorded data from four females exposed to further males without being allowed to lay eggs after their FC (observations (b), (d), (e) and (g); Table 1); only in (g) the female remated, but after copulating with a small male that, according to Cordero *et al.* (unpublished MS), should have transfered a small spermatophore. In the experiment, FR mated for a second time after laying between 21.6 % and 48.9 % of their lifetime egg production; however, the numbers of eggs laid by FR and equivalent FNR were not different (Table 2). Therefore, although copulation inhibits female sexual receptivity in captivity, at least for a number of days, the specific factor(s) controlling the length of the period during which the female remains sexually unreceptive need to be investigated.

Female reproductive behavior and selection on male mating behavior.

Three reproductive characteristics of female *C. xami* indicate that there must be selection in favor of males best able to copulate with recently emerged females. First, females are capable of copulating and being fertilized the day they emerge from the pupae (which usually happens early in the morning; Cordero and Jiménez, personal observations). Second, copulation inhibits sexual receptivity for a number of days (Table 1 and Fig. 3). And third, females exhibit a pattern of oviposition with a peak in the first 1-3 days after copulation, with a subsequent continuous decrease in the number of eggs laid (Jiménez & Soberón, 1988-89). Therefore, when a male copulates successfully with a virgin female and inhibits her sexual receptivity, he expects to be rewarded with the paternity of most of the eggs laid by the female during her life, since the period of female unreceptiveness will correspond to the period in which oviposition rates are higher.

The decrease in sexual receptivity of females after mating is partially responsible of the scarcity of receptive females and probably contributes to the high incidence of unsuccessful courtships observed in the field (Cordero, 1993). However, females show some degree of polyandry. The figures obtained in this study are probably good estimates of the number of copulations resulting in spermatophore transfer and, probably, insemination. The number of successful copulations by females is very important for the study of mating system evolution since it gives an indication of the potential for sperm competition and cryptic female choice.

Many studies of insect mating systems consider the number of copulations an important component of male fitness (Thornhill & Alcock, 1983). However, the evolutionary importance of male mating success is a function of male mating costs, female remating patterns, cryptic female choice mechanisms, and sperm competition. Although we have no data on sperm competition in *C. xami*, we know that mating inhibits female sexual receptivity in captivity for a number of days (if it is the first mating of the female, these days correspond to the days in which oviposition rates are higher), lessening the importance of sperm competition. Besides, although up to three spermatophores were found in females, 78.6 % of them (Fig. 1) had only one or no spermatophore in their *corpus bursae*. Therefore, the number of matings seems to be an important male fitness component in *C. xami*.

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Table 1. Summary of observations suggesting that mating induces a period of female unreceptivity.

FC: first copulation; SC: second copulation.

I. Observations in which no female rematings were observed

(a) Two virgin females (3 and 2 d old) mated in captivity the first time they were exposed to males; after laying an unrecorded number of eggs, they were taken to the field, 21 days after their FC, and presented to territorial males (in this way we have induced copulations of laboratory reared virgin females with territorial males in the field); the females were courted, but none remated.

(b) One 2 d old mated female was exposed to a virgin male, one, two, three and five days after her FC (the male was different in each trial), but did not remate. The female was then allowed to lay at least 17 eggs six days after her FC, and, on the 19th day after her FC, it was taken to the field and presented to a territorial male who courted her without success.

(c) Four virgin females mated in captivity the first time they were exposed to virgin males; after laying eggs during two or three days, they were individually introduced in mating cages with two (n = 3) or three (n = 1) virgin males, nine days after their FC and after laying 33, 53, 121 and 55 eggs, respectively; no female rematings were observed.

(d) One virgin female mated with a male (possibly due to perturbation by the observer, this mating was shorter than normal copulations and finished like an "interrupted copulation"†) that had mated for the first time the day before; without being allowed to lay eggs, the female was exposed to her first mate the two days after her FC and to a different male the following three days, but did not remate.

(e) One 2 d old virgin female was the second mate of the day of one of her brothers (this male had mated four times, one of them "very brief", in the two previous days); this mating lasted only two minutes. The female was not given the opportunity to lay eggs and did not mate in any of the opportunities to mate she had in the three following days; in all opportunities, two other females and one virgin male were in the mating cages (afterwards, the three males copulated between two and four times).

II. Observations in which female rematings were observed

(f) Three virgin females mated in captivity the first time they were exposed to virgin males. Two of the females (3 and 6 d old, and not allowed to lay eggs and allowed to lay an unknown number of eggs, respectively) were exposed, two days after their FC, to three males each and did not remate; the third female (12 d old), after laying 101 eggs (78.2 % of these survived to the adult stage), was exposed to four males five days after her FC and mated for a second time.

(g) One 2 d old virgin female mated with one small male (wing length = 1.27 cm; mean wing length of 145 field males was 1.64 ± .1 cm, range: 1.36 - 1.89 cm) that had mated once in each of the two previous days; four days after her FC, and before laying any eggs, the female mated with a 14 d old male (wing length = 1.53 cm) that had already mated twice, 7 and 5 days before.

† Another new observation of interrupted copulation, similar to those reported in Cordero (1993), occurred in the absence of any obvious external perturbation, about 3 min after the beginning of mating; dissection of the female showed that no spermatophore was transfered. In both cases, the interrupted copulation was the second of the male (they had copulated successfully the day before) and the first of the female.

Table 2. Number of eggs laid by females that did not remate and by females mated twice, during the first *X* days after their first copulation, and, in the case of twice mated females, before second copulation.

		X = 3	*X* = 4	*X* = 6	*X* = 8
(a) ♀♀ that did not remate	Mean ± SD	64.5 ± 13.7	86.8 ± 15.6	119.5 ± 19	152.1 ± 26.6
	Median	63	82	118	146
	Min.-Max.	41-91	61-118	86-157	110-200
	N	13	13	11	11
(b) ♀♀ remated	Mean ± SD	65.4 ± 10.6	84 ± 9.7	135.7 ± 6.1	162
	Median	67	82	137	-
	Min.-Max.	54-77	73-96	129-141	-
	N	7	6	3	1
(c) ♀♀ that mated a 2nd occasion after ovipositing *X* days after first copulation: number of eggs laid up to *X*	♀: # eggs laid	*29.7.118:* 76	*3.7.118:* 73 *1.11.1:* 77 *3.4.19:* 87	*36.11.1:* 137 *11.4.27:* 141	*39.7.118:* 162
(d) Number of (a) that laid more eggs than (c) during the first *X* days after their first copulation	# of ♀♀ (Min.-Max. number of eggs laid)	2 (81-91)	12 (74-118)† 11 (79-118) 4 (96-118)	2 (147-157)	5 (168-200)

† The number of (a) that laid more eggs was different for each female that remated after ovipositing 4 d.

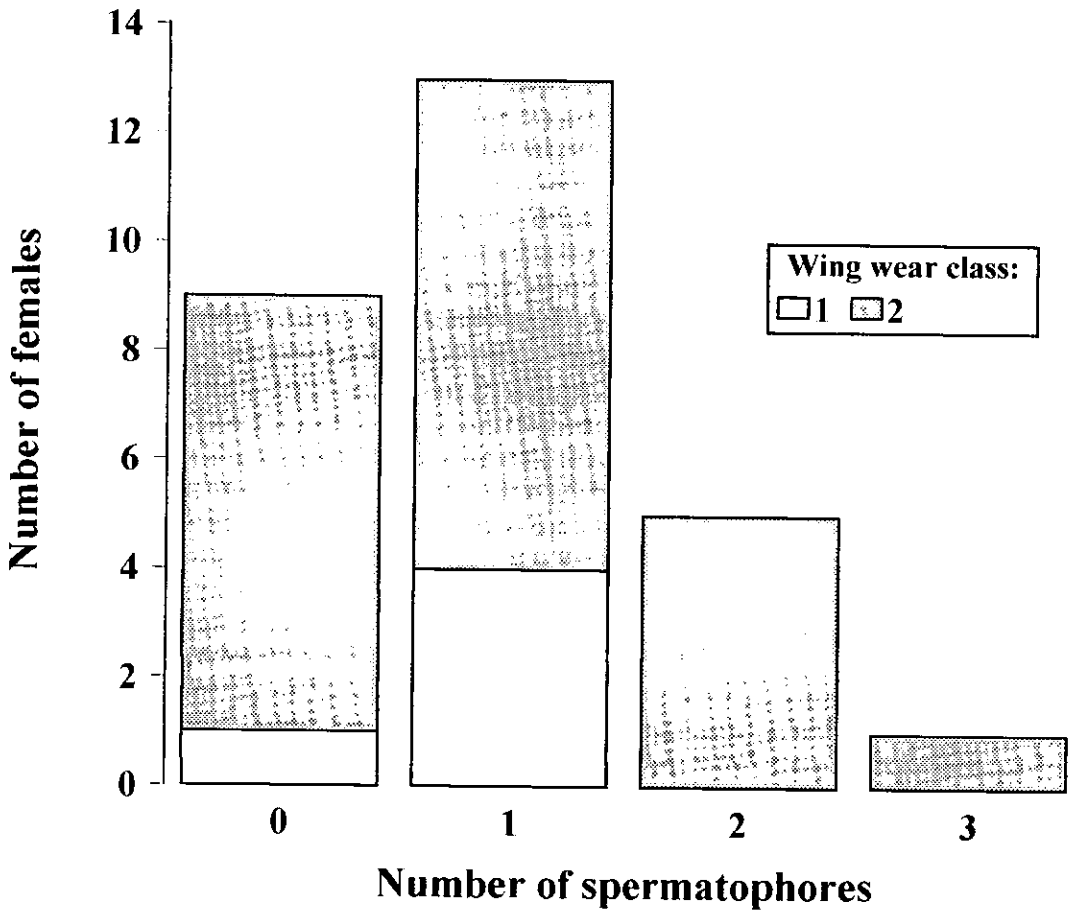

Fig. 1. Distribution of females with different number of spermatophores and the relationship between number of spermatophores and wing wear category.

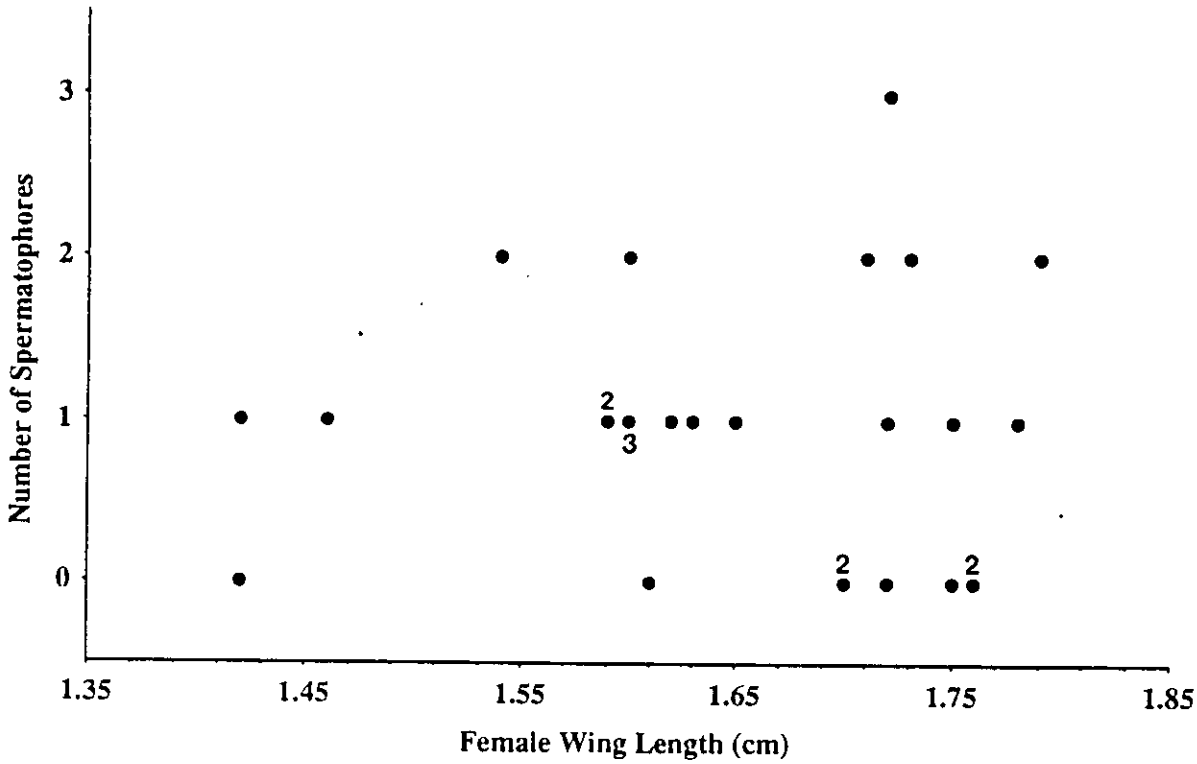

Fig. 2. Relationship between female wing length and number of spermatophores in *corpus bursae*. As indicated in the figure, three points represent two females and one point three females.

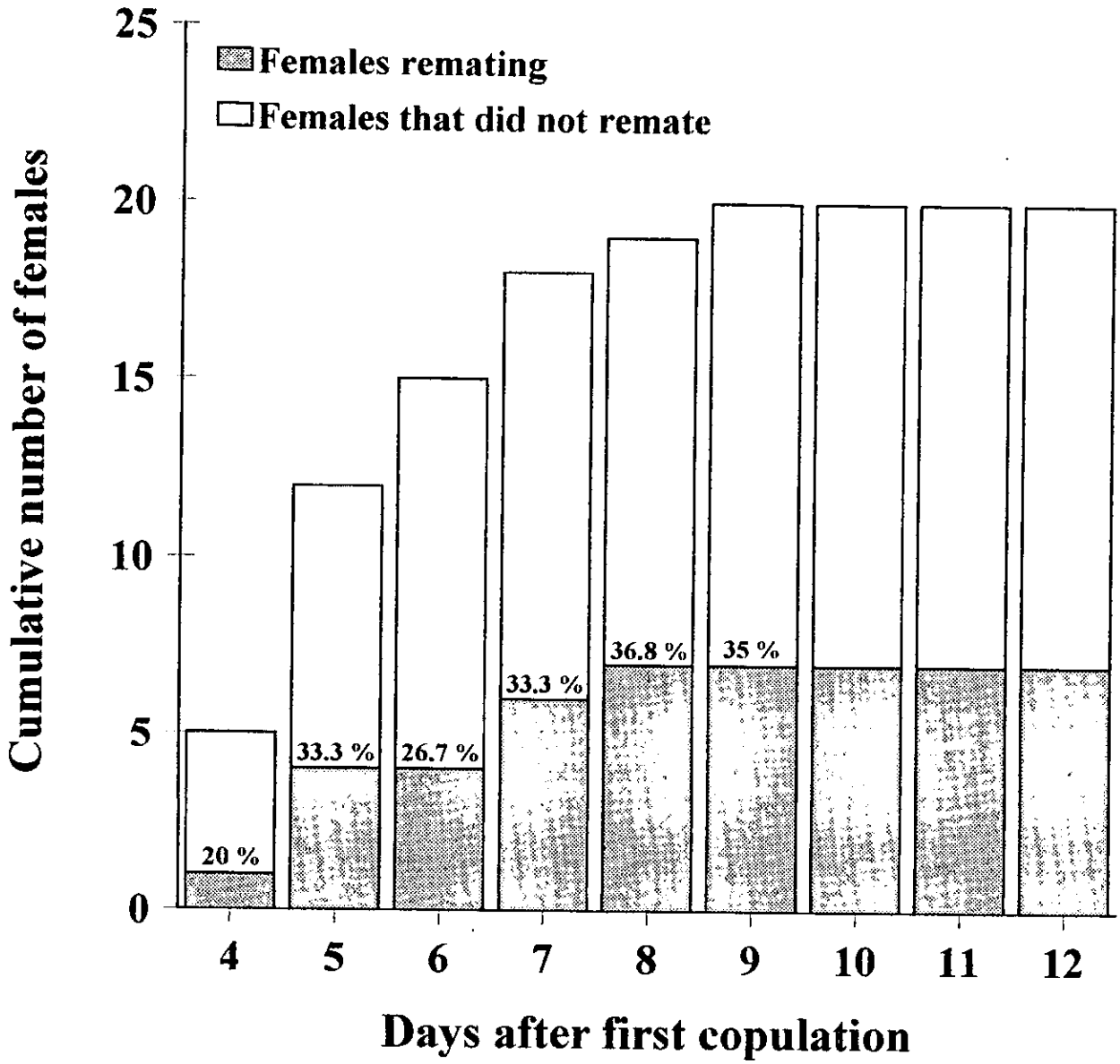

Fig. 3. Female remating as a function of number of days after first copulation.

CAPÍTULO 3

The number of copulations of territorial males of the butterfly *Callophrys xami* (Lycaenidae)

Abstract. The distribution of copulations between males and territories in the butterfly *Callophrys xami* (Lycaenidae) was evaluated in the field. Copulation rate was very low (.0014 copulations / male / h). There was high variance between males in the number of copulations. Data from the few males observed copulating more than once (n = 3) suggests a sexual selection advantage for big, long lived males. Variation among territories in the number of resident males, frequency of occupation and number of copulations suggests variation in territory quality. Frequency of occupation and number of matings were not correlated with the territory characteristics measured; furthermore, there were not between-years correlations in frequency of occupation and number of copulations in the territories studied in two different years. The location of territories may be important in determining territory quality.

KEY WORDS: *Callophrys xami*, Lycaenidae, copulation, mating success, territoriality, sexual selection

INTRODUCTION

The fitness of male insects is difficult to determine in the field (Thornhill & Alcock, 1983). Although the number of copulations has been used frequently as a measure of male fitness (Thornhill & Alcock, 1983), it is not possible to be confident about such measure without knowledge of male mating costs, female copulation frequency, sperm competition patterns (Smith, 1984) and postcopulatory female choice criteria (Eberhard, 1996). However, some studies suggest that the number of copulations achieved by a male is an important fitness component at least in some species. One line of evidence supporting this suggestion is the fact that several aspects of the male phenotype seem to be specific adaptations to increase the number of copulations (reviews in Darwin, 1871; Thornhill & Alcock, 1983; Choe & Crespi, 1997).

In the butterfly *Callophrys xami* Reakirt (Lycaenidae) the number of copulations seems to be an important fitness component for males since they spend all their active adult lifetime defending territories that lack concentrations of larval and adult food resources (Cordero & Soberón, 1990) and that are used only as mating stations (Cordero & Soberón, 1990; Cordero, 1993). Besides, laboratory observations indicate that copulation inhibits female sexual receptivity for a number of days (if it is the first mating of the female, these days correspond to the days in which oviposition rates are higher), and dissection of field collected females indicates a trend towards monandry (see discussion).

In this paper we report field observations of copulations by territorial males of the butterfly *C. xami*. The distribution of copulations between different males and between different territories is described and some factors possibly affecting such distributions are discussed.

METHODS

The study was conducted in the Pedregal de San Angel ecological reserve, maintained by the Universidad Nacional Autónoma de México in the south of Mexico City. This zone is characterized by volcanic soil, rough topography, markedly seasonal rainfall regime, and xerophytic shrubby vegetation. *C. xami* is a multivoltine butterfly that can be found throughout the year at relatively low numbers, reaching its highest density from October to January (Soberón *et al.*, 1988). The main larval food plant in the study area is the perennial *Echeveria gibbiflora* (Crassulaceae), an abundant species (Soberón *et al.*, 1988; Larson *et al.*, 1994).

Study periods were chosen to coincide with population density "peaks" (Soberón *et al.*, 1988; personal observation); observations were made between November 1 and December 20, 1989 and between November 10 and December 6, 1990. Most territorial males observed were individually marked on the wings with felt-tip pens and their right forewing length was measured through the mesh of the net with a calliper (in the laboratory, male wing length is correlated with adult body weight at emergence: $r = 0.91$, $p < 0.001$, $n = 28$; Cordero, unpublished data). Individuals were assigned to one of three wing wear categories: (1) similar to a recently emerged adult (wings mostly green with intact margins), (3) very worn male (wings mostly brown with worn margins), and (2) all individuals intermediate between (1) and (3). Longevity was defined as the number of days elapsed between the first and the last observation of the male. Territory limits were determined as explained in Cordero & Soberón (1990). We measured the (i) maximum length and the (ii) "cross" length (length of the perpendicular axis crossing through the middle point of i) of territories; territory area was approximated as (i) x (ii); the ratio (i) / (ii) was used as a measure of territory "shape".

The study period of each year was divided in two parts. During the first part we measured the frequency of occupation of each territory (= number of days the territory was occupied by a territorial male / number of days the territory was censused), determined the identity of each male defending the territory, and recorded all copulations observed. We studied 25 territories in 1989 and 19 in 1990. One observer walked along transects joining several territories two times per day between 1000 and 1500 h, the daily territorial defense period (DTDP; Cordero & Soberón, 1990), during 31 days in 1989 and during 11 days in 1990, and observed each territorial male (if present) for at least two minutes. The observation period was longer if, for example, the male was interacting with a conspecific male or courting a female. The average number of days (± SD) each territory was

censused was 26.2 ± 1.8 (median = 26; range: 22 -30 d) in 1989, and 10.2 ± .6 (median = 10; range: 9 - 11) in 1990.

During the second part of each study period we estimated the probability of copulating twice in a day (previous work indicated that the maximum number of successful copulations per day that a male can achieve is two, since a male's first copulation of the day lasts 32 min on average, while the second copulation of the day lasts several hours; Cordero, 1993). We made focal observations of territorial males throughout the DTDP and recorded all copulations observed, during nine days in 1989 and 12 days in 1990. The number of males with focal observations was 15 in 1989 and 16 in 1990; the total number of hours of focal obsevations was 200 h in 1989 (40 five h periods of focal observations) and 130 h in 1990 (26 five h periods of focal observations). The number of days of focal observations per male varied from 1 to 6 in 1989 (mean ± SD = 2.7 ± 1.7, median = 2), and from 1 to 3 in 1990 (1.6 ± .8, median = 1). Focal observations were made in 14 territories in 1989 and in 11 territories in 1990. The number of days of focal observations per territory varied from 1 to 6 in 1989 (2.9 ± 1.6, median = 2), and from 1 to 3 in 1990 (2.4 ± .7, median = 3). All observations were made during fine, sunny days (*C. xami* is not active under cloudy conditions). All summary statistics are given as mean ± standard deviation.

RESULTS

Throughout the study periods of 1989 and 1990, we observed territorial males (Cordero, 1997) and sexually receptive females (Figure 1.a). All complete courtships resulting in copulations observed (n = 15) began inside territories and involved territorial males; we did not observe the moment at which 12 copulations began (Figure 1.b), however, all of them involved territorial males. Copulations were observed between 1100 and 1500 h in 1989 and between 1230 and 1500 h in 1990 (Figure 1.b).

Distribution of copulations among males

We observed a total of 27 copulations (Table 1). Although we marked and observed 159 territorial males (99 in 1989 and 60 in 1990), only 21 males were observed copulating (12 in 1989 and 9 in 1990). One of the males observed copulating in 1989 and two of the males observed copulating in 1990 were not marked, therefore, 141 marked territorial males (88.7%) were not observed copulating during the study. Three males were observed copulating more than once (two, three and four times). Only one male was observed copulating two times in a day (this was the male that mated four times in 1989). We suspect that another male mated twice in one day (this was the male that mated three times in 1989), since this male was observed arriving to the territory at 1111 h

and copulating at 1137 h for more than 268 min (observation was interrupted at 1605 h); therefore, it is possible that this male copulated before the beginning of observations (focal observations began at 1004 and sometimes males began territory defense before 1000 h) and that the long copulation observed was the second of the day (as mentioned in Methods, the male's first copulation of the day lasts on average 32 min, while the second copulation of the day lasts several hours). Therefore, only in one (possibly two) of the 40 five h periods of focal observations in 1989 we observed two copulations; no male copulating twice in day was observed in any of the 26 five h periods of focal observations in 1990.

As expected from the different sampling methods employed during the first and second part of each study period, the proportion of marked males observed copulating in the first part of the study (10 / 144 = 6.9%; 8 / 92 in 1989 and 2 / 52 in 1990) was lower than the proportion observed copulating during the second part (10 / 31 = 32.3%; 4 / 15 in 1989 and 6 /16 in 1990) (Table 1). Seven of the nine copulations performed by the three males observed copulating more than once were observed during the second part of the study (including the two copulations performed in the same day by a male).

The rates of copulation calculated from the focal observations were similar in both years of study: .04 copulations / h (= 8 copulations / 200 h of focal sampling) in 1989 and .046 copulations / h (= 6 copulations / 130 h of focal sampling) in 1990; the overall copulation rate for both years was .042 copulations / h. Mating rates per male calculated from the focal observations were almost identical in both years of study: .0027 copulations / male / h (= 8 copulations / 15 males / 200 h) in 1989 and .0029 copulations / male / h (= 6 copulations / 16 males / 130 h) in 1990; the overall copulation rate per male for both years was .0014 copulations / male / h (= 14 copulations / 31 males / 330 h).

Characteristics of males

A total of 99 territorial males in 1989 and 60 in 1990 were individually marked. No significant differences between years were found in wing length (1989: 1.64 ± .1 cm, max.—min.: 1.36—1.89, n = 90; 1990: 1.65 ± .09 cm, max.—min.: 1.4—1.83, n = 55; t = -.29, p = .77), longevity (1989: 4.8 ± 5.1 days, median = 2, max.—min.: 1 - 20, n = 99; 1990: 4.9 ± 5.9, median = 2, max.—min.: 1 - 28, n = 57; Mann-Whitney U = 2819, p = .99) and wing wear at the moment of being marked (1989: median = 1, max.—min.: 1—3, n = 90; 1990: median = 1, max.—min.: 1—3, n = 57; U = 2419.5, p = .51). There was no correlation between wing length and longevity (1989: r_S = .13, p = .22, n = 90; 1990: r_S = .11, p = .43, n = 52).

Due to the sampling methods employed in this study, we cannot look for a relationship

between male traits and number of copulations in the data. However, the characteristics of the three males observed copulating more than once suggest that male size and longevity could be positively correlated with copulation success. The male that was observed copulating more times (four) during both years of study was also the biggest male observed in both years (wing length = 1.89 cm); this male was also the only one observed copulating twice in a day. The longevity of this male was 14 days, longer than that of 89.9% of the males observed in 1989. The male that was observed copulating three times in 1989 was bigger (wing length = 1.72 cm) than 73.3% of the males observed that year. The longevity of this male was 11 days, longer than that of 85.9% of the males observed in 1989. This male probably copulated twice in a day (see previous section). The male that was observed copulating two times in 1990 was bigger (wing length = 1.72 cm) than 74.5% of the males observed that year. The longevity of this male was 18 days, longer than that of 94.7% of the males observed in 1990. Therefore, the characteristics of the multiply mated males indicate that a study of the effect of wing length and longevity on male mating success would be particularly interesting in this butterfly (see Appendix).

Distribution of copulations among territories

The 17 copulations of 1989 and the 10 copulations of 1990 were observed in seven territories each year, although only three of these were the same in both years (Table 1). There was wide variation in territory characteristics (Table 2). In order to explore the relation between territory measurements (maximum length, "cross" length, [maximum length / "cross" length] and area) and the frequency of occupation of the territory and the number of copulations observed in the territory, only the data obtained during the first part of the study periods were analized. This decision was made considering that during the second part of both study periods the sampling effort was very heterogeneous (the number of days of focal observations per territory varied from 1 to 6 in 1989 [CV = 55.2%] and from 1 to 3 in 1990 [CV = 29.2%]), whereas during the first part of both study periods it was much more homogeneous, and, therefore, comparable, between territories (the number of days each territory was censused varied from 22 to 30 in 1989 [CV = 6.9%], and from 9 to 11 in 1990 [CV = 5.9%]).

Average frequency of occupation of territories during the first part of the study periods was .48 ± .29 (median = .5; max.—min.: .04—1) for 1989 and .6 ± .37 (median = .8; max.—min.: 0—1) for 1990. The frequency of occupation of territories was not correlated with any of the territory measurements (Spearman correlations, all $p \geq .26$). Average number of copulations in territories during the first part of the study periods was .36 ± .76 (median = 0; max.—min.: 0—3) for 1989 and .24 ± .44 (median = 0; max.—min.: 0—1) for 1990. There were not significant differences in

maximum length, "cross" length, [maximum length / "cross" length] and area between territories in which copulations were observed and territories in which no copulations were observed (Mann-Whitney U tests, all $p > .14$) in the first part of both study periods. The frequency of occupation of territories in which copulations were observed was higher than that of territories in which no copulations were observed in 1989 (territories in which copulations were observed: $.7 \pm .18$, $n = 6$; territories in which no copulations were observed: $.4 \pm .28$, $n = 19$; $U = 21.5$, $p = .024$), but no difference was detected in 1990 (territories in which copulations were observed: $.81 \pm .13$, $n = 4$; territories in which no copulations were observed: $.53 \pm .4$, $n = 13$; $U = 18$, $p = 36$). However, the 1989 difference is not significant if we perform a sequential Bonferroni adjustment of significance levels (Rice, 1989), using as a family of tests (Chandler, 1995) the five U tests of 1989, and using $\alpha = .1$ as suggested by Chandler (1995) ($k = 5$, $\alpha / k = .02$).

Twelve territories were observed in both years. Considering only the data collected during the first part of both study periods, there were no significant between-years correlations in the frequency of occupation of these territories ($r_S = .55$, $p = .078$, $n = 11$) or in the number of copulations (Gamma correlation, $\gamma = -.09$, $p = .87$, $n = 11$) observed in these territories. Therefore, the "quality" of a territory in a given year was not a predictor of that in the next. In fact, the territory that in 1989 had the maximum number of observed copulations (five or, probably, six; Table 1) and the second highest frequency of occupation (0.94; maximum = 1), was not occupied by a territorial male in any of the more than 10 days in which it was censused in 1990.

DISCUSSION

Male copulation frequency

As it seems to be common in insects exhibiting lek territoriality (*e. g.* Alcock, 1983, 1987; Alcock & O'Neill, 1986; Table 3), the average rate of copulations observed in *C. xami* was low: .0014 copulations / male / h. Low copulation rates are expected since this mating system seems to be selected when receptive females are scarce and widely dispersed (Thornhill & Alcock, 1983; Rutowski, 1991); as it has been discussed previously (Cordero & Soberón, 1990), such conditions seem to apply to the population of *C. xami* in the Pedregal de San Angel.

This study suggest that there was relatively high variance in copulation success between males. First, although most males were not observed copulating, some males copulated up to four times, including one (probably two) male that was observed copulating two times in a day. Second, one third of the copulations observed (nine out of 27) were performed only by three males. Although we were not able to obtain estimates of male lifetime reproductive success, these results, together with information indicating that females tend to be monandrous (in a sample of 28 field collected

females, 78.6 % had only one or no spermatophore in their *corpus bursae*, and the mean number of spermatophores found in non-virgin females was 1.37 ± 0.6 [Cordero & Jiménez, unpublished data]), suggests that there is high variance in male fitness and, therefore, that the opportunity for sexual selection in males is high.

Sexual selection may be acting in favor of an increase in male size (wing length) and longevity if the mating advantage suggested by the wing length and longevity of the few males that mated more than once is real. Although the relationships between male phenotypic traits and copulation success still needs clarification, the information available on the mating behavior of *C. xami* permits some brief speculations about the possible advantages for big, long lived males. The advantage for long lived males may result from the correlation between male longevity and the length of territory tenure, since males defend mating territories probably during all their adult lifetime (Cordero & Soberón, 1990). There are two possible explanations for the effect of male size. First, larger males may obtain and maintain better territories as a result of a fighting advantage. The long and complicated aggressive interactions (Cordero & Soberón, 1990) and the observations of aggressive displacements of previous residents from territories (Cordero, 1997), suggest that territorial contests are not "arbitrarily" settled in *C. xami*, and that characteristics influencing fighting ability, such as body size or correlated traits, may be the target of intrasexual selection. Second, females may prefer bigger males as mates. For a female *C. xami* it could be advantageous to discriminate in favor of bigger males because bigger males transfer bigger ejaculates, and ejaculates could be nuptial gifts (Cordero, unpublished data). Although we have no direct data on female choice, we have observed many unsuccessful courtships and even some copulations interrupted, probably before sperm transfer, which could have been result of female discrimination (Cordero, 1993; Cordero and Jiménez, unpublished data).

Number of copulations and territory characteristics

The substantial variation observed between territories in frequency of occupation, numbers of males and number of copulations (Table 2) suggests that territories of *C. xami* vary in quality. However, none of the territory variables measured affected the frequency of occupation or the number of copulations. In species with non-resource based territoriality, such as *C. xami*, it has been proposed that female "rules of movement" may be responsible for territory location and quality (Bradbury, 1985; Cordero & Soberón, 1990; Rutowski, 1991; Wickman *et al.*, 1995). Although female movement in *C. xami* has not been studied, observations suggest that territories are located in the confluence of natural or manmade trails, which are probably used by females for their displacement through the habitat (Cordero & Soberón, 1990). Therefore, differences in territory

quality may result from the specific location of territories with respect to areas of high probability of female transit, which may vary with time (as suggested by the lack of between-years correlations in occupation frequency and number of copulations in territories).

Male copulation success in other butterflies

Field estimates of male copulation success are scant. In Table 3 we summarize the information on the mating behavior of butterflies in which male copulation success and/or phenotypic traits associated with male copulation success have been studied in the field. Unfortunately, a formal quantitative comparison is prevented by the different methods employed to estimate copulation success (Table 3).

The copulation success of males has been shown to be affected by a variety of factors, such as weather conditions (Davies, 1978), adult emergence date (Elgar & Pierce, 1988), body size (Deinert *et al.*, 1994), longevity (Elgar & Pierce, 1988), mating experience (Suzuki & Matsumoto, 1992) and female mate choice (Rutowski, 1981-83). A positive effect of body size on male mating frequency has been found in three (four if the case of *C. xami* is true) species (Table 3): *Jalmenus evagoras* (Elgar & Pierce, 1988), *Heliconius hewitsoni* (Deinert *et al.*, 1994) and *Pieris napi* (Wiklund & Kaitala, 1995). The first two species exhibit pupal mating, a mating system that involve direct male-male competition (the same as lek polygyny, the mating system of *C. xami*), whereas the last species exhibit scramble competition polygyny, a mating system with indirect male-male competition. These data suggest that body size (or correlated traits) confer advantages in different male competition settings. However, in other two butterfly species (Table 3), *Coenonympha pamphilus* (Wickman, 1987) and *Atrophaneura alcinous* (Susuki & Matsumoto, 1992), showing lek polygyny and scramble competition polygyny, respectively, no relation between male size and mating success was found. These observations are in accord with other studies that indicate that male size and resource holding power are correlated in some butterfly species (Rosenberg & Enquist, 1991) but not in others (Alcock, 1994). In the three species in which it has been investigated, a correlation between male longevity and number of copulations achieved has been found (*Atrophaneura alcinous* and *J. evagoras*) or is suspected (*C. xami*). These species have different mating systems, pertain to different families, and have very different adult body sizes (*Atrophaneura alcinous* is much bigger than the two lycaenids).

Appendix

A prospective comparison of all marked males observed copulating (CM) during the first and second parts of both study periods, with all marked males not observed copulating (NCM) supports

this suggestion. CM had longer wing length (CM: 1.71 ± .1 cm, median = 1.72, max. - min.: 1.51 - 1.89, n = 14; NCM: 1.64 ± .1, median = 1.65, max. - min.: 1.36 - 1.88, n = 131; Mann-Whitney U = 569, P = .0197) and lived longer (CM: 10.1 ± 5.6 days, median = 9, max. - min.: 2 - 20, n = 18; NCM: 4.1 ± 5, median = 2, max. - min.: 1 - 28, n = 138; U = 397, P = .000001) than NCM, but the degree of wing wear at the moment of being marked was not different (CM: 1.4 ± .7, median = 1, max. - min.: 1 - 3, n = 15; NCM: 1.6 ± .7, median = 1, max. - min.: 1 - 3, n = 132; U = 845.5, P = .29). The mean number of copulations of the CM was 1.3 ± .8 (median = 1, max. - min.: 1 - 4, n = 18). We stress that these comparisons are based in data obtained from a heterogeneus, non-random, and probably biased sampling of males.

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Table 1. Distribution of copulations observed in different territories in each part of the study periods of 1989 and 1990. T: territory. TNTO: Total number of territories observed.

1989			1990		
	Number of copulations:			Number of copulations:	
T	First part	Second part	T	First part	Second part
3-4N	1	3[1]	3-4N	0	1
IV	0	2	IV	1	1*
Pnm	1	*	Pnm	1	0
d	2	3[2]	a	1	1
Id	3	0	8-9	1	1
V	1	0	A	0	1
‡	1	*	ICh2	0	1
TNTO	25	14	TNTO	19	11

[1] Territory in which a male copulated twice in a day.
[2] Territory in which a male probably copulated twice in a day.
* Territory in which no focal observations were made.

Table 2. Characteristics of territories of male *Callophrys xami* butterflies. Min.: minimum value; Max.: maximum value.

	1989			1990		
	Median	Min.	Max.	Median	Min.	Max.
Maximum length (m)	3.5[b]	2.32	7.1	4.2[d]	2.32	7.1
"Cross" length (m)	2.8[b]	1.35	4.85	2.7[d]	1.5	4.85
Area (m^2)	9[b]	4.54	33.61	8.7[e]	4.56	33.61
Number of males	4[c]	1	12	2[b]	0	7
Frequency of occupation[a]	0.46[c]	0.08	1	0.59[b]	0	0.94
Number of copulations	0[b]	0	5	1[f]	0	2

[a] Number of days the territory was occupied by a territorial male / number of days the territory was censused during the first and second parts of the study.
[b] N = 19. [c] N = 25. [d] N = 16. [e] N = 18. [f] N = 12.

FIGURE CAPTION

Fig. 1. Temporal patterns of copulation by males of the butterfly *Callophrys xami*. (a) Weekly distribution of the number of copulations observed. (b) Number of copulations observed at different times of the day. The time at which "copulations in course" began in not known.

Table 3. Mating behavior of butterflies in which male copulation success and/or phenoty- pic traits associated to male copulation success have been studied in the field[a]

Species	MS	♂ MF	♂ TCMF	♀ MF[b]	♀ TCMF	Reference
Papilionidae						
Papilio polyxenes	LT	.13 ± .49 (0-3)[c]	ST[C]	1.3 ± .54 (0-3)	WW[C]	Lederhouse (1981, 1982)
Atrophaneura alcinous	SC	.43 ± 1.31[d] (0-5)	ED[C], L[C], ME[C], WL[NC]	1.0	--	Suzuki & Matsumoto (1992)
Nymphalidae						
Coenonympha pamphilus	LT	.019[e] .083	TB[C], WL[NC]	.97 ± .05 (0-3)	--	Wickman (1987)
Danaus plexippus[f]	SC	2.98 ± 2.65 (0-11)[g]	--	3.50 ± 1.22 (1-6)[h]	--	Oberhauser (1989)
Euphydryas editha	SC	--[i]	ED[NC]	1.27 + .46 (1-2)[j]	--	Baughmann (1991)
Heliconius hewitsoni	PM	--	BL[C], WL[C], WW[NC]	1.0	--	Deinert *et al.* (1994)
Pieridae						
C. philodice eriphyle	SC	--	G[C]	1.21 (0-3)[k]	WW[PC]	Watt *et al.* (1986)
Colias eurytheme	SC	--	G[C]	(3)[l]	--	Watt *et al.* (1986)
Pieris napi[m]	SC	--	PW[C]	2.03 ± .11 (1-5)[n]	--	Wiklund & Kaitala (1995)
Lycaenidae						
Jalmenus evagoras	PM	.97 ± 2.56 (0-7)[g]	L[C], ED[C], WL[C]	1.0	--	Elgar & Pierce (1988)
Callophrys xami	LT	.0027 (0-4) .0029 (0-2)[o]	WL[PC], L[PC]	1.37 ± .60 (0-3)	WL[NC], WW[NC]	This study and Cordero (1998)

Footnotes to Table 3.

[a] MS: male mating system according to the classification of Thornhill & Alcock (1983). LT: lek polygyny. PM: pupal mating. SC: scramble competition polygyny. MF: mating frequency. TCMF: traits correlated (^{C}), possibly correlated (^{PC}) or not correlated (^{NC}) with MF. BL: body length. ED: adult emergence date. G: genotype. L: longevity. ME: mating experience. PW: pupal weight. ST: MF depends on specific territory. TB: territorial behavior (in species with territorial and non-territorial males). WL: wing length. WW: wing wear.

[b] Mean ± SD (range) of spermatophore number of mated females.

[c] Mean ± SD (range) for the second brood of 1975.

[d] Mean lifetime number of copulations ± SE (range).

[e] Number of copulations/ male/ census. Upper figure: non-territorial males; lower figure: territorial males.

[f] Studied in a big outdoors mating cage.

[g] Mean lifetime number of copulations ± SD (range).

[h] Pliske (1973), cited in Eberhard (1985), estimated a mean number of spermatophores (maximum) = 2.13 (8).

[i] Relative number of matings estimated by marking male genitalia with powdered fluorescent dye.

[j] Data from Ehrlich & Ehrlich (1978), cited in Drummond (1984).

[k] Mean (range) (Drummond, 1984).

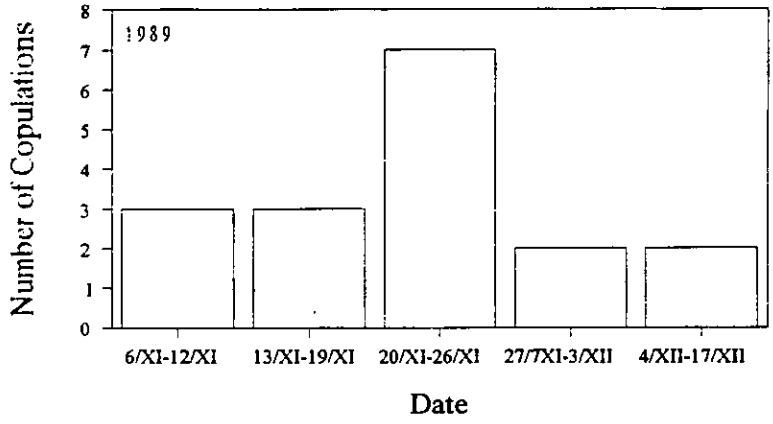
[l] Maximum number of spermatophores (Gwynne, 1984).

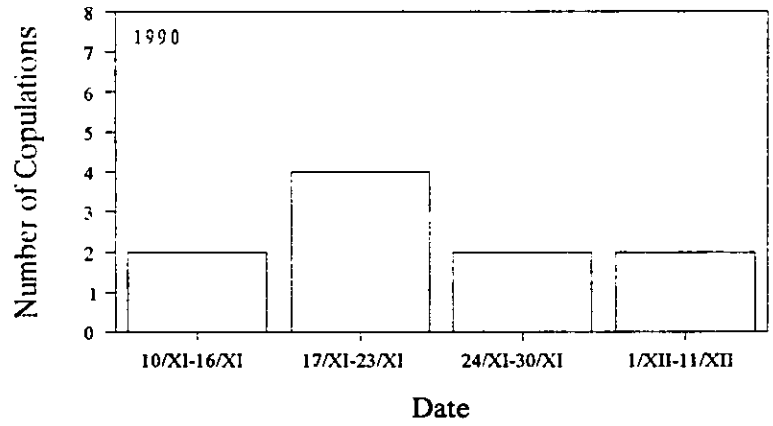
[m] Butterflies were raised in captivity and released in the field.

[n] Mean ± SE (range).

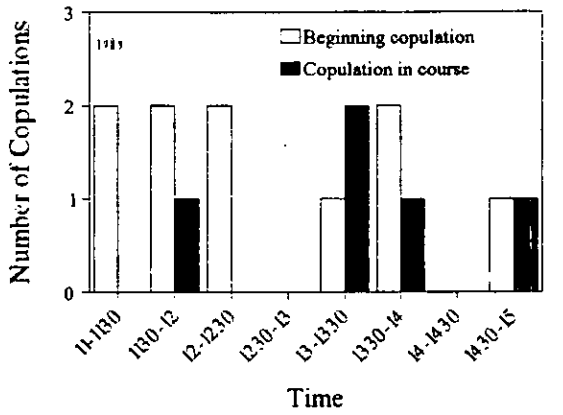
[o] Number of copulations / focal male / hour of focal observation (minimum number of copulations per male - maximum number of copulations per male). Upper figure: 1989 study period; lower figure: 1990 study period.

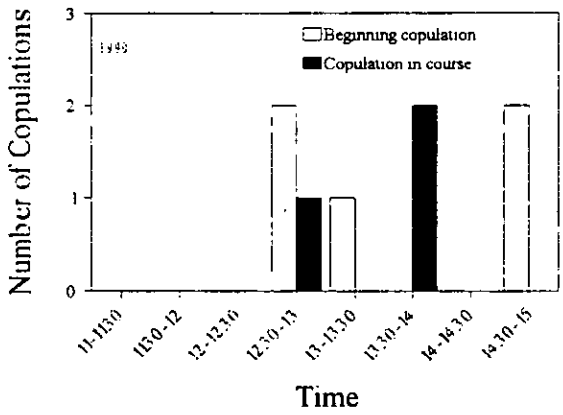
(a)


1989
Number of Copulations
0
1
2
3
4
5
6
7
8
6/XI-12/XI
13/XI-19/XI
20/XI-26/XI
27/7XI-3/XII
4/XII-17/XII
Date


1990
Number of Copulations
0
1
2
3
4
5
6
7
8
10/XI-16/XI
17/XI-23/XI
24/XI-30/XI
1/XII-11/XII
Date

(b)


Number of Copulations
3
2
1
0
Beginning copulation
Copulation in course
11-11:30
11:30-12
12-12:30
12:30-13
13-13:30
13:30-14
14-14:30
14:30-15
Time


Number of Copulations
3
2
1
0
Beginning copulation
Copulation in course
11-11:30
11:30-12
12-12:30
12:30-13
13-13:30
13:30-14
14-14:30
14:30-15
Time

APÉNDICE 3.1

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WHY DO SOME MALE *CALLOPHRYS XAMI* (LYCAENIDAE) SHIFT THEIR TERRITORIES?

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ABSTRACT. In a Mexican population of the butterfly *Callophrys xami* at least 13% of the males defended two or more territories sequentially. There were two observed causes of territory shifts by males: aggressive displacement from their territories by other males (n = 2), and spontaneous shift to a different territory (n = 3); however, in 26 territory shifts the causes were not determined. Evidence suggests that territories were in short supply during the study and, therefore, more territory shifts may have been the result of aggressive displacement. The spontaneous shifts suggest that some males may move in search of a better territory after occupying one of low quality.

Additional key words: behavioral variation, male competition, territoriality.

In several butterfly species, males defend territories that are employed exclusively for male display, mate location and courtship (Rutowski 1991). Variation in territorial behavior in butterflies has been studied mainly in the context of alternative mate location strategies within a species (Davies 1978, Dennis 1982, Wickman 1985, 1988, Alcock & O'Neill 1986, Dennis & Williams 1987, Alcock 1994), although some authors have also discussed the basis for differences between species in territorial vs. nonterritorial mating systems (Alcock 1985, Dennis & Shreeve 1988, Cordero & Soberón 1990, Wickman 1992).

Although intraspecific variation in the number of territories sequentially defended by male butterflies has been documented (Alcock 1985, Knapton 1985, Alcock & O'Neill 1986), it has been specifically discussed in only one study (Robbins 1978). In some species, males spontaneously shift territory as a consequence of their normal migratory movements (Baker 1972). In non-migratory species there are at least two hypotheses to explain territory shifts; these hypotheses and some of their predictions are summarized in Table 1.

In this paper, variation in the number of territories sequentially occupied by individual males of *Callophrys xami* Reakirt (Lycaenidae) is reported, and some of its possible causes and consequences are explored.

MATERIALS AND METHODS

The study was conducted in a 146.8 ha ecological preserve within the main campus of the Universidad Nacional Autónoma de México, in Mexico City. This area is part of the Pedregal de San Angel, and is characterized by volcanic soil, rough topography, markedly seasonal rainfall, and xerophytic shrubby vegetation.

Callophrys xami is a multivoltine butterfly that in the Pedregal de San

TABLE 1. Two hypotheses to explain why males of non-migrant butterfly species might shift territories that they already occupy, and some predictions of these hypotheses.

Hypothesis A: Males shift territories as a result of being aggressively displaced from their previous territories by intruder males.
Prediction A1: Aggressive displacement of territorial males should be observable.
Prediction A2: Successful territory holders (monoterritorial males) should be males with high resource holding power and, therefore, they should tend to be larger, more agile or more experienced than less successful territory holders (polyterritorial males).
Prediction A3: Polyterritorial males, as a result of their displacement from high quality territories, should have a lower copulation success than monoterritorial males.
Prediction A4: The incidence of territory shifts as a result of aggressive displacement should be higher when male density and, therefore, competition for territories is high.
Hypothesis B: Males shift territories because they evaluate their current territories and voluntarily move in search of better ones.
Prediction B1: Voluntary (spontaneous) territory shifts should be observable in territorial males.
Prediction B2: Polyterritorial males should shift, on average, towards territories of higher quality (i.e., those with higher copulation rates).
Prediction B3: Polyterritorial males, as a result of having spent some time in territories of poor quality, should have a lower copulation success than monoterritorial males.
Prediction B4: Male density should be inversely correlated to the probability of finding an unoccupied territory of high quality, and therefore the cost of voluntary territory shift should be lower when density is low, and the probability of changing territory should be higher.

Angel can be found at varying densities throughout the year (Soberón et al. 1988). The population reaches peak density from October to January, although it is never abundant (Soberón et al. 1988). The main larval food plant is the perennial *Echeveria gibbiflora* D. C. (Crassulaceae), which is abundant in the area (Soberón et al. 1988). Males are territorial and defend areas with well defined topographical limits, located beside or on natural or manmade trails; these areas lack concentrations of receptive females and larval or adult food resources (Cordero & Soberón 1990). Males actively defend their territories by means of different types of aggressive flights, for an average of five h per day (between 1000 and 1500), and spend the rest of the time feeding and resting outside territories (Cordero & Soberón 1990). Territories are occupied year after year and function as mate location and courtship stations (Cordero & Soberón 1990, Cordero unpubl. data). Other details of courtship behavior are given in Cordero (1993).

A total of 159 territorial males was captured, individually marked on the wings with indelible felt-tip pens and their right forewing length measured with a caliper through the mesh of the net. Individuals were assigned to one of three wing-wear categories: 1 = similar to a recently emerged adult

(wings mostly green); 3 = very worn male (wings mostly brown with worn margins); and 2 = individuals intermediate between 1 and 3.

Observations were made between 1 November and 20 December in 1989, and between 10 November and 6 December in 1990. The number of territories observed was 25 in 1989 and 19 in 1990; the number of days a territory was visited varied between 25 and 38 in 1989 and between 14 and 24 in 1990. Observations were made in two ways: by walking along transects joining groups of territories at least two times per day, on 31 days in 1989 and 11 in 1990, and observing each territory for a brief time; and by continuous observations through the daily territorial period in a group of occupied territories, during nine days in 1989 and 13 days in 1990.

RESULTS

Most marked males were observed defending only one territory (86/99 males in 1989 and 52/60 in 1990; hereafter, monoterritorial males). Twenty-one males were observed sequentially occupying more than one territory (hereafter, polyterritorial males); these males represented 13.2% of all marked males. Thirteen males occupied two territories, six males occupied three, and two males occupied four. Therefore, a total of 31 territory shifts was detected; however, the exact date of shifts was only determinable for 26 events. The median number of days polyterritorial males occupied each territory was 1 (1.5 in fourth territory, n = 2); however, the range varied from <1 day to 14 days in their first territory (n = 20), to 1 to 2 days in their fourth territory (n = 2) (Table 2). Only one of the 55 marked males observed more than one day in 1983–1985 occupied more than one territory, probably as a result of aggressive displacement (Cordero & Soberón 1990). Territories seem to be in short supply for the males of this butterfly, at least during peaks of male density. In 14 of 17 cases, the site that a male had left was occupied by a different male the same day or the day after.

Direct support for Prediction A1 (Table 1) was provided by two cases in 1989, in which the cause of territory shift clearly was aggressive displacement of the polyterritorial male by an intruder (for description of aggressive interactions see Cordero & Soberón 1990). Two other cases in 1989 probably involved aggressive displacement and resulted in a territory shift. In the first case an aggressive interaction was observed after which a male not previously in the territory began or continued defending it; less than an hour later, the male that had been defending this territory for the three previous days was observed defending a new territory. In the second case, a male was observed for over an hour defending a territory, and then suddenly a different male was in residence; the first male was found defending a different territory 4.5 hours later.

One way of testing Prediction A2 is by comparing the wing length (a measure of size and, possibly, resource holding power) and wing wear (a possible measure of age and experience) of males that are polyterritorial as a result of aggressive displacement with that of monoterritorial males; however, the small number of aggressive displacements observed in this study prevents statistical analysis. In one of three observations of aggressive displacement, the winning male was bigger (1.65 vs. 1.48 cm) and older (2 vs. 1), and in another it was smaller (1.55 vs. 1.62 cm) and younger (1 vs. 3) than the displaced male; data for the third case were not known. Of the two cases of probable aggressive displacement observed in 1989, the winning male was bigger in one (1.72 vs. 1.69 cm) and smaller (1.49 vs. 1.63 cm) in the other. These scant observations neither support nor contradict Prediction A2.

Since virtually all males observed during the course of this and previous studies (since 1983) were territorial or were apparently trying to get a territory, the proportion of territories occupied in a given day was used as a measure of male density (Fig. 1). In 1989, the proportion of territories occupied decreased through the study period ($r_S = -0.887$, $P < 0.001$, $n = 35$), but in 1990 no significant differences were observed in the proportion of territories occupied ($r_S = -0.305$, $P > 0.05$, $n = 17$). Territory shifts were observed throughout the study periods in both years (Fig. 1). Contrary to Prediction A4, aggressive displacement was observed or suspected at both high and low densities in 1989.

Regarding Hypothesis B (Table 1), we observed three cases of spontaneous territory shifts (Prediction B1). In 1989, territorial male *c* moved from territory 3–4S to the contiguous territory 3–4N while inspecting a heterospecific butterfly, and perched in 3–4N without being detected by male *b* (who had been defending 3–4N since the previous day); after two minutes *c* aggressively displaced *b* and defended this "new" territory for the rest of that day as well as the next. No copulations were observed in territory 3–4S, in any of the eight days it was occupied by a male; four copulations were observed in territory 3–4N in the 23 days it was occupied by a male. Also in 1989, territorial male *m* moved spontaneously from territory V to territory IV (about 15 m away) aggressively displaced the previous resident and defended territory IV for one hour, returning afterwards to territory V. Male *m* occupied territory V four more days and later defended territory IV again on two days; this male was observed defending two other territories before defending territory V for the first time. One copulation was observed in the 31 days territory V was occupied; two copulations were observed in the 32 days territory IV was occupied. Finally, in 1990, territorial male *30a* moved from territory E to territory F′ (which was unoccupied), about 25 meters away, and defended it for one day. This male was observed again defending territory

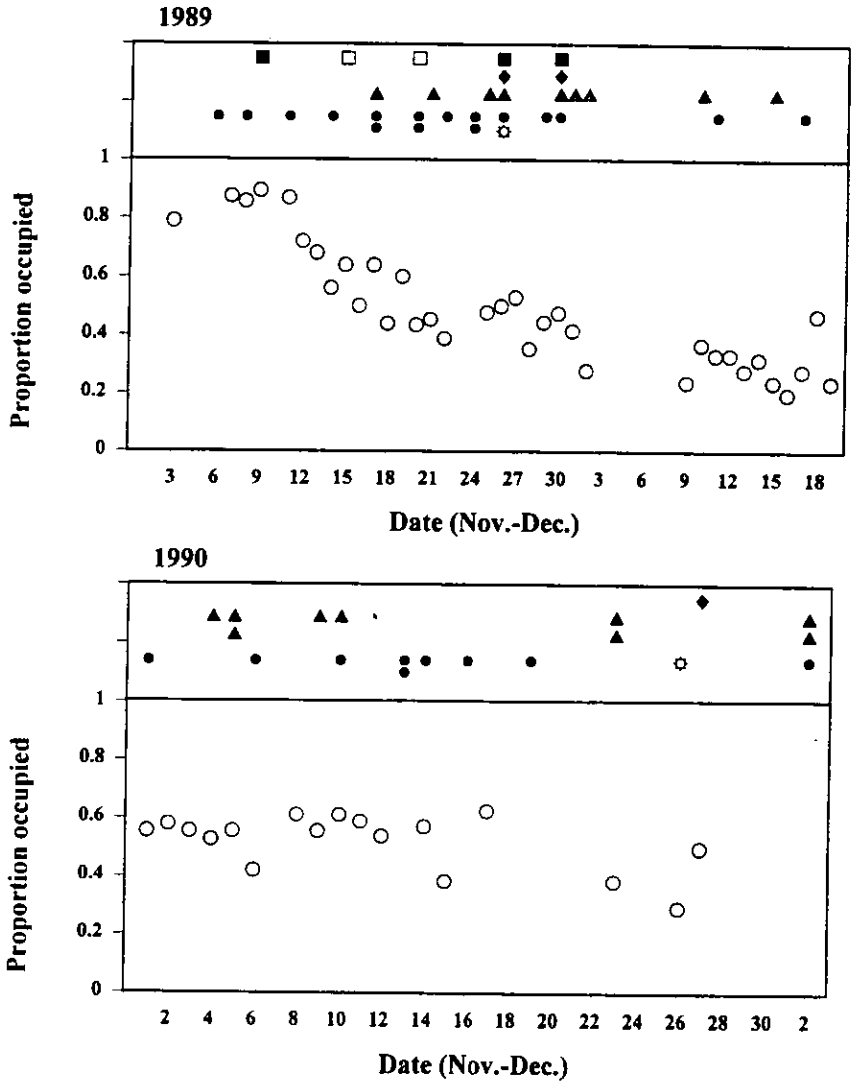

FIG. 1. Proportion of territories occupied by males, territory shifts and matings observed during the study periods of 1989 and 1990. In 1989, only those days in which 17 or more territories were surveyed are included; in 1990, only those in which 13 or more territories were surveyed are included. Key: *solid squares*: observed aggressive displacements; *empty squares*: suspected aggressive displacements; *diamonds*: spontaneous territory shifts; *triangles*: territory shifts due to unknown causes; *solid circles*: matings by monoterritorial males; *sunbursts*: matings by polyterritorial males; *empty circles*: proportion of territories occupied

TABLE 2. Summary of male characteristics. WL: wing length (cm). WW: wing wear category. L: longevity (days). T_1, T_2, T_3 and T_4 are, respectively, the number of days polyterritorial males defended their first, second, third and fourth territories. Cop: number of copulations.

	WL	WW	L	T1	T2	T3	T4	Cop
All males:								
mean ± SD	1.64 ± 0.1	1.56 ± 0.73	4.8 ± 5.4	2.4 ± 3	2 ± 2	2.7 ± 2.5	1.5 ± 0.7	0.15 ± 0.5
median	1.65	1	2	1	1	1	1.5	0
range	1.36–1.89	1–3	1–28	<1–14	<1–9	1–7	1–2	0–4
N	145	147	156	20	20	8	2	159
Males shifting via aggressive displacement:								
g	1.62	3	7	4	<1	—	—	1
y	1.76	1	1	<1	1	—	—	0
Males shifting spontaneously:								
c	1.65	2	2	<1	2	—	—	0
m	1.55	1	17	1	1	5	2	0
30a	1.59	2	7	4	1	1	—	0

E on two days, four days after defending territory F'; afterwards he occupied territory A for one day. No copulations were observed in any of the six and four days territories E and F', respectively, were occupied. The fact that two spontaneous shifts were toward territories which apparently had higher copulation rates is in agreement with Prediction B2. The behavior of the last two males suggest sampling of territories, an idea implicit in Hypothesis B.

In agreement with Prediction B4, the two spontaneous territory shifts witnessed in 1989 occurred when male density was low (Fig. 1). In both years, spontaneous shifts were observed in the second half of the study period and after most of the copulations were observed (Fig. 1), suggesting that a decreasing encounter rate with females may be used by males as a cue for voluntarily leaving the territory.

Only two polyterritorial males were observed copulating, both in their second territory; these males were observed defending two territories and the causes of their territory shifts are unknown (one of these males was aggressively displaced from his second territory a few minutes after mating finished, and returned to his first territory).

DISCUSSION

In *Callophrys xami* some males shift territory because they are aggressively displaced from their territories by other males, or because they move spontaneously to a different territory. Given that the cause of 84% of the territory shifts detected was unknown, the relative importance of each of these causes cannot be determined.

The direct observations of aggressive displacement indicate that competition for territories is an important cause of shifts between territories. Rapid re-occupation of abandoned territories also suggests intense competition for territories. Competition happens in spite of the availability of unoccupied territories (Fig. 1), suggesting that competition varies in space at a local scale, probably in response to limited male movement and differences in territory quality, and, temporarily, due to local changes in male density and territory quality.

The existence of spontaneous territory shifts indicates that factors other than aggressiveness are responsible for some of the shifts. One possibility (Hypothesis B) is that males shift towards territories of higher quality (i.e., where mating rates are higher). We have insufficient data to test this possibility; however, the two observed copulations of polyterritorial males occurred in their second territories. Furthermore, two spontaneous shifts were towards territories where copulation rates seemed to be higher.

If the quality of prospective territories is difficult to determine for a male butterfly, males may simply tend to move to a different territory in

the hope of finding a better one. The time spent in a territory that is eventually abandoned may be necessary to determine its low quality or it may reflect a territory quality changing (decreasing) with time. Under these conditions we would expect to observe some cases of males shifting territory and returning to the previous one after some time, as was observed in two cases. Under this scenario, a smaller, and therefore more difficult to detect, difference between the average quality of pairs of territories sequentially occupied by males changing spontaneously might be expected. Intensive studies are needed to analyze the possible effects of territory characteristics on territory shifts.

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CAPÍTULO 4

A SURVIVAL COST OF MATING IN MALES OF A POLYGYNOUS BUTTERFLY

ABSTRACT

Despite the high investement made by male butterflies in the production of ejaculates, male survival costs of mating have not been conclusively demonstrated. I present the results of three laboratory experiments designed to measure the effect of mating on male lifespan in the polygynous butterfly *Callophrys xami* (Lycaenidae). In experiment 1, the lifespan of virgin males was not significantly different from that of multiply-mated males. To approach more natural, resource-limited conditions, in experiment 2, I used small males resulting from the experimental food limitation of last instar larvae; small virgin males lived significantly more days than small multiply-mated males. Since ecological costs of mating (disease transmission, predation risk, etc.) were excluded in these experiments, diminished male lifespan was a product of physiological costs of sexual interactions, which include costs of precopulatory courtship, copulation, copulatory courtship, and ejaculate production. To evaluate the effect of the rates of mating and of spermatophore production on male survival, in experiment 3, I compared the longevity of pairs of males that produced a similar amount of spermatophore, but that mated different numbers of times, mated at different rates, and that produced spermatophores at different rates; the lifespan of "low mating rate" males was not different from that of "high mating rate" males. My results suggest that the cost of ejaculate production is probably one important cause of lifespan reduction, however, the role of other possible physiological costs (costs of precopulatory courtship, copulation, and copulatory courtship—although no obvious copulatory courtship has been observed in this species) in lifespan reduction is still unknown.

INTRODUCTION

As a result of sexual differences in parental investment, in most animal species, the function relating fitness to number of copulations increases at a higher rate for males than for females (Trivers 1972; Thornhill & Alcock 1983; Brown et al. 1997). In general, the number of copulations that maximizes female fitness is smaller than that maximizing male fitness, since female reproduction is more limited by time and resources than by access to mates. In fact, in several species, female fitness decreases as the number of copulations increases as a result of, for example, an increased death risk when copulating or because of time budget constraints (Thornhill & Alcock 1983; Brown et al. 1997). In contrast, if mating is not costly to males, we expect male fitness to increase with the number of successful matings achived,

since, in general, male reproductive success is limited by access to females' eggs (Trivers 1972; Thornhill & Alcock 1983; Brown et al. 1997). However, probably in most species, during copulations males incur costs that may result in a fitness function with an intermediate maximum (Daly 1978; Dewsbury 1982; Drummond 1984; Oberhauser 1989; Hayashi 1993; Bissoondath & Wiklund 1995; Cordts & Partridge 1996). Mating is costly if produces an increase in mortality or a decrease in future reproductive success (Partridge 1987; Lessells 1991). Moreover, mating costs paid by an individual male are affected by its own condition and that of its mates.Therefore, male fitness functions may vary depending on individual conditions and ecological factors.

Male butterflies are good subjects for studying mating costs because they invest a significant amount of limiting resources in the production of each ejaculate (Rutowski et al. 1983; Svärd & Wiklund 1989; Boggs 1990; Bissoondath & Wiklund 1995). Furthermore, a considerable amount of time is invested in each copulation, and time seems to be a limiting resource for males, since they have relatively short lifespans (Scott 1986), and their flight activity requires good weather conditions (*e.g.* Wickman 1985), available only a few hours per day. However, previous experimental studies with butterflies have failed to detect a direct negative effect of mating on male lifespan (Svärd 1985; Oberhauser 1989). Indirect evidence of a survival cost was found in the Pierid *Tatochila sterodice* by Shapiro (1982): mated males refrigerated at 2° C had significantly shorter lifespans than refrigerated virgin males.

In this paper I report the results of three experiments designed to measure the effect of mating on adult male lifespan in the polygynous butterfly *Callophrys xami* Reakirt (Lycaenidae). Comparisons of the lifespan of virgin *vs.* multiply mated males, and of males mating at different rates, show that, at least under some circumstances, mating is costly for males of this butterfly. *C. xami* is particularly interesting for this type of study, since spermatophore production has been shown to be physiologically costly, judging from the inability of small males and recently mated males to produce big ejaculates (Cordero 1998), and from the increased investment of time in a copulation made by recently mated males (Cordero 1993, 1998).

GENERAL METHODS

Species Studied

Male *C. xami* defend territories that function as mating stations (Cordero & Soberón 1990; Cordero 1993). Although copulations are rarely observed in most males, some males mate more than

once (up to four times) (Cordero 1998). In a field sample of 28 females (Cordero 1998), 32.1% had no spermatophores (they were probably virgin), 21.4% had more than one spermatophore, and mated females ($N = 19$) had an average ±SD of 1.37 ± 0.6 spermatophores in their bursa (this is a measure of female butterfly mating frequency).

Rearing Methods

The butterflies used in these experiments were reared from eggs laid by females collected in the field or reared in the laboratory. Following the method described by Jiménez & Soberón (1988/89), the larvae were fed with fresh pieces of the perennial *Echeveria gibbiflora* (Crassulaceae), the main food plant in the study area (Soberón et al. 1988). Females were collected in the Pedregal de San Ángel ecological reserve of the Universidad Nacional Autónoma de México (UNAM), located in Mexico City. Butterflies were weighed on the day of emergence, after meconium had been discarded, and individually marked on the wings. Adult butterflies were fed *ad libitum* a 10% glucose solution every morning.

General Experimental Procedures

To investigate the effect of mating on adult male lifespan I performed three paired experiments. In each experiment, pairs of virgin males matched for body weight, date of adult emergence, and "genotype" (by using brothers or cousins) were selected. One male of each pair was randomly assigned to the mating treatment and the other was kept virgin for life (in experiment 3 males were randomly assigned to the "low mating rate" treatment or to the "high mating rate" treatment; see below). In the morning, after males and females were fed *ad libitum* a 10% glucose solution, paired males were introduced in contiguous "mating cages" (cylindrical cages made of mesh cloth, 58 cm high and 26 cm diameter; Jiménez & Soberón 1988/89) without food; the virgin male (experiments 1 and 2) was kept alone and virgin females were introduced, at predetermined intermating periods, in the cage of the male mating multiply (of both males, but not necessarily at the same moment, in experiment 3). The number of females introduced in cages varied between one and five depending on the availability of females in our laboratory cultures. Mating cages were hung outdoors in the Pedregal de San Angel a maximum of five hours (between 1000 and 1500 h, the time period at which wild males are active in the field, mainly defending mating territories; Cordero & Soberón 1990), during sunny days; most matings began within the first two hours of each trial. If the mating cages are not outdoors and if it is not sunny the butterflies remain motionless most of the time and never mate (Cordero and Jiménez, personal observation). When males and females are outdoors in mating cages they also remain motionless most of the time, however,

in some moments, males fly short distances and eventually contact females, usually standing on the net of the cage, in which case they began courting them by vigorously fluttering their wings in front of the females (Cordero and Jiménez, personal observation; for a complete description of courtship see Cordero 1993). Therefore, the number of opportunities for mating (= trials) of a given male is the number of occasions the male was with one or more females in a mating cage hung outdoors during a fine day. Five or more minutes after copulations began, mating couples, together with their paired males, were gently transported to the laboratory within their mating cages and hung below fluorescent lights; this does not affect the course of copulation and decreases the movement of unmated females, decreasing the probability of disturbance of the mating couple (Jiménez & Cordero, personal observation). After copulation finished, the mated female was frozen and the unmated females were moved to a different mating cage or to a wood cage (see Jiménez & Soberón 1988/89) with other females until being used for further mating trials; males were kept alone in the laboratory until their next mating trial.

The interval between successive copulations was not completely controlled since sometimes males did not mate in a given trial for unknown reasons (although, from some anecdotic observations, I suspect female rejection may be an important cause). This also resulted in variation between males in the number of opportunities for mating (in experiment 1, mated males had an average ±SD of 6.3 ± 3.2 [3 - 12] trials in 5.4 ± 3.2 [3 - 12] days, but mated only 3.3 ± .8 [2 - 5] times; in experiment 2, small mated males had 4.1 ± 1.7 [2 - 7] trials in equal number of days, but mated only a median of 3 [2 - 3] times; see results for number of trials in experiment 3). The cages of males of each pair were kept side by side and exposed to similar conditions during the period of days in which the multiply-mating males were being exposed to females. After this period, to save laboratory space, both males of each pair were kept in the same cage within the laboratory until one male died; subsequently, to keep conditions uniform, the surviving experimental male was kept until death with another male introduced in the cage one day after the death of the other experimental male. Virtually no male-male behavioural interactions occur in cages kept in the laboratory with more than one male (Cordero and Jiménez, personal observation).

After copulating, females were frozen. Spermatophores transferred during copulations were dissected out in saline solution and weighed in a CAHN electrobalance (model 4700 for experiment 1 and model C-31 for experiment 3; in experiment 2 we did not obtain spermatophore weight.). Since a male's second copulation of the day lasts several hours (Cordero 1993, 1998), when a female was a male's second mate of the day, she was refrigerated until the next morning, a few hours after the couple separated, and after at least part of the sperm had migrated to the spermatheca (Cordero, unpublished observations). In these cases the spermatheca was dissected out and its weight or, in the cases in which

spermatheca weight was not available, the mean weight of the sample of weighed spermathecae, added to spermatophore weight. We did not weigh empty spermathecae, but, since these structures are very small and thin, we consider their weight negligible.

Since sex ratios at emergence are heavily female-biased in our laboratory cultures (Jiménez and Cordero, unpublished data), it was difficult to obtain sufficient matched pairs of males. Therefore, not all pairs entered the experiments the same day (the pairs entered experiments 1, 2 and 3 over a period of 14, 12 and 26 days, respectively) and sample sizes were small. A few males did not mate more than once as required, further reducing sample sizes.

Statistics

Statistical analyses were made using STATISTICA for Windows 4.3, StatSoft Inc. All summary statistics are given as mean ± standard deviation (when pertinent, minimum - maximum values are given in parenthesis) for variables normally distributed, and as median and minimum - maximum values for variables whose distribution departed from normality.

EXPERIMENT 1: VIRGIN MALES *VS.* MULTIPLY MATED MALES

Methods

In this experiment, I compared the lifespan of virgin males with that of multiply mated males. Sample size was 16 pairs of males matched for body weight (Paired t test: $t = -1.57$, $P = .14$; Table 1), date of adult emergence (in 15 pairs both males emerged on the same day and in one pair the virgin male emerged one day after the mated male), and "genotype" (in 12 pairs males were brothers and in four pairs males were unrelated). Multiply mated males were mated for the first time when they were 2 ± 1.1 (1 - 5) days of age.

Results

Multiply mated males were mated an average of three times (Table 1) and produced a total amount of spermatophore equivalent to 2.7±.72 (1.35-3.93)% of their body weight at emergence. There was no significant difference in lifespan between virgin males and mated males ($P = .63$; Table 1).

EXPERIMENT 2: SMALL VIRGIN MALES *VS.* SMALL MULTIPLY MATED MALES

In the previous experiment, larvae were reared at a warm and relatively constant temperature, food was provided continuously and larvae ate *ad libitum*. Furthermore, adults were kept in captivity, free from natural enemies, and provided daily with food and water. In the laboratory males are motionless most of the time (Cordero and Jiménez, personal observation) and probably expend little energy. Therefore, the butterflies in experiment 1 were kept under highly favorable conditions which are rarely met in the wild, and this may underestimate the mating costs paid by males under field conditions. To approach more natural, resource-limited, conditions, in this experiment I used small males resulting from a resource limitation during the last larval instar.

Methods

In this experiment I compared the lifespan of small virgin males with that of small multiply mated males. I obtained small males by starving last instar larvae (4th), after a few days of feeding (last instar larvae normally fed for about seven days), to enforce early pupation (Parlange 1991). The females used in this experiment also came from the same resource-limited culture. "Small" males were significantly smaller than males that fed *ad libitum* as larvae. For example, the males of this experiment were significantly smaller than the males of experiment 1 (virgin males + multiply mated males: 52 ± 7 mg; small virgin males + small multiply mated males: 35 ± 5 mg; $t = 8.76$, $df = 46$, $P < .000001$). Sample size was eight pairs of males matched for body weight (Wilcoxon matched pairs test: $T = 5$, $P = .13$; Table 1), date of adult emergence (in five pairs both males emerged on the same day and in three pairs the small virgin male emerged one day after the small multiply mated male), and "genotype" (in two pairs the males were brothers, in five pairs males were cousins, and in one pair males were unrelated). Small multiply mated males were mated for the first time when they were 1.9 ± 1.4 (1 - 5) days of age.

Results

Three small multiply mated males mated twice and five mated three times. Thirty eight days after the last experimental mating, all five remaining individuals died accidentally due to dehydration, after they had already outlived their paired male (four individuals were small virgin males and one a small multiply mated male). Therefore, the accidental mortality affected the magnitude of the difference between males of these pairs, but not the sign of such differences. In agreement with the hypothesis that at least some aspects of mating are costly, small virgin males lived 19 more days (72% more) than small multiply mated males ($P = .025$; Table 1).

EXPERIMENT 3: COMPARISON OF MALES MATED AT DIFFERENT RATES

The experimental method employed in the previous experiments does not permit to estimate the relative importance of the different possible components of mating costs (costs of precopulatory courtship, copulation, copulatory courtship, and ejaculate production; see discussion). As a first attempt to evaluate the importance of the rates of mating and of spermatophore production on male survival, in this experiment I compared the lifespan of pairs of multiply mated males that produced a similar total amount of spermatophore, but that mated different numbers of times, at different rates and that produced spermatophores at different rates.

Methods

In this experiment, I varied the number of matings, the mating rate, and the rate of spermatophore production, while keeping constant the total amount of spermatophore produced by both males. To do this I used the fact that spermatophore weight decreases in recently mated males, and then increases as time since last mating increases (Cordero 1988). By manipulating intermating interval, I was able to obtain matched pairs of males that produced similar total amounts of spermatophore, but mated different numbers of times at different rates and, therefore, had different rates of spermatophore production. In this experiment sample size was 12 pairs of males matched for body weight (Paired t test: $t = -.24$, $P = .81$; Table 1), date of adult emergence (in 10 pairs both males emerged on the same day and in two pairs the the "high mating rate" male emerged one day before the "low mating rate" male), and "genotype" (in eight pairs the "high mating rate" male and the "low mating rate" male were brothers and in four pairs they were unrelated). "Low mating rate" males were mated for the first time when they were 1.5 ± .7 days old (1 - 3), and "high mating rate" males were mated for the first time when they were 1.6 ± .5 days old (1 - 2) ($t = -.32$, $P = .75$). "Low mating rate" males had 4.9 ± 1.5 (3 - 8) mating trials and mated in 67 ± 27 (37.5 - 100)% of these opportunities, while "high mating rate" males had 5.75 ± 2 (4 - 10) mating trials and mated in 76 ± 25 (25 - 100)% of these opportunities; these differences were not significant (number of opportunities: $t = -1.36$, $P = .2$; percentage of opportunities resulting in mating: $t = -1.2$, $P = .25$).

Results

As expected from the experimental design: (a) there was no significant difference in the total amount of spermatophore produced by both types of males (Paired t test: $t = -.81$, $P = .43$; Table 1); (b)

"high mating rate" males mated one more time that "low mating rate" males (Wilcoxon matched pairs test: $T = 0$, $P = .003$; Table 1); (c) "high mating rate" males had twice the mating rate (= total number of copulations performed by the male / age of the male when mating for the last time) of "low mating rate" males ("low mating rate" males: .54 ± .13 copulations / d; "high mating rate" males: 1.1 ± .4 copulations / d; $t = -5.1$, $P = .0003$); and (d) "high mating rate" males had almost twice the rate of spermatophore production (= total spermatophore produced by the male / age of the male when mating for the last time) than "low mating rate" males ("low mating rate" males: .2 ± .1 mg of spermatophore / d; "high mating rate" males: .38 ± .2 mg of spermatophore / d; $t = -3.6$, $P = .004$). "Low mating rate" males produced a total amount of spermatophore equivalent to 2.2 ± .77% of their body weight at emergence, and "high mating rate" males produced an equivalent of 2.4 ± .87% ($t = -.54$, $P = .6$). No significant difference in lifespan was observed between "low mating rate" males and "high mating rate" males ($P = .46$; Table 1).

DISCUSSION

Multiple mating is generally advantageous for male insects (Thornhill & Alcock 1983; Drummond 1984; Brown et al. 1997), although the relationship between fitness and number of copulations is not simple due to sperm competition, cryptic female choice, and mating costs (Daly 1978; Dewsbury 1982; Thornhill & Alcock 1983; Drummond 1984; Parker 1984; Hayashi 1993; Eberhard 1996; Brown et al. 1997). In this study I have shown that in *C. xami* there is a survival cost of multiple mating for small (resource-limited as a last instar larva) males. The survival costs observed in this laboratory study are probably experienced by some males under natural conditions, since in the field some males mate up to four times (Cordero 1998), and some males seem to be as small as the resource-limited males used in experiment 2 (as judged from wing length measurments; personal observations). I think that the probability that the decrease in longevity of the mated males of this experiment is due, at least partially, to a cost of other type of male-female social interaction, such as competition for food or space, is low since males and females were fed *ad libitum* before the mating trial, there was not food in the mating cages during the trials and males and females were kept together only during the trial; furthermore, during mating trials males and females remained motionless most of the time, sometimes close to each other (Cordero and Jiménez, personal observation). However, sometimes females flew a brief distance and alighted on or very close to the male, disturbing him and making him fly (Cordero and Jiménez, personal observation).

Since in my experiments mating costs of ecological origin (disease transmission, predation risk,

etc.; Partridge 1987; Cordts & Partridge 1996) were excluded, the negative effect on male lifespan was a product of physiological costs of mating (Partridge 1987; Cordts & Partridge 1996). Physiological costs of sexual interactions may include costs of precopulatory courtship, costs of copulation, costs of copulatory courtship (Eberhard 1996), and costs of ejaculate production (Svärd & Wiklund 1989; Bissoondath & Wiklund 1995). The experimental method employed in this study does not allow me to distinguish the relative importance of each of these physiological costs of mating. However, in *C. xami* the act of copulating does not seem to be very costly *per se* and there is no evidence of costly copulatory courtship (personal observations); furthermore, precopulatory courtship in mating cages is less complex (excludes one of the, possibly, most energetically expensive phases) and of shorter duration than in the field (Cordero 1993). Therefore, the costs of ejaculate production are, possibly, one important cause of the reduction in male lifespan observed in experiment 2.

The spermatophores produced by male Lepidoptera represent a relatively large material investment which may decrease the resources available for somatic maintenance and future reproduction (Boggs 1990; Bissoondath & Wiklund 1995). This effect is exacerbated in many butterfly species, including *C. xami*, that build ejaculates from materials obtained during the larval stage, since in these species adults are not able to replenish, through feeding, most of the resouces spent in an ejaculate. This reproductive cost could result in a reduction in the lifespan of mating males if the resouces spent in an ejaculate are, at least partially, the same than those used for maintenance and repair. The results of my experiments suggest that males with small reserves of resources to invest in courting, mating and transfering an ejaculate (experiment 2) pay higher costs of mating than males fed *ad libitum* when larvae (experiments 1 and 3).

An inverse relationship between male lifespan and lifetime mating frequency has been found in some moths (Drummond 1984), but not in a butterfly species. The survival cost of mating shown in my study is, to my knowledge, the first direct evidence of a trade-off between these fitness components in males of a butterfly. Indirect evidence of a survival cost was found in the Pierid *Tatochila sterodice* by Shapiro (1982): mated males refrigerated at 2° C had significantly shorter lifespans than refrigerated virgin males (the butterflies in this study were unfed as adults). However, as Drummond (1984) says, "[r]elating the rigors of cold storage in the laboratory to actual conditions in nature is difficult (although *Tatochila* is an alpine genus)". Although the sample is very small, it is interesting to note that, across species, a negative effect of mating on lifespan does not seem to be related to the relative investment RI made by virgin males in a spermatophore (RI = 100 × weight of first spermatophore / male body weight). Whereas in *C. xami* (RI = 1.3—1.6%, minimum—maximum averages from three different experiments;

Cordero 1998) I found a negative effect, the number of matings does not affects male lifespan in *Pararge aegeria* (RI = .37%; Svärd 1985) and *Danaus plexippus* (RI = 5—10%, minimum—maximum; Oberhauser 1988).

The lack of a significant difference in lifespan in experiment 1 could be the result of non-mating males also paying the costs of spermatophore production (Oberhauser 1989); this could happen if males accumulate materials necessary to produce spermatophores in their accessory glands and if these materials are not available for other functions (maintenance and repair, for example) even if males do not mate. However, the fact that several days after mating are necessary to build spermatophores of a size similar to that of the last spermatophore produced (Cordero 1998), and the long duration of second matings of the day (Cordero 1993, 1998) and of matings ocurring two days after a day in which a male mated twice (Cordero 1998), suggests that male *C. xami* do not store enough materials in their accessory glands to produce several spermatophores, but that they must translocate resources and synthesize substances to replenish their reserves. Other factor which could have been, at least partially, responsible of the lack of a significant difference in male lifespan in experiment 1 is that the butterflies were kept under highly favorable conditions which are rarely met in the wild, and these conditions may conceal the mating costs paid by males under field conditions.

Although the rates of mating and spermatophore production of "high mating rate" males were the double of those of "low mating rate" males, and although "high mating rate" males mated on average four times and "low mating rate" males three times, there were not differences in lifespan in experiment 3. These results may not indicate a lack of mating costs, but that the functions describing the (negative) effect of copulation rate, spermatophore production rate and number of copulations on male lifespan are not linear, and that even in "low mating rate" males these variables where high enough to result in a similar decreased lifespan (Table 1), or that even in "high mating rate" males these variables where small enough to produce no decrease in male lifespan. The fact that the butterflies used in experiment 3 were kept under highly favorable conditions which are rarely met in the wild, and that these conditions may conceal the mating costs paid by males under field conditions, could also contribute to the lack of a significant difference in male lifespan.

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Table I. Statistics of Experimental Males (a)

Experiment	Treatment	*n*	Body weight (mg)	Num. of copulations	Total spermatophore (mg)	Lifespan (d)	*P*
1	Virgin	16	51.1 ± 6.5 (41-67)			45.6 ± 22.6 (11-77)	.63 (b)
	Mated	16	53.1 ± 7.3 (42-69)	3.3 ± .8 (2-5)	1.43 ± .38 (.69-1.94)	48.8 ± 15.5 (20-81)	
2	Small virgin	8	36.5 (29-43)			45.5 (33-47)	.025 (c)
	Small mated	8	33 (27-42)	3 (2-3)		26.5 (13-47)	
3	"Low rate"	12	51.5 ± 6.1 (39-58)	3 ± .6 (2-4)	1.14 ± .45 (.39-1.85)	29 ± 11 (11-43)	.46 (d)
	"High rate"	12	51.7 ± 6.5 (36-60)	3.9 ± .8 (2-5)*	1.26 ± .56 (.74-2.7)	25.6 ± 14.7 (10-49)	

(a) Average ± SD or median value is given for variables with normal and not-normal distributions, respectively; minimum-maximum values are given in parenthesis.
(b) Paired t test, $t = -.49$.
(c) Wilcoxon matched pairs test, $T = 2$.
(d) Paired t test, $t = .76$.
* One HMR-male mated seven times, however, two of his copulations lasted only 2 and < 12 min, even when they were the second copulation of the day (these copulations normally last several hours; Cordero 1993).

CAPÍTULO 5

SPERMATOPHORE PRODUCTION IS PHYSIOLOGICALLY COSTLY FOR MALES OF A POLYGYNOUS BUTTERFLY

Abstract

1. In many insect species, males invest significant amounts of time and resources in the production of spermatophores and other ejaculate elements. Such reproductive investment may constrain the evolution of male mating strategies because it may be costly.

2. Previous studies with the polygynous butterfly *Callophrys xami* (Lepidoptera: Lycaenidae) have shown that at least in some conditions multiple mating decreases male lifespan, and suggest that the physiological cost of spermatophore production is one important cause of lifespan reduction. Three predictions from the hypothesis that spermatophore production is physiologically costly were experimentally tested.

3. In agreement with prediction 1, there was a direct correlation between the amount of "non-renewable" resources (resources available only for larvae) stored by the male (body weight was used as an estimate of the amount of these resources) and spermatophore weight.

4. In agreement with prediction 2, recently mated males produced smaller spermatophores than males that had longer intermating intervals.

5. In agreement with prediction 3, there was a time cost for males mating twice in one day, since second copulations of the day lasted several hours, whereas first copulations of the day lasted half an hour, and the males are, therefore, unable to mate successfully more than twice in a day.

6. The inability of small males and recently mated males to produce big ejaculates, and the increased investment of time in a copulation made by recently mated males, support the hypothesis that spermatophore production is physiologically costly for males.

Key words: Spermatophore, Male investment, Cost of reproduction, *Callophrys xami*, Lycaenidae.

Introduction

In many insect species males invest a substantial amount of time and resources in the production of spermatophores and other ejaculate elements. Such reproductive investment may be costly (*i. e.*, it may produce an increase in mortality or a decrease in future reproductive success; Partridge 1987; Lessells 1991; Cordts and Partridge 1996) and, therefore, act as a constraint for the evolution of male reproductive strategies. Male reproductive investment may also affect the relative intensity of sexual

selection acting on the sexes, since it may be used by females as a source of resources for their self-maintenance and protection, and for egg provisioning and protection (Thornhill and Alcock 1983; Dussourd *et al.* 1989; Brown *et al.* 1996).

During copulation, male butterflies produce an ejaculate that consists of accessory gland products plus one spermatophore, which contains sperm and more accessory gland substances (Hinton 1964; Drummond 1984). Accessory gland substances are made up of resources that are potentially valuable for females, such as proteins (Marshall 1985; Drummond 1984; Boggs 1990; Oberhauser 1992; Bissoondath and Wiklund 1995). Male investment of resources in ejaculates tends to be substantial and shows interspecific variation (Rutowski *et al.* 1983; Svard and Wiklund 1989; Bissoondath and Wiklund 1995, 1996). The production of large ejaculates may increase male fitness for different reasons. First, in several species the transfer of an ejaculate induces a decrease in female sexual receptivity and an increase in the rates of ovogenesis and oviposition, with the magnitude of both effects correlated with ejaculate size (Sugawara 1979; Drummond 1984; Oberhauser 1989; Svard and Wiklund 1989; Kaitala and Wiklund 1995; Bissoondath and Wiklund 1996); therefore, ejaculate size increases male fitness by decreasing the intensity of sperm competition and increasing the male's rate of offspring production. Second, ejaculate constituents could be used by females to increase the quantity and quality of the male's offspring (Rutowski *et al.* 1987; Dussourd et al. 1989; Boggs 1990). Third, ejaculate size may be used by females for their cryptic choice of males (LaMunyon and Eisner 1994; Wedell 1996).

Despite the advantages of producing big ejaculates, the substantial investment of resource and time made by male butterflies suggests that the physiological cost of ejaculate production may be one important cost of mating. From the hypothesis that ejaculate production is physiologically costly to males the three following predictions have been developed (Svard and Wiklund 1989; Bissoondath and Wiklund 1995, 1996). First, there should be a positive correlation between the amount of "non-renewable" resources (*i. e.* those resources available only in larval food, such as proteins; see Boggs [1990] for exceptions) stored by the male and ejaculate weight. Second, recently mated males should produce smaller ejaculates than males that had longer intermating intervals. This is because a longer intermating time provides the male with more time to translocate materials, and to synthesize and store ejaculate substances. Third, recently mated males should take longer to transfer an ejaculate than males that had longer intermating intervals. This is because more time is needed to translocate materials, and to synthesize and store the minimum quantity of substances needed for the production of a functional ejaculate. This is costly because time is a limiting resource for male butterflies as a result of the dependence of flight activity on specific ("fine") weather conditions (Wickman 1987), generally

available a few hours per day, and the relatively short lifespan of adults (an average of one week in temperate species according to Scott [1986]).

In this paper, we report the results of experiments designed to test the three above predictions about the physiological costs of spermatophore production with males of the butterfly *Callophrys xami* Reakirt (Lycaenidae). This butterfly is an adequate species to test these predictions because males exhibit a polygynous mating system (Cordero and Soberón 1990; Capítulos 1 and 3), and previous studies have shown that multiple mating decreases male lifespan under some circumstances and suggest that the physiological cost of ejaculate production could be one important cause of lifespan reduction (Capítulo 4). In these experiments we used male body weight as an estimate of the amount of non-renewable resources stored.

Materials and methods

We tested the predictions with three multiple mating experiments. In each experiment, males of different weights were mated to virgin females two or more times, at different intermating intervals, and the weight of the secretions transferred and the duration of copulations were measured. In each experiment we obtained the relationship between spermatophore weight and male weight at emergence, our measure of non-renewable resources (Prediction 1), spermatophore weight and time since last mating (Prediction 2), and duration of copulation and time since last mating (Prediction 3). In each of the three experiments we obtained data for all the three predictions and, therefore, each experiments could be considered a replicate. However, we analyzed the data of each experiment separately since the experiments were performed at different dates, with butterflies pertaining to different laboratory cultures, and with different sample sizes. Furthermore, in experiment I males were mated only twice, whereas in experiments II and III males were mated between two and five times. We repeated the same experiment three times because sample size in a given experiment was always small for logistic reasons. Sex ratios at emergence are heavily female-biased in our laboratory cultures (Jiménez and Cordero, unpublished data) and there is immature mortality, therefore the number of butterflies that we needed to rear to reach a "big" sample of males (say $N > 50$) in a relatively short period of time (2-4 weeks) was not possible to obtain. Furthermore, a few males did not mate more than once as required, further reducing sample sizes.

We reared butterflies from eggs laid by females collected in the field or reared in the laboratory, following Jiménez and Soberón (1988/1989). We collected females in the Pedregal de San Angel ecological reserve of the Universidad Nacional Autónoma de México (UNAM), in Mexico City. Butterflies were weighed on the day of emergence, after meconium had been discarded, and individually

marked on the wings. Adult butterflies were fed *ad libitum* a 10% sugar solution every morning. Before and between matings, males were individually kept in the laboratory in "mating cages" (cylindrical cages made of mesh cloth, 58 cm high and 26 cm diameter); virgin females were kept in groups in mating cages and wood cages (see Jiménez and Soberón 1988/89).

Matings were effected by introducing one male with one or more virgin females in a mating cage hung outdoors in the Pedregal de San Angel, between 1000 and 1500 h, during sunny days (we call this event a "mating trial"). If the mating cages are not outdoors and if it is not sunny the butterflies do not mate (personal observation). Five or more minutes after copulations began, mating couples were gently transported to the laboratory within their mating cages and hung below fluorescent lights; this does not affect the course of copulation and decreases the movement of unmated females, decreasing the probability of disturbance of the mating couple (Jiménez & Cordero, personal observation). After copulation finished, the mated female was frozen and the unmated females were moved to a different mating cage or to a wood cage (see Jiménez & Soberón 1988/89) with other females until being used for further mating trials; males were kept alone in cages in the laboratory until their next mating trial. Copula duration was measured in most matings observed. Copulations that were the second mating of the day for the male generally finished late in the night or early in the morning of the following day (Capítulo 1; see Results), after we left the laboratory; in these cases we underestimated copula duration. The interval between successive copulations was not completely controlled since sometimes males did not mate in a given trial for unknown reasons (although, from some anecdotic observations, we suspect female rejection may be an important cause), and this resulted in variation between males in the number of mating trials. However most mating trials resulted in a copulation and there was low variance in the number of mating trials per male.

In experiment I, 29 males were mated twice; intervals between copulations were varied from 0 (*i.e.*, both matings in the same day) to 13 days, and three males were mated twice in a day. In experiment II, 16 males were mated between two and five times; intervals between copulations were varied from 0 to seven days, and six males were mated twice (their first two matings) in a day. In experiment III, 31 males were mated between two and five times; intervals between copulations were varied from 0 to five days, and 18 males were mated twice in a day. One male was mated seven times, but two of them were very brief (2 and < 12 min) second copulations of the day that preceded a third copulation of the day (these two second copulations are not included in the analyses); the duration of the other 16 second copulations of the day was not recorded in this experiment (see results).

After copulating, females were freezed. The corpus bursae containing the ejaculate was dissected

out in saline solution, blotted to remove excess liquid, and weighed in a CAHN electrobalance (model 4700 for experiments I and II, and model C-31 for experiment III); the spermatophore was then dissected out from the bursae and weighed. Besides the spermatophore, the bursa copulatrix of a mated female also contains other secretions ("accessory fluid" or "accessory substances"). However, since there is evidence that, at least in some Lepidoptera, females contribute secretions to the "accessory fluid" (Hinton 1964; Drummond 1984), these substances pose a problem to studies of male investment through ejaculates. Unfortunately, in most species male investement has been evaluated by weighing only the complete "ejaculate" (= total contents of the corpus bursae). We were not able to distinguish female secretions, if any, from the male contribution to the accessory fluid and, therefore, we only use spermatophore weight as an estimate of male investment. Since a male's second copulation of the day lasts several hours (Capítulo 1), in the cases in which a female was the second mate of the day for a male, the female was freezed until the next morning, few hours after the couple separated, and after at least part of the sperm had migrated to the spermatheca (unpublished observations). In these cases the spermatheca was dissected out and its weight or, in the cases in which spermatheca weight was not available, the mean weight of the weighed spermathecae, added to spermatophore weight. We did not weigh empty spermathecae, but, since these structures are very small and thin, we considered its weight negligible. In few other cases, females that were not the male's second mate of the day were freezed a number of hours after separation of the couple; in these cases the weight of the spermatheca was also added to spermatophore weight.

Multiple regression analysis was used to investigate the effect of male traits and male mating patterns on the weight of spermatophores. In experiment III, in first copulations, the distribution of male age (♂A) and, in second copulations, time between first and second copulation (T_{F-S}), could not be normalized with any standard transformation. In these cases, the effect of ♂A and T_{F-S} on spermatophore weight was investigated with Spearman correlation. Also in experiment III, in third copulations, time between second and third copulation (T_{S-T}) and male age were correlated. In these cases, the effect of T_{S-T} on spermatophore weight was investigated with simple regression. All summary statistics are given as mean ± standard deviation (when pertinent, minimum—maximum values are given in parenthesis).

Results

Male investment in spermatophores

The average relative investment of resources (RI = [spermatophore weight / male body weight] × 100) in

first spermatophores made by males varied from 1.3 % in experiment III to 1.6 % in experiment I, with an individual maximum of 3 % in experiment III, for their first spermatophores produced (Table 1). The total RI made by males during their lifetime copulations varied from an average of 2.4 % in experiment III to 2.7 % in experiment II, with an individual maximum of 4.5 % in a male that mated five times in experiment III (Table 1). There was substantial individual variation in spermatophore weight of first and subsequent copulations (Table 1), and in the rates of "recovery" in subsequent copulations (see below).

In experiment I, no spermatophore was transfered during a second copulation that lasted 10 min (this male was excluded from the analyses). In experiment II, no spermatophore was transfered by a virgin male during a copulation that lasted 15 min. We tried unsuccessfully to remate this male on the day that he mated for first time; however, the following day this male transferred two spermatophores during a copulation that lasted 31 min. In experiment III, one male had one brief copulation that lasted about seven minutes, four of which the female was apparently trying to separate from the male in the same way as in the "interrupted copulations" described in Capítulo 1, and that did not result in the transfer of an spermatophore (this male had mated the previous day during 20 minutes with a female that was allowed to lay eggs before being dissected; there were spermatophore remains in the bursa of this female). Another two males did not transfer spermatophores during their second copulations: in one case we did not timed the copulation, and in the other case the copula lasted 38 min (however, in this case the female had deformed ductus bursae and corpus bursae). These two males transfered spermatophores in their other three and two matings, respectively. In experiment III, three males transfered two spermatophores during their first copulations; the weight of each of these pairs of spermatophores was added and considered as one spermatophore in the analyses.

Prediction 1: There should be a direct correlation between the amount of non-renewable resources stored by the male and spermatophore weight

As mentioned in the introduction, in our studies we utilized male body weight as a measure of the amount of non-renewable resources (*i.e.* those resources available only in larval food) stored. Experimental results relevant to this prediction are presented in Table 2. In agreement with prediction 1, the spermatophore weight was positively related to male body weight in: (a) first copulations of males in experiment I (in experiment III we found a marginally significant effect, $P = .054$); (b) second copulations in experiments I and III; and (c) considering the total production of spermatophore in all their copulations, in experiments I and III (in experiment II, the multiple regression including male body weight and total number of copulations was marginally significant, $P = .058$, and the coefficient for male

body weight had a $P = .05$; however, a second multiple regression including male body weight and mating rate was not significant). There was no effect of male body weight on the mass of the spermatophores transfered during third copulations (Table 2).

Prediction 2: Recently mated males should produce smaller spermatophores, in comparison with males that had longer intermating intervals

Statistical analyses to test this prediction are presented in Table 2. In agreement with this prediction, in comparison with the weight of the previous spermatophore, subsequent spermatophores generally showed a decrease in weight and this decrease was greater when the intermating interval was shorter (Table 3). The positive relationship between time since last mating and spermatophore weight (Table 2) indicates that previously mated males required time to replenish accessory gland substances, and that a number of days is required before males are able to transfer spermatophores similar in mass (in some cases even bigger) to those transfered in previous copulations (Table 3).

A positive effect of time between first and second copulation ($T_{F\text{-}S}$) on the weight of spermatophores transferred during second copulations was observed in all experiments (Table 2; experiment 3: $r_S = .59$, $P = .001$). $T_{F\text{-}S}$ also affected the weight of spermatophores produced during third copulations in experiment III ($r_S = .53$, $P = .0098$, $n = 23$) but not in experiment II (in experiment I males mated only twice). A positive effect of time between second and third copulation ($T_{S\text{-}T}$) on the weight of spermatophores transferred during third copulations was observed in experiments II (Table 2) and III ($r_S = .86$, $P = .00001$). However, the rates of male "recovery" between second and third copulation were much higher than those between first and second copulations and, in fact, the weight of third spermatophores produced one or more days (experiment III) or two or more days (experiment II) after the second copulation was, in most cases, higher than that of second spermatophores (Table 3).

Implicit in prediction 2 is a negative effect of mating rate (MR = total number of copulations / male age when mating for the last time) on total spermatophore mass. As expected, in experiment III, but not in II, MR affected negatively total spermatophore mass (Table 2). In experiment I, since males mated only twice, $T_{F\text{-}S}$ is an inverse measure of MR and, as expected, affected positively the total amount of spermatophore produced (Table 2).

Prediction 3: Recently mated males should take longer to transfer an spermatophore

Experimental results to test this prediction are summarized in Table 4. The average duration of first and subsequent copulations occurring in different days was similar. However, in agreement with prediction 3,

in experiments I and II, second copulations of the day were much longer than all other copulations. In experiment III we did not measure the duration of 16 second copulations of the day for a male (we only timed two brief second copulations, 2 and < 12 min, that occurred previous to a third copulation of the day), however, they were noted to last until night. We only timed one of the two third copulations of the day for a male (483 min), although the other was noted to last until the nigth (*i.e.*, several hours). These differences in copula duration have also been observed in the field (C. Cordero, personal observation).

In experiment I, the three males that mated two times in the same day were mated for a third time. The duration of the these three copulations suggests an inverse effect of mating rate on the duration of third copulations: one male mated for 26 min three days after his first two copulations, whereas two males that mated two days after their first two copulations mated for 170 and 180 min.

Discussion

Physiological costs of spermatophore production

Mating is costly if it produces an increase in mortality or a decrease in future reproductive success (Partridge 1987; Lessells 1991). The substantial investment of resources and time made by male butterflies (Rutowski *et al.* 1983; Svard and Wiklund 1989; Bissoondath and Wiklund 1995, 1996) suggests that one important cost of mating is the physiological cost of ejaculate production (Svard and Wiklund 1989; Bissoondath and Wiklund 1995; Bissoondath and Wiklund 1996). Previous studies with the polygynous butterfly *C. xami* have shown that, in some conditions, multiple mating decreases male lifespan, and suggest that the physiological cost of spermatophore production is one possible cause of lifespan reduction (Capítulo 4). The three predictions from the hypothesis that spermatophore production is physiologically costly tested in this study were supported by the results. In agreement with prediction 1, in most cases there was a positive correlation between the amount of non-renewable resources (those available only in larval food) that were stored by the male (we used body weight as a measure of these resources) and spermatophore weight (Table 2). In agreement with prediction 2, recently mated males produced smaller ejaculates than males that had longer intermating intervals (Tables 2 and 3). In agreement with prediction 3, the copula duration of males mating for the second time of the day (i. e. recently mated males) was much longer (several hours) than that of males that had longer intermating intervals (males mated one or more days after their previous mating copulated for about half an hour). Furthermore, two males that mated two days after the day of their first two copulations, mated for 170 and 180 min.

Mating decreases female sexual receptivity in *C. xami* (Capítulo 2), and it is possible that in this

species, as in many other Lepidoptera (Sugawara 1979; Drummond 1984; Rutowski *et al.* 1987; Dussourd *et al.* 1989; Oberhauser 1989; Svard and Wiklund 1989; Boggs 1990; LaMunyon and Eisner 1994; Kaitala and Wiklund 1995; Bissoondath and Wiklund 1996), spermatophore size is positively correlated with the length of the period during wich females are sexually unreceptive, and, if sperm competition is an important selective pressure, with male fitness. Therefore, the inability of small males and recently mated males of *C. xami* to produce "big" ejaculates, and the increased investment of time in a copulation made by recently mated males, could reduce their future reproductive success. In the near future we will experimentally investigate the relation between ejaculate mass and mated female sexual receptivity, to test the hypothesis that spermatophore size is correlated with success in sperm competition avoidance.

Since the production of spermatophores entails benefits and costs to male butterflies, in virgin males selection should favor the production of spermatophores of the size that maximizes the difference between such benefits and costs (denoted here by S_V). In the case of mated males, selection should also favor the production of an ejaculate of the size (S_M) that maximizes the difference between the benefits and costs of ejaculate production during their subsequent copulations. Although S_V and S_M may be the same, they may be different due to "strategic" reasons. Svard and Wiklund (1986). S_V and S_M may also be different as a result of physiological constraints (Oberhauser 1988). In species in which males have a high probability of remating before the time necessary to reach S_M, the rate of replenishment of ejaculate material will constrain male mating potential. This constraint may be expressed in at least two ways. First, males may need to wait some time before mating successfully again; this may be favored, for example, if there is a minimum ejaculate size for successful mating. In *Danaus plexippus*, the only butterfly species studied in this respect, no evidence of such delayed remating exists (Oberhauser 1988), although the decrease in courtship performance observed in some other Lepidoptera may result in delayed remating (Rutowski 1979; Drummond 1984). In *C. xami*, although the results of our experiments show that spermatophore weight increases with time since last mating, at least some males do not delay remating, even when they have recently mated and, therefore, are only able to transfer small spermatophores during prolonged copulations. This behaviour is not an artifact of the conditions experienced in captivity, since it is also exhibited in the field, where we have observed some males mating twice in a day (Capítulo 3; personal observation), and several males mating and then, in the same day, courting further females. However, it is interesting to note that several mated males did not remate in the first opportunity they had. For example, in experiment I, 14/29 males did not mate for a second time in the first opportunity they had, but after a number (1—9) of "failed" opportunities; and 8/29 males

had opportunity of mating twice in a day, but only three remated in their second opportunity of the day (four remated after 1—8 "failed" opportunities, and another did not remate in any of the 12 opportunities it had). Unfortunately we do not have the detailed observations necessary to determine if these males "voluntarily" delayed remating until they had replenished their depleted reserves of seminal constituents, or if they were rejected by the females (although, from some anecdotic observations, we suspect that female rejection may be an important cause).

Second, in butterflies that do not delay remating until they can produce a large spermatophore, like at least some male *C. xami* do, matings in which the male transfers a small ejaculate will generally have a low success, given the positive effect of ejaculate size on intermating interval of females, and the last male sperm precedence typical of many Lepidoptera. Therefore, males transfering small ejaculates will sire a small proportion of the female's offspring, and will have the risk of become "stepfathers" (*sensu* Wickler 1985) of some of them. As previously mentioned, in *C. xami* and in many other Lepidoptera species (Drummond 1984; Svard 1985; Rutowski and Gilchrist 1986; Svard and Wiklund 1986; Kaitala and Wiklund 1995), mating duration is inversely correlated with time since last mating of males. The increase in mating duration of recently mated males may result of the time needed to replenish sperm and accessory products or may be a mate guarding mechanism. In the last case, a recently mated male transfering an ejaculate of suboptimal size may force a female to resume oviposition behaviour after mating, instead of "foraging" for further matings (Kaitala and Wiklund 1994), if he makes her "lose" time in a long copulation. For a female in this situation it may be better to engage in oviposition instead of consuming more of her precious time in further copulations, even if she did not receive an ejaculate of optimal size. The fact that in some species ejaculates are transferred well before the end of lengthy copulations (Drummond 1984; Svärd and Wiklund 1988; Oberhauser 1989) is consistent with this hypothesis. This idea implies that copula duration is determined by males, as it has been suggested for some butterflies (Drummond 1984; Wickman 1985; but see Capítulo 1).

Cryptic male choice?

The evolution of ejaculate size may also be affected by other factors. If the value of a female for a male is positively correlated with female weight (because female weight is positively correlated with the number of eggs produced) and, negatively, with the probability of experimenting sperm competition within that female (and, therefore, with her mating status and her probability of remating), and with female age (because older females have a lower reproductive value and a higher chance of being already mated), it could be adaptive for males to modulate ejaculate size in relation to the mating status (virgin

vs. mated, and how recently mated), weight and age of females (Dewsbury 1982). Two predictions of this "cryptic male choice" hypothesis are that: (a) there should be a direct correlation between ejaculate weight and female weight; and (b) there should be an inverse correlation between ejaculate weight and female age. Our experiments were not specifically designed to test these predictions. However, a prospective analysis (not shown) of the effect of female weight (♀BW) and age (♀A) on spermatophore weight found only one significant positive effect of ♀BW in the copulations of virgin males of experiment I (multiple regression: $R_{adj}^2 = .66$, $F_{4,23} = 14$, $P < .00001$, $\beta[♀BW] = .31$, $P = .017$). The only other significant effect found was contrary to that expected from prediction (b), since ♀A had a positive effect on spermatophore weight in the second copulation of experiment I ($r = .42$, $P = .03$). Therefore, no evidence of cryptic male choice was found in this study; however, it must be remembered that our experiments were not intended to put this hypothesis to test.

Female costs of mating

Although this study was designed to test the existence of spermatophore production costs in males, we also observed a female cost of mating. The increased duration of second copulations (and third copulations, when the second was of very short duration) of the day (Table 4) is costly for females, probably more than for males, because time is a limiting resource for them as a result of the dependence of their flight activity on fine wheather conditions, generally available a few hours per day, and the relatively short lifespan of adults. For example, if we consider that the average lifespan of one week, proposed for temperate butterflies by Scott (1986), is a good approximation to the average longevity of female *C. xami*, a female that is the second mate of the day for a male, and that mate early in the morning, may lose almost one of the seven days available for egg production (~ 14%, a time consuming process for females; personal observation). This percentage must vary depending on the age of the female (a young female would have more to lose than an "old" female that already laid most of her eggs). Also, a time cost of mating probably exists for females when copulating with males whose two previous copulations occurred one or two days before (see last paragraph of Results).

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Table 1. Spermatophore weight (SW; mg), male body weight at emergence (♂BW, mg), relative male investment (100 x SW/♂BW), female body weight at emergence (♀BW; mg), and female age at mating (♀Age; days) of experimental subjects. cop.: copulation(s). Average ± SD, minimum - maximum values are given in parenthesis; *n*: number of experimental males.

SW	[SW/♂BW] x 100	♀BW	♀Age
	Experiment I (♂ BW: 54 ± 9.9 [22-75]; *n* = 28)		
1st cop.: .83 ± .25 (.48-1.29)	1.6 ± .5 (.9-2.7)	63.2 ± 8.9 (46-77)	3.4 ± 2.1 (1-7)
2nd cop.: .56 ± .27 (.1-1.08)	1.1 ± .5 (.2-1.9)	63 ± 14 (15-87)	3.8 ± 2 (1-10)
1st + 2nd cop.: 1.39 ± .43 (.67-2.17)	2.6 ± .9 (1.1-4.3)		
	Experiment II (♂ BW: 53.1 ± 7.3 [42-69])		
1st cop.: .73±.25 (0-1.02; *n* = 14)	1.48 ± .36 (1.16-2.4; *n* =13)	61.3 ± 8.1 (40-71)	2.6 ± 1.4 (1-6)
2nd cop.: .41 ± .15 (.25-.74; *n* = 15)	.78 ± .27 (.45-1.3; *n* = 15)	67.3 ± 14.3 (34-86)	4.3 ± 5.4 (1-23)
3rd cop.: .33 ± .15 (.18-.66; *n* = 13)	.63 ± .25 (.38-1.1; *n* = 13)	64.9 ± 9.8 (56-92)	6.2 ± 8 (1-30)
1st +...+ 5th cop.: 1.43 ± .38 (.69-1.94)	2.71 ± .72 (1.35-3.93; *n* = 16)		
	Experiment III (♂ BW: 50.8 ± 6 [36-60])		
1st cop.: .67 ± .35 (.02-1.52; *n* = 29)	1.3 ± .67 (.05-3; *n* = 29)	60.8 ± 8.8 (42-75)	3.3 ± 4.7 (1-19)
2nd cop.: .26 ± .17 (0-.6; *n* = 26)	.48 ± .3 (0-1; *n* = 25)	61 ± 9.2 (35-75)	4.1 ± 3.9 (1-17)
3rd cop.: .29 ± .15 (.06-.58; *n* = 23)	.6 ± .3 (.1-1.1; *n* = 22)	63.7 ± 7.1 (50-75)	3.1 ± 3.1 (0-12)
1st +...+ 5th cop.: 1.22 ± .49 (.39-2.7)	2.4 ± .85 (.81-4.5; *n* = 29)		

Table 2. Multiple regressions analyses of the effect of male traits and male mating patterns on the weight of spermatophores. ♂BW: male body weight at emergence (mg); ♂A: male age at mating (days); T_{F-S}: time between first and second copulations (days); NC: total number of copulations; MR: mating rate (= NC / ♂A when mating for the last time); T_{S-T}: time between second and third copulations (days). In the second copulation of experiment II and in the second and third copulations of experiment II, ♂A and T_{F-S} (or T_{S-T}) were correlated and, therefore, we present the multiple regressions including ♂A or T_{F-S} (or T_{S-T}).

Variables included in the multiple regression	Significant coefficients
Experiment I	
1st cop.: ♂BW + √♂A ($R_{adj}^2 = .59$, $F_{2,25} = 20.7$, $P < .00001$)	β[♂BW] = .33, $P = .013$; β[√♂A] = .73, $P = .000003$
2nd cop.: (a) ♂BW + ♂A ($R_{adj}^2 = .51$, $F_{2,25} = 14.8$, $P < .00006$)	β[♂BW] = .42, $P = .005$; β[♂A] = .71, $P = .00003$
(b) ♂BW + T_{F-S} ($R_{adj}^2 = .55$, $F_{2,25} = 17.21$, $P < .00002$)	β[♂BW] = .42, $P = .004$; β[T_{F-S}] = .73, $P = .00002$
1st + 2nd cops.: ♂BW + T_{F-S} ($R_{adj}^2 = .4$, $F_{2,25} = 9.8$, $P < .0007$)	β[♂BW] = .46, $P = .006$; β[T_{F-S}] = .58, $P = .0008$
Experiment II	
1st cop.: ♂BW + √♂A ($R_{adj}^2 = .03$, $F_{2,11} = 1.2$, $P > .34$)	—
2nd cop.: (a) ♂BW + ♂A ($R_{adj}^2 = .31$, $F_{2,12} = 4.1$, $P < .045$)	β[♂A] = .55, $P = .031$
(b) ♂BW + T_{F-S} ($R_{adj}^2 = .51$, $F_{2,12} = 8.3$, $P < .005$)	β[T_{F-S}] = .7, $P = .003$
3rd cop.: (a) ♂BW + ♂A ($R_{adj}^2 = .16$, $F_{2,10} = 2.1$, $P > .17$)	—
(b) ♂BW + T_{S-T} ($R_{adj}^2 = .42$, $F_{2,10} = 5.3$, $P > .027$)	β[T_{S-T}] = .61, $P = .021$
1st +...+ 5th cops.: (a) ♂BW + NC ($R_{adj}^2 = .25$, $F_{2,13} = 3.6$, $P < .0583$)	β[♂BW] = .51, $P = .05$; β[NC] = .52, $P = .045$
(b) BW + MR ($R_{adj}^2 = .01$, $F_{2,13} = 1.1$, $P > .37$)	—
Experiment III	
1st cop.: ♂BW ($R_{adj}^2 = .1$, $F_{1,27} = 4.1$, $P < .054$)	β[♂BW] = .36, $P = .054$
2nd cop.: ♂BW + ♂A ($R_{adj}^2 = .47$, $F_{2,21} = 11.2$, $P < .0005$)	β[♂BW] = .48, $P = .0047$; β[♂A] = .49, $P = .0044$
3rd cop.: ♂BW + √♂A ($R_{adj}^2 = .71$, $F_{2,19} = 26.8$, $P < .000001$)	β[√♂A] = .91, $P = .000001$
1st +...+ 5th cops.: ♂BW + NC + MR ($R_{adj}^2 = .42$, $F_{3,25} = 7.7$, $P < .0009$)	β[♂BW] = .53, $P = .002$; β[NC] = .46, $P = .006$; β[MR] = -.39, $P = .02$

Table 3. Percent change in spermatophore weight (SW) produced in subsequent copulations, as a function of number of days since last mating. Δ_{F-S}: percent change between first and second spermatophores; Δ_{S-T}: percent change between second and third spermatophores; T_{F-S}: time between first and second copulations (days); T_{S-T}: time between second and third copulations (days). "-" indicates a decrease, and "+" an increase, in spermatophore weight between two consecutive copulations. Average ± SD, minimum and maximum values are given in parenthesis.

	Experiment I	*Experiment II*	*Experiment III*
Δ_{F-S}			
$T_{F-S} = 0$	-82.5 ± 8.4 % (-73.6, -90.3) (n = 3)	-63.3 ± 7.2 % (-54.3, -71.1) (n = 6)	-80.1 ± 6.1 % (-67.7, -90.8) (n = 10)
$T_{F-S} = 1$	-56.4 ± 11 % (-48.1, -71.4) (n = 4)	-48.8 ± 1.8 % (-47.5, -50) (n = 2)	+6.3 ± 82.9 % † (-55.6, +115) (n = 5)
$T_{F-S} = 2$	-43.4 ± 7.4 % (-35.4, -50) (n = 3)	-28.8 % (n = 1)	-64.1 ± 22.6 % (-38.8, -94.2) (n = 7)
T_{F-S} = 3-13 (Exp. I); 3-4 (Exp. II); 3 (Exp. III)	-12.7 ± 26 % (-50, +37.5) (n = 18)	-28.7 ± 28.5 % (-46, +4.2) (n = 3)	+39.5 % (n = 1)
Δ_{S-T}			
$T_{S-T} = 0$	—	—	-72.5 ± 11.1 % (-65.2, -88.9) (n = 4)
$T_{S-T} = 1$	—	-37.3 ± 18.3 % (-12, -61.1) (n = 8)	+79 ± 75.1 % (-3.2, +214.3) (n = 7)
$T_{S-T} = 2$	—	+26.6 ± 20.6 % (+11.4, +50) (n = 3)	+47.2 ± 103.8 % (-20.4, +166.7) (n = 3)
T_{S-T} = 7 (Exp. II); 4-5 (Exp. III)	—	+85.2 % (n = 1)	+151 ± 154.7 % (+16.7, +286.7) (n = 4)

†If we exclude the two individuals that increased their spermatophore weight between first and second copulations we had: -55.5 ± 1.9 % (-51.8, -55.6) (n = 3).

Table 4. Duration of copulations of experimental males.

Copulation	Duration of copulations (min) (average ± SD, minimum - maximum): *Experiment I*	*Experiment II*	*Experiment III*
First	35.9 ± 10.2 (14-57) (n = 28)	32.9 ± 9.8 (15-48) (n = 15)	26.2 ± 7.1 (18-46) (n = 26)
Second (same day as the first)	760.7 ± 68.8† (717-840) (n = 3)	394.8 ± 75.5† (283-449) (n = 4)	—‡
Second (≥ 1 day after first)	33.6 ± 8.7 (13-50) (n = 25)	32.4 ± 6.7 (26-47) (n = 8)	23.8 ± 15.1 (7-74) (n = 18)
Third	—	30.8 ± 6.6 (17-45) (n = 13)	20.6 ± 5.5§ (11-33) (n = 18)
Fourth	—	32.8 ± 5.4 (28-40) (n = 5)	23.6 ± 4.6 (17-30) (n = 9)

† These figures are minimum estimates since we did not measure the exact duration of these copulations and statistics were calculated with the minimum possible durations (see Methods).

‡ Sixteen second copulations of the day were not measured in this experiment (we only timed two second copulations of very short duration: 2 and < 12 min); however, as in the previous experiments, they were much longer than other copulations.

§ Two third copulations that ocurred the same day as the first and the second copulation (in both cases after a very short second copulation; see note ‡) are not included in this average; one of these copulations lasted 483 min and the other was not timed, but it was noted to last until the nigth (and, therefore, for several hours).

III. COMENTARIOS ACERCA DE LA EVOLUCIÓN DEL SEMEN DE LOS INSECTOS

CAPÍTULO 6

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Ejaculate Substances that Affect Female Insect Reproductive Physiology and Behavior: Honest or Arbitrary Traits?

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Males of many insect species transfer, within the ejaculate, substances that render females sexually unreceptive and promote ovulation and oviposition. In this paper, I propose hypotheses for the evolutionary origin of these substances and discuss how selection may have modified them subsequently. Two hypotheses are considered. According to the handicap hypothesis, receptivity inhibition substances (RIS) and ovulation and oviposition stimulating substances (OSS) are used by females to evaluate the quality of the ejaculate received, the last being a function of the genetic or phenotypic quality of sperm, and of the nutritional or protective (to the female or her offspring) quality of its chemical constituents. This hypothesis predicts that RIS and OSS must be reliable indictors of ejaculate quality, reliability being the result of the high RIS/OSS production costs and specific chemical composition. The second hypothesis proposes that RIS/OSS are selected only in regard to their effectiveness as stimulators through Fisher's sexual selection runaway process. According to this hypothesis, RIS/OSS are not necessarily are reliable indicators of sperm or ejaculate chemical constituents quality (other than ability to stimulate), and must show a high species-specificity (rapid evolutionary divergence). Empirical evidence is reviewed, and the kind of information necessary to evaluate the relative importance of each hypothesis is indicated.

1. Introduction

During copulation, males of many insect species transfer, within the ejaculate, substances that render females sexually unreceptive and promote oocyte maturation and egg laying (Leopold, 1976; Thornhill & Alcock, 1983; Chen, 1984). These receptivity-inhibition (RIS) and oviposition-stimulant (OSS) substances are secreted by the accessory glands and/or by other glandular parts of the male reproductive tract (Leopold, 1976; Chen, 1984). After transfer into the female reproductive tract, RIS and/or OSS enter the hemolymph and affect her nervous and/or secretory systems, at least in some species (this aspect has not been investigated in many species; Table 1).

RIS and OSS have been considered beneficial from the female's point of view because once she has been provided with an ejaculate, a behavioral change from mating related activities (mate location, pheromonal "calling", courtship, etc.) to oviposition and/or foraging will save time, energy, and, in some cases, reduce mortality risks (Leopold, 1976; Thornill & Alcock, 1983; Chen, 1984; Eberhard, 1985; Boggs, 1990). For the male, the production of these substances has also been considered favorable because he will benefit if the female concentrates on laying eggs fertilized by his spermatozoids and, in some species, provisioned with the nutrients or protected with the deterrent substances that he transferred through the ejaculate, instead of "losing" time and energy, and putting herself at risk, in further copulations (Thornhill & Alcock, 1983; Eberhard, 1985). Sperm competition and its typical last-male sperm precedence pattern (Smith, 1984) will further increase the benefits from the male's point of view.

The evolution of these substances has not been analyzed in detail previously. In this paper, I discuss the possible selective pressures responsible for the evolution of RIS and OSS (I refer to both as RIS/OSS). I shall show that the relative importance of the hypotheses advanced cannot be decided from the

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available empirical data. However, the hypotheses suggest new avenues of research.

2. Evolutionary Origin of RIS and OSS

Starting with the hypothetical primitive state in which females do not receive any RIS/OSS at all, it is necessary to consider the possible origin of these substances and the benefits accrued from their use as *cues* for a physiological and behavioral change in females. I assume that, for the reasons outlines in the Introduction, before RIS/OSS appeared, females would have been selected to exhibit a reduction in sexual receptivity (i.e. to enter into a refractory period) and an increase in oocyte maturation and/or oviposition tendency because of the stimulation (auditory, mechanical, pheromonal or visual) provided by a courting or copulating partner (Thornhill & Alcock, 1983; Eberhard, 1985).

In several (probably most) insect species, some copulations do not result in the transfer of an ejaculate, or an ejaculate of adequate size (Lea, 1968; Thornhill & Alcock, 1983; Eberhard, 1985; Oberhauser, 1988; Svard & Wiklund, 1989). Females with the ability to discriminate against these mates (i.e. not exhibiting receptivity inhibition and/or oviposition stimulation after copulation) would be favored by selection (at this point *female choice* pressures arise). Male insects transfer with the ejaculate several chemicals, some of which could be detected internally by the female; females using these chemicals as cues (with appropriate response thresholds) for becoming unreceptive and beginning (or increasing) egg maturation and oviposition, would have a selective advantage because they would become unreceptive only after mating with males that transferred an ejaculate of adequate size.

An alternate evolutionary scenario for the origin of RIS/OSS may be derived from Wickler's (1985) ideas on the evolutionary origin of seminal nuptial gifts (nutritious or protective substances transferred by males through ejaculates). Wickler argues that in species whose females are frequently inseminated by more than one male, sperm competition selects for ejaculates with increased sperm quantity. Once sperm number is above that required by females for fertilization, selection for the "digestion" of excess sperm may arise, giving origin to seminal gifts. After this, RIS/OSS may evolve from other seminal fluid chemicals, enabling females to synchronize their reproductive processes with the obtainment of a gift.

Independent of the way in which RIS/OSS originated, once the use of such substances has arisen, selective pressures could affect their subsequent evolution in different ways. Two hypotheses on the evolutionary elaboration of RIS/OSS are considered here. The first is an application of the Handicap Principle (Zahavi, 1975, 1991; Grafen, 1990). The second is based in applications of Fisher's model of

TABLE 1.

Summary of characteristics of receptivity inhibition substances (RIS) and ovogenesis/oviposition stimulant substances (OSS)

(a) *Receptivity inhibition substances (RIS)*†

Characteristic	Yes	?
RIS produced by accessory glands or equivalent tissues does exist	24	2
RIS is a peptid or a protein	7	2
There is evidence of a positive correlation between RIS quantity and ejaculate quantity	3	—
There is a positive correlation between RIS quantity and refractory period length (dosage-dependent effect)	8	1
The filling of the female bursa affects the onset and/or length of the refractory period	—	12
Sperm quantity or mechanical stimuli affect the onset and/or length of the refractory period	3	1
Females refractory for life to subsequent insemination	11	1

(b) *Ovogenesis/oviposition stimulant substances (OSS)*‡

Characteristic	Yes	?	No
OSS produced by accessory glands or equivalent tissues does exist	20	—	5
OSS is a peptide or a protein	3	5	1
OSS increases oocyte maturation	9	—	1
OSS increases oviposition	15	—	1
OSS effect is dosage-dependent	4	3	2
Testicular secretions act as OSS	4	—	1
Sperm acts as OSS	3	—	—

Entries are the number of species in which the characteristic is present (Yes), has not been demonstrated but it probably exists (?), or it has been looked for but not found (No). (See Appendix for details).

† Data based on information about 26 species.

‡ Data based on information about 25 species.

runaway sexual selection by female choice to the evolution of intromittent genitalia (Eberhard, 1985).

3. RIS/OSS as Signals

In species in which (i) males vary continuously in the quantity/quality of sperm and/or accessory gland secretions transferred (the factors that determine the quality of an ejaculate are discussed in the last three paragraphs of this section), and (ii) female reproductive success is directly affected by these variables, females using the "primitive" RIS/OSS discussed above could only discriminate against males not transferring any ejaculate at all or transferring ejaculates of size smaller than a minimum, by means of a threshold mechanism (this minimum could be a constant or a function of, for example, female nutritional state; Gwynne, 1990; Simmons & Gwynne, 1991). Possible examples of this may be found in *Drosophila funebris* and *Aedes aegypti*. Experiments with these two species have shown that injection of different quantities of OSS produce similar effects in females (Leahy, 1967; Baumann, 1974; see Appendix). The assumptions behind this interpretation are that in the experiments the quantity injected was always higher than the putative threshold, and that the quantities injected corresponded to doses delivered by males.

As an alternative, females with the ability to evaluate ejaculate quantity/quality, and, accordingly, to decide the length of the refractory period, the response threshold for male attractiveness (quantitative behavioral responses), and/or the degree of increase in egg maturation rate and/or oviposition rate (quantitative physiological responses) would be favored by selection. In accord with this idea, refractory period length and egg maturation and oviposition rates are directly correlated with RIS and OSS quantities transferred in several species (Table 1), and in three species evidence suggests that RIS quantity is directly correlated with ejaculate quantity (Table 1). It is also possible that in at least some of the cases in which a female threshold response seems probable (see previous paragraph), quantities smaller than those experimentally injected will produce a gradual female response.

According to the above ideas, the evolution of RIS/OSS is the evolution of a communication system between males and females. Males transfer signals (= RIS and OSS) to females, and females use these signals to evaluate quantity/quality of sperm and/or accessory gland secretions and behave accordingly, i.e. decide a refractory period length, response threshold for male attractiveness, or an egg maturation and/or oviposition rate. (The possibility that RIS/OSS are used by females to evaluate male genetic quality will not be considered here; however, the evolution of a communication system of this sort could be explained with a modified version of the handicap hypothesis developed in the next section). Systems in which signals are used by the receiver to assess the quality of the signaler and behave accordingly, are open to cheating. Signalers producing deceptive signals that make receivers perceive them to be of better quality than they are, would be favored by selection. In the case of RIS/OSS, cheating means the production by males of RIS/OSS that, by their quantity/quality, induce a longer refractory period, an increase in the response threshold for male attractiveness, and a higher increase in egg maturation and/or oviposition rates, than those favored by selection acting on females on the basis of the amount of sperm or other ejaculate components received. In other words, if females were able to assess directly the quantity/quality of the ejaculate received, they would have been selected to enter into a shorter refractory period, to have a lower threshold for male attractiveness and/or they would have shown a lower increase in egg maturation and oviposition rate.

Cheating is probably in the case of RIS/OSS, because females not receiving enough sperm would be selected to stop ovipositing and to remate *before* depleting their sperm stores (Parker, 1970; Dewsbury, 1982). On the other hand, males would be selected to induce a longer refractory period, and to increase the female's threshold for male attractiveness and the egg maturation and oviposiiton rates, high enough for their sperm to be totally used. This last prediction could be quantitatively modified because of "marginal value" reasons if there were diminishing returns in the relationship between male fitness and refractory period length and egg maturation/oviposition rate.

However, there is evidence that in many species males generally transfer enough sperm to fertilize most of the female's eggs (Thornhill & Alcock, 1983; Smith, 1984), even if males have recently mated (Rutowski *et al.*, 1987; Oberhauser, 1989; Markow *et al.*, 1990; Smith *et al.*, 1990). In these cases conflict is also possible because, for females, it may be better to receive more than the sperm necessary to fertilize their eggs, for one or more of the following reasons:

(i) Females may digest sperm cells and use them as a nutrient source (Parker, 1970; Sivinski, 1984).

(ii) Females of some species may need "excess" sperm to compensate for sperm mortality (Tsubaki & Yamagishi, 1991; Yamagishi *et al.*, 1992).

(iii) Females may exert within-ejaculate choice of best quality sperm (Sivinski, 1984; Birkhead & Moller, 1993). "Best quality" has different meanings that depend on the particular species: Sperm without genetic or phenotypic defects (Sivinski, 1984), "vigorous" sperm, particularly if sperm vigor correlates with offspring fitness, or young sperm (known to be less likely to produce embryos with developmental problems; W. G. Eberhard, personal communication; Baker & Bellis, 1993). Sperm preference may be also related to gender choice of offspring ("excess" sperm may be needed for sex ratio manipulation in species with sex chromosomes; Sivinski, 1984).

In species in which males transfer seminal nutritious or protective gifts (Thornhill & Alcock, 1983; Simmons & Parker, 1989), the possibilities for male–female conflict are also great. Seminal nutritious gift size has been shown to depend on male size and/or how recently the male mated and/or male diet, in most species studied (Svard & Wiklund, 1986, 1989; Oberhauser, 1988; Marshall & McNeil, 1989; Gwynne, 1990; Markow *et al.*, 1990; Hayashi, 1993). Ejaculate chemical composition is also affected by male condition (Marshall, 1985; Conner *et al.*, 1981). The conflict arises because males are selected to induce a refractory period long enough, a threshold for male attractiveness high enough, an egg maturation and/or oviposition rate increase high enough, for most of their sperm to be used, whereas females are selected to obtain a suitable supply of nutrients.

4. RIS/OSS as Handicaps

As discussed above, RIS/OSS are signals which, at least in some species, may be used by females to evaluate ejaculate quality. Once arisen, a signal of this kind may develop along at least three different evolutionary paths. One is discussed at length in Section 5. The other two depend on the evolutionary response given to cheating. On the one hand, females may respond to "cheat" RIS/OSS by ceasing to use them as signals. In this case a different RIS/OSS may evolve, or the female may evolve an alternate ejaculate evaluation system. An example of this could be the evolution of methods for the direct measurement of ejaculate volume, such as the stretch receptors in the spermatheca or bursa of some butterflies (Sugawara, 1979). However, it is important to note that even in these cases of "direct" measurement, cheating by males is possible. For example, males may tranfer large but empty spermatophores or sperm diluted in a cheap and innocuous fluid.

On the other hand, RIS/OSS may evolve according to the handicap principle (Zahavi, 1975, 1991; Grafen, 1990). According to this principle, evolutionarily stable signals used for signaler assessment by receivers must be "honest, costly and costly in a way that relates to the true quality revealed" (Grafen, 1990). In our case, honesty means that females can obtain good estimations of ejaculate quality by using RIS/OSS quantity and/or quality. The honesty of the signals is guaranteed, according to the handicap theory, because female choice selects signals that are costly to produce, and costlier for males able to produce only small size/low quality ejaculates. These costs must be a function of the resources invested in the obtainment of raw materials, and in the synthesis and storage of RIS/OSS.

The commonly observed dosage-dependent effect of RIS/OSS and the evidence indicating that, in at least some species, RIS/OSS quantity is directly correlated with ejaculate quantity (Table 1), suggests that RIS/OSS may be "honest" signals in the sense that they enable females to make a good estimate of ejaculate quantity (and, possibly, quality) received.

There is evidence suggesting that RIS/OSS may be costly. In most cases in which their chemical nature has been investigated (Table 1 and Appendix), RIS/OSS have been shown to be nitrogenous based substances, and nitrogen is a common limiting resource for insects (Boggs, 1990). The fact that replenishment of RIS/OSS after mating takes time may also be an evidence of its costliness (Hayashi, 1993). However, without a detailed physiological and ecological knowledge of particular species, it is difficult to evaluate the exact magnitude of these costs.

The last property of an evolutionarily stable RIS/OSS according to the handicap principle ("costly in a way related to the quality revealed") concerns the design (chemical composition) of the RIS/OSS: "design . . . is, through the nature of the cost paid, a direct reflection of underlying quality . . ., and therefore of the message conveyed by the signal" (Guilford & Dawkins, 1993). According to this, if RIS/OSS are used by females to assess ejaculate quality, then males must be "using up" part of the resources necessary to produce a high quality ejaculate, in the elaboration of RIS/OSS.

In the case in which females assess the nutritious quality of ejaculates, we would expect to observe RIS/OSS elaborated with the most important nutrient(s) transferred with the ejaculate. In most cases investigated, RIS/OSS are nitrogen-rich substances (Table 1; see also Leopold, 1976; Chen, 1984), and it has been shown that nitrogen is probably the most

common male nutritional contribution to the female (Boggs, 1981; Gwynne, 1988; Markow *et al.*, 1990). (However, Dr. W. G. Eberhard has pointed out to me that this property of male contributions may be an artifact of what researchers have been looking for and of their experimental techniques). A possible species to evaluate this hypothesis is the orthopteran *Melanoplus sanguinipes* whose accessory glands produce oviposition stimulant proteins as well as proteins that are used to provision eggs (Pickford *et al.*, 1969; Friedel & Guillott, 1976, 1977). However, it must be considered that the quantity of ejaculate protein incorporated in the eggs is very small (to the point of raising doubts about its nutritional role in one of the authors that originally considered ejaculate proteins as paternal investment; Cheeseman & Gillot, 1989). It would also be interesting to analyze the RIS/OSS of species in which the main male nutrient contribution is not nitrogen.

In the cases in which females evaluate sperm quantity/quality, it is more difficult to predict the chemical composition of RIS/OSS. For this, it will be necessary to determine the limiting resources for sperm production and/or for the production of substances necessary for sperm transfer, maintenance and activation (Leopold, 1976). The enzyme EST 6, which is transferred through ejaculate in *Drosophila melanogaster*, may be a good candidate for exploring this question (Gromko *et al.*, 1984).

The case of defensive chemicals transferred though ejaculates provides us with a possible application of the handicap principle to the evolution of a different kind of sexual chemical signal. In several lepidopterans, chemicals obtained through larval or adult diet (pyrrolizidine alkaloids) defend adults from predators; males transfer to females part of these substances through spermatophores and derive courtship pheromones from them (Brown, 1984; Conner *et al.*, 1981; Dussourd *et al.*, 1988, 1989). In these species, male alkaloids are transferred by the female to her eggs, and in *Utetheisa ornatrix* it has been shown that eggs are protected against predators as a result (Dussourd *et al.*, 1988, 1989). Dussourd *et al.* (1988, 1989) have proposed that males advertise their alkaloid-donating capacity through their (pyrrolizidine alkaloid derived) courtship pheromone, based in the fact that the quantity of pheromone secreted is proportional to male systemic alkaloid content, "which itself is proportional to the fraction of alkaloid transmitted in mating" (Dussourd *et al.*, 1988). The apparent honesty of the male pheromones of these species calls for an explanation which could be quite similar to that proposed for RIS/OSS in this section. Of course, RIS/OSS may also exist in these species. In fact, these substances could *also* be used to evaluate the quantity of defensive chemicals received by the female. In this case, we predict that RIS/OSS would be derived from pyrrolizidine alkaloids.

5. Fisherian Runaway in RIS/OSS Evolution

Eberhard (1985) consideres that, as mentioned earlier, females are selected to associate reproductive processes (e.g. tendency to remate or to oviposit) with stimuli associated with copulation (e.g. transference of RIS/OSS by males). Under these conditions, males which produce better stimuli (e.g. RIS which produce a longer refractory period or OSS which produce a bigger increase in egg oviposition rate) will be favored. Females that select these males will have the advantage of producing male offspring which will produce a better stimulus. In Eberhard's words: "the stimulus itself (and the ability to increase its effectiveness) becomes an advantage *per se*, regardless of any possible association with other aspects of male quality" (Eberhard, 1985: 71). In other words, the stimulus will be "arbitrary" in the sense that males which produce it are not necessarily the males which produce the best ejaculates, i.e. under this scheme, RIS/OSS are not used by females to assess quantity and/or quality of ejaculate, although incidental correlations cannot be eliminated.

Applying the above ideas to RIS/OSS, we would expect these substances not to have evolved in order to give an accurate measure of the quantity and/or quality of sperm and/or nutritious or otherwise beneficial accessory gland secretions transferred by males. In some species, RIS render females sexually unreceptive for the rest of their life (e.g. several species of *Aedes*; Craig, 1967; but see Young & Downe, 1982, on *Aedes aegypti*); whereas in other species OSS does not seem to exhibit a dosage-dependent effect (Table 1). In these cases, the Fisherian hypothesis could apply because females do not seem to be using these substances to evaluate the quantity and/or quality of sperm and/or nutritious substances in the ejaculate. However, in *A. aegypti* receptivity inhibition is not immediate and females could be evaluating males and giving a graduated response in terms of the length of time elapsed before the beginning of sexual refractoriness (W. G. Eberhard, personal communication).

According to this hypothesis, the dosage-dependent effect may be the result of a dosage-dependent stimulatory effectiveness of RIS/OSS (W. G. Eberhard, personal communication). Against this prediction, there is evidence suggesting that, in at least some species, RIS/OSS are used by females to assess ejaculate quality (see Section 4). Eberhard (1985)

stresses that in species in which females use male traits (RIS and/or OSS in our case) to assess male quality (sperm and/or nutritious substances in the ejaculate), runaway sexual selection would be "restrained" because of selection in favor of females that discriminate against males giving deceptive signals. As we have seen (Section 4), female responses to deceptive signals may evolve according to the handicap principle.

Another prediction of this theory is that male stimulatory traits subject to runaway sexual selection by female choice will evolve in a rapid and divergent way (Eberhard, 1985). Eberhard discusses in detail the reasons to expect such a rapid and divergent evolution of male traits (Eberhard, 1985: 71–73). In agreement with this prediction, OSS and RIS are species-specific in several Diptera (Chen, 1976, 1984; Fuyama, 1983). On the other hand, in three sibling species of the *D. melanogaster* species complex (*D. simulans*, *D. mauritania* and *D. sechellia*) the RIS of *D. melanogaster* was equally effective in inducing a refractory period (Chen *et al.*, 1988). Although the chemical structure of *D. sechellia* RIS was slightly different from that of *D. melanogaster*, it was also active in this last species (Chen & Balmer, 1989 in Spencer *et al.*, 1992). Lack of specificity has also been found in other Diptera (Leahy, 1967; Nelson *et al.*, 1969). In the case of the *Drosophila* species complex, it is important to consider the fact that these species are very closely related, and some experts consider these "slight" differences as a proof of, rather than evidence against, rapid divergence (Chen, 1984; Chen *et al.*, 1988). Clearly, more research is needed before any generalization about species-specificity of RIS/OSS is drawn.

6. Concluding Remarks

The two hypotheses advanced provide a series of testable predictions that must be empirically evaluated to define their relative importance for the evolution of RIS/OSS. Particularly important is the necessity of studying more non-dipteran species in detail, and the study of groups of related species to assess the degree of species specificity of these substances. The measurement of ejaculate quality, the relationship between RIS/OSS quantity and ejaculate quantity/quality, the possible costs of RIS/OSS production, and their chemical nature in species in which the main nutrient transferred is not a protein, are other areas in which empirical investigation is needed.

In some species RIS/OSS are not the only factors affecting refractory period length, response threshold for male attractiveness, and egg maturation and oviposition rates (Table 1). We can speculate that, at least in some cases, these multiple mechanisms of receptivity inhibition and oviposition stimulation could be a female evolutionary response to deceptive RIS/OSS, or elaboration of male signals under runaway selection. Recent models show that the handicap principle is not incompatible with these multiple mechanisms, if we consider the possibility of imperfect perception of RIS/OSS quantity/quality (Johnstone & Grafen, 1992). On the other hand, in some cases RIS/OSS may be complemented by these other factors as has been proposed for *Aedes aegypti* (Leopold, 1976). In this species the initial stimulus not to remate may be mechanical (the filling of the bursae with seminal fluid); this stimulus may "give time" for the RIS to act.

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APPENDIX

TABLE A1

Insect species in which the existence of a female sexual Receptivity Inhibition Substance (RIS) in the ejaculate secretions produced by male accessory glands (or other glandular parts of the male reproductive tract) has been looked for

Species	Characteristic	Reference
ORTHOPTERA		
Requena verticalis†	1?, 3, 4	Gwynne (1986, 1990)
Unnamed Zaprochilinae†	1?, 4	Simmons & Gwynne (1991)
DIPTERA		
Aedes aegypti	1, 2, 5?, 7?	Craig (1967), Gwadz (1972), Leopold (1976), Young & Downe (1982)
Aedes albopictus, *A. atropalpus*, *A. mascarensis*, *A. scutellaris*, *A. polynesiensis*, *A. sierrensis*, *A. togai*, *A. triseriatus*, *A. vittatus*, *Culex pipiens*, *Anopheles quadrimaculatus*	1, 5?, 7	Craig (1967)
Drosophila melanogaster	1, 2, 4, 6	Leopold (1976), Chen (1984), Gromko *et al.* (1984), Chen *et al.* (1988).
Drosophila simulans, *D. mauritania*, *D sechellia*	1, 2	Chen *et al.* (1988)
Drosophila funebris	1, 2, 4	Baumann (1974)
Lucilia cuprina	1, 3, 4	Smith *et al.* (1989, 1990)
Musca domestica	1, 2, 3, 4, 6?	Riemann *et al.* (1967), Leopold *et al.* (1971*a*, *b*)
Stomoxys calcitrans‡	1, 4	Morrison *et al.* (1982)
Delia antiqua	1, 2?, 4?	Spencer *et al.* (1992)
Hylemya brassicae (= *Delia radicum*)	1	Swailes (1971)
Glossina morsitans	1, 2?, 6	Gillot & Langley (1981)
Mayetiola destructor	1, 4, 6	Bergh *et al.* (1992)

† In the two orthopteran species female nutritional state affects the onset and/or length of the refractory period.
‡ Blood meal by males is necessary for the full effect of RIS to be expressed.
Key for characteristics: 1. RIS produced by accessory glands or equivalent tissues exists. 2. RIS is a peptide or a protein. 3. There is evidence suggesting a positive correlation between RIS quantity and ejaculate quantity. 4. There is a positive correlation between RIS quantity and refractory period length (dosage-dependent effect). 5. The filling of the female bursa affects the onset and/or length of the refractory period. 6. Sperm quantity or mechanical stimuli affect the onset and/or length of the refractory period. 7. Females refractory to subsequent insemination for life. "?" after the characteristic indicates that the effect has not been demonstrated but it probably exists.

TABLE A2

Insect species in which the existence of an Oogenesis or Oviposition Stimulant Substance (OSS) in the ejaculate secretions produced by accessory gland (or other glandular parts of the male reproductive tract) has been looked for

Species	Characteristic	Reference
ORTHOPTERA		
Locusta migratoria	NO 1, 6	Leopold (1976)

continued

TABLE A2 *continued*

Species	Characteristic	Reference
Melanoplus sanguinipes	1, 2, NO 3, 4	Pickford *et al.* (1969), Friedel & Gillott (1976), Leopold (1976)
Schistocerca gregaria	1, 2?, 3, 4, 5?, NO 6, NO 7	Leopold (1976)
Teleogryllus commodus	NO 1, 6	Leopold (1976)
COLEOPTERA		
Acantoscelides obectus	1, 2?, 3, NO 4, 7	Leopold (1976), Chen (1984)
Tenebrio molitor	NO 1	Leahy (1967)
Tenebrio obscurus	NO 1	Leahy (1967)
DIPTERA		
Aedes aegypti	1, 2, 3, 4, NO 5	Hiss & Fuchs (1972), Leahy (1967)
Aedes albopictus	1, 3, 4	Leahy (1967), Chen (1984)
Culex pipiens	1, 2?, 4, 6	Leahy (1967)
Anopheles gambiae	1, 4	Leopold (1976)
Drosophila melanogaster	1, 2, 3, 4, 5	Leopold (1976), Chen (1984), Chen *et al.* (1988)
Drosophila funebris	1, NO 2, 3, 4, NO 5	Baumann (1974), Chen (1984)
Drosophila suzukii, D. pulchrella	1, 3, 5	Fuyama (1983)
Lucilia cuprina	1, 4, 5?, 6	Smith *et al.* (1989)
Musca domestica	1, 2?, 4	Leopold (1976)
Stomoxys calcitrans	1, 3	Venkatesh & Morrison (1980), Morrison *et al.* (1982)
Glossina morsitans	NO 1	Gillott & Langley (1981)
Mayetiola destructor	1, 4, 5?	Bergh *et al.* (1992)
Delia antiqua	1, 2?, 4	Spencer *et al.* (1992)
Hylemya brassicae (= *Delia radicum*)	1, 4	Swailes (1971)
LEPIDOPTERA		
Bombyx mori	1, 3 and/or 4, 5	Yamaoka & Hirao (1977)
Trichoplusia ni	1, 4, 7	Leopold (1976)
Zeiraphera diniana	1, 4, 7	Leopold (1976)

Key for characteristics: 1. OSS produced by accessory glands or equivalent tissues exists. 2. OSS is a peptide or a protein. 3. OSS increases oocyte maturation. 4. OSS increases oviposition. 5. OSS effect is dosage dependent. 6. Testicular secretions act as OSS. 7. Sperm act as OSS. "NO" before the characteristic indicates that the effect has been looked for but not found. "?" after the characteristic indicates that the effect has not been demonstrated but it probably exists.

CAPÍTULO 7

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Short Communication

On the Evolutionary Origin of Nuptial Seminal Gifts in Insects

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KEY WORDS: nuptial gift; seminal substances; female choice; sexual selection.

Males of several insect species transfer to the female nutritious substances within the ejaculate known as *nuptial seminal gifts* (Simmons and Parker, 1989). The function of these substances varies among species from mating effort [risk and energy involved in obtaining fertilizations (Gwynne, 1984)], including sperm protection (Wedell, 1993), to paternal investment (Gwynne, 1988). The possible paternal investment function has been the subject of some controversy (Wickler, 1985, 1986, 1994; Gwynne, 1986; Simmons and Parker, 1989): critics argue that since sperm precedence may create stepfathers, the main function of nuptial gifts is related to mating effort (Wickler, 1985, 1986). Other authors suggest that both functions may occur within a species, and their relative importance may vary depending on ecological conditions (Gwynne and Simmons, 1990; Oberhauser, 1989, 1992).

Despite the controversy on the selective pressures responsible for the maintenance and elaboration of nuptial gifts, most researchers accept Wickler's (1985) idea on the evolutionary origin of seminal gifts (see also Simmons and Parker, 1989). According to this hypothesis, seminal nuptial gifts are the result of (i) selection for high sperm counts as a result of sperm competition (i.e. intrasexual selection), followed by (ii) selection on females for digesting "excess" sperm (excess with respect to the quantity needed for successful fertilization) and (iii) subsequent evolutionary elaboration of the ejaculate in response to female choice based on gift quantity (Fig. 1a).

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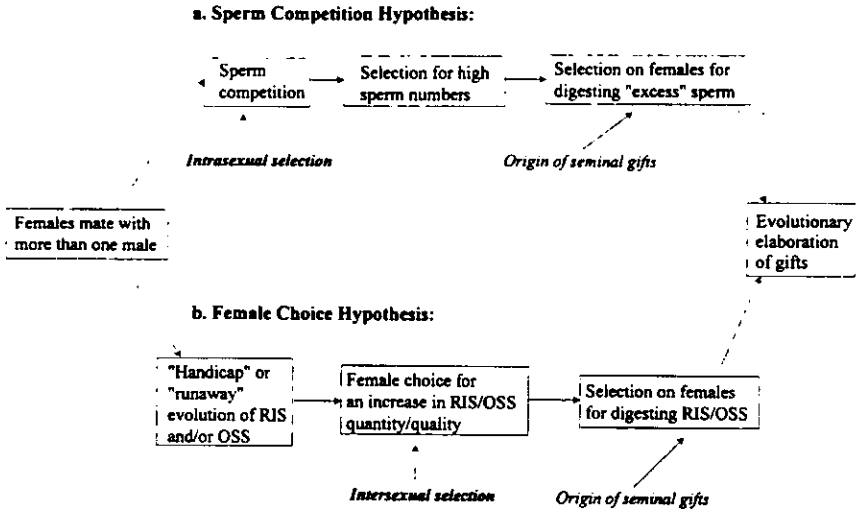

Fig. 1. Schematic outline of the hypotheses proposed to explain the evolutionary origin of nuptial seminal gifts: arrows indicate the temporal sequence of evolutionary innovations. (a) Wickler's hypothesis; (b) hypothesis advanced in this paper.

Recent ideas on the evolution of ejaculate chemicals that inhibit female sexual receptivity (RIS) or stimulate oogenesis or oviposition (OSS) in several groups of insects (Cordero, 1995; Eberhard and Cordero, 1995; Eberhard, 1996) provide the foundation for an alternative hypothesis for the evolutionary origin of nuptial seminal gifts. This hypothesis is based on the idea that some ejaculate chemicals constitute part of a male–female communication system (Cordero, 1995). Males transfer signals (RIS and OSS) to females, and females detect and assess these signals, using them to set a refractory period length or an egg maturation or oviposition rate. Biological signals employed in assessment between males and females may evolve according to the handicap principle (Zahavi, 1975) or to the runaway process (Fisher, 1958).

Handicaps. According to the handicap hypothesis, RIS and OSS evolved as a honest male–female communication system (Cordero, 1995). Females use RIS and OSS to evaluate (correctly, on average) the quality of the received ejaculate [which is a function of the genetic and phenotypic quality of sperm cells and of the quantity and quality of the ejaculate substances necessary for sperm activation, nutrition, and protection (Cordero, 1995)]. Females then decide how long to remain sexually unreceptive or the onset and rate of oogenesis and oviposition. Male honesty, expressed as a positive correlation between RIS and OSS quality and ejaculate quality, could evolve through the handicap principle (Zahavi, 1975; Grafen, 1990). According to this model, evolutionarily stable

signals (in the present case, RIS and OSS) used for assessment of signaler quality (=ejaculate quality) by receivers (females) must be honest (i.e., they should provide accurate information on ejaculate quality). The honesty of the signals is guaranteed, according to the handicap model, because female choice selects signals that are costly to produce, especially for males just able to produce low-quality ejaculates. The costs must be related to the quality revealed, ejaculate quality in our case, and must be a function of the gathering of raw materials for, and of the synthesis and storage of, RIS and OSS (Cordero, 1995).

As predicted, there is evidence (reviewed by Cordero, 1995) that RIS and OSS are costly and made up, in substantial proportions, of common limiting resources (*v. gr.* nitrogen). The commonly observed dosage-dependent effect of RIS and OSS, and data from some insects indicating RIS quantity is directly correlated with ejaculate quantity, suggests that RIS and OSS are used by females of at least some species to assess ejaculate quality (Cordero, 1995).

Runaway Evolution. According to the runaway hypothesis, RIS and OSS are selected by female choice only because of their effectiveness as stimulators (Cordero, 1995). In this model, females, as a result of time and resource-economy pressures, are selected to associate reproductive processes (in the present case, tendency to remate or to accelerate ovogenesis and oviposition) to the transference of RIS or OSS by males. Under these conditions, males producing chemicals which, by their nature, act as better stimuli (RIS which induce a longer period of sexual refractoriness or OSS which produce a bigger increase in oogenesis and oviposition rates) will be favored. Females that select these males will have the advantage of producing sons that will provide a better stimulus for their mates. In other words, the ability to increase the effectiveness of the stimulus will become an advantage per se, regardless of any association with other aspects of male quality (Eberhard, 1985, 1996). Under this scheme, RIS and OSS are "arbitrary" in the sense that they are not used by females to assess ejaculate quality, given that males producing the best (most stimulating) RIS and OSS will be only average in ejaculate quality (and other respects). In other words, there is no correlation between the effectiveness of RIS and OSS as stimulators, and the ejaculate quality, measured as the genetic and phenotypic quality of sperm cells and the quantity and quality of the ejaculate substances necessary for sperm activation, nutrition, and protection.

The frequently shown dosage-dependent effect of RIS and OSS of insects can also be explained by this model, given that it could be a female mechanism for discriminating among males purely on the basis of the (quantity-related) stimulatory effectiveness of RIS and OSS. Another prediction from the runaway model is a rapid and divergent evolution of the male stimulatory traits (Eberhard, 1985, 1996), an evolutionary pattern observed in several Diptera (Cordero, 1995; Eberhard, 1996).

Origin of Nuptial Seminal Gifts. The hypothesis for the evolutionary origin

of nuptial gifts derived from the above ideas is as follows: (i) selection for an increase in RIS or OSS quantity/quality as a result of female choice (i.e., intersexual selection) for honest chemical signals or purely stimulatory substances (Cordero, 1995), followed by (ii) selection on females for "digesting" RIS or OSS and (iii) subsequent elaboration of the nuptial gift through female choice and/or paternal investment selective pressures (Fig. 1b). During phase iii, new RIS or OSS could evolve according to the handicap hypothesis, in order to permit the evaluation of ejaculate quality (at this point, also a function of its nutritious content) by females (Cordero, 1995).

This hypothesis explains why in most, probably all, known cases, seminal gifts are accessory gland secretions (for recent examples and references see Bissoondath and Wiklund, 1995), not sperm cells (Thornhill and Alcock, 1983, p. 381; Ladle and Foster, 1992; but see Sivinski, 1984; Pitnick and Markow, 1994). The change from use of sperm to use of another type of ejaculate material as a nutrient source by females is an evolutionary step not explicitly addressed by Wickler's hypothesis. The basic difference between the two hypotheses lies in the proposed cause for the initial increase in ejaculate quantity: *intrasexual selection* (sperm competition) in the case of Wickler's idea and *intersexual selection* (female choice of RIS and OSS) in the case of the hypothesis advanced here (Fig. 1).

[The alternative explanation, that RIS and OSS are devices evolved by males in order to manipulate to their favor the physiology and behavior of females— as suggested by the seminal substances of some species, which apparently "mimic" messenger molecules from the female's own body—even if this manipulation decreases female fitness, does not provide an explanation for an increase in the quantity of RIS and OSS conducive to the evolution of seminal gifts. In such situations, natural selection on females could act as a brake on the possible sexually selected advantages to the female of favoring males with more powerful chemical inducers (Eberhard and Cordero, 1995).]

Much more study is necessary for evaluation of the relative importance of Wickler's hypothesis and that developed here. For example, we could use the comparative method (Harvey and Pagel, 1991) to measure the probability of seminal gift evolution given that the focal taxa already exhibit either sperm competition [spermatheca form, elasticity, and number, mating system, and courtship structure could be used to estimate potential sperm competition in groups not very well studied (Walker, 1980; Smith, 1984)] or RIS and OSS (detailed experiments are needed to determine this, although reliable morphological, physiological, of life history correlates could exist).

However, at this point we cannot perform this comparative test because we are not able to develop estimations of the number of times RIS, OSS, and seminal gifts have evolved independently. There are two reasons for this. On the one hand, the number of insect groups in which the existence of RIS and

OSS has been investigated is small relative to the number of insect taxa [about 4 orders, 21 genera, and 39 species (see Cordero, 1995)], although it is possible that these chemicals exist in several other groups. On the other hand, many species have not been tested for the presence of gifts, and the relatively recent discovery of seminal gifts in one of the best-known animal genera [*Drosophila* (Pitnick *et al.*, 1991)] suggests that many more insects could produce these substances.

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