El aprendizaje en insectos fitófagos: Diferencias en procesamiento de la información referente a rasgos de la planta hospedera y en expresión génica entre dos áfidos con distinto grado de especialización.

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RESUMEN

La especialización ecológica es un tema central en el estudio de las relaciones insecto-planta. La hipótesis de limitaciones neuronales (HLN) propone que los especialistas perciben, procesan y usan menos información y responden más eficientemente hacia los estímulos del hospedero, que los generalistas. Las limitaciones en la capacidad de aprendizaje pueden estar relacionadas con la ocurrencia de interferencia retroactiva (IR), la cual está asociada a defectos en la recuperación de información previamente aprendida debido a un nuevo evento de aprendizaje; su ocurrencia involucraría competencia entre respuestas en lugar de pérdida de memoria, además de bajos niveles de expresión del gen *foraging*. Además, el reconocimiento de la planta hospedera y posiblemente la evolución de la especialización sobre ésta, depende fuertemente de la percepción de señales químicas, la que involucra sensilas que contienen proteínas de unión a odorantes (Odorant-binding proteins, *Obp*). Se ha demostrado una relación entre diferencias en la expresión génica de estas

proteínas y la especialización ecológica. Esta tesis explora en el áfido generalista, *Myzus persicae sensu stricto*, y en la subespecie especializada en tabaco, *Myzus persicae nicotianae*: i) la existencia de limitaciones en el aprendizaje y, si estas existen, la ocurrencia diferencial de IR en el especialista, durante las etapas de reconocimiento a distancia y de reconocimiento sobre la superficie, de la planta hospedera; ii) la existencia de pérdida de memoria en áfidos, ante la eventual ocurrencia de IR; iii) la existencia de diferencias en la expresión génica del ortólogo para el gen *foraging* en el complejo *Myzus persicae* (*MpFor*), gen asociado a aprendizaje, y iv) la existencia de una relación entre número de sensilas olfativas, expresión relativa de *Obps* y la especialización ecológica de *M. p. nicotianae*.

La hipótesis de esta tesis propone una menor capacidad de aprendizaje, un mayor impacto de la IR, una menor expresión del gen *MpFor* y un menor número de sensilas olfativas y una mayor expresión de *Obps* en el áfido especialista, comparado con el generalista. Además propone a la competencia entre respuestas como explicación más robusta para la ocurrencia de IR, en lugar de la pérdida de memoria.

Las diferencias en las capacidades de aprendizaje entre los taxones de áfidos fueron evaluadas a través de bioensayos olfatométricos y registros de su conducta sobre la superficie foliar, tanto en sus respectivos hospederos de crianza (RH) como en hospederos alternativos (AH), usando áfidos con o sin experiencia en el AH. La expresión del gen *MpFor* fue evaluada mediante el método semicuantitativo de transcripción reversa de la reacción en cadena de la polimerasa (RT-PCR). Las hipótesis sobre la ocurrencia de IR fueron evaluadas mediante bioensayos olfatométricos con individuos que experimentaron transferencias al AH y retornos al RH de forma alternada, además de olfatometrías con exposición a dos hospederos simultaneamente. Finalmente, el número de sensilas asociadas a las rinarias primarias, el número de rinarias secundarias y la expresión de genes *Obp* fueron comparados también entre ambos taxones de áfidos.

Tras su experiencia sobre el AH, el áfido especialista perdió su preferencia olfativa original por los volátiles del RH, mientras que en el generalista ésta se mantuvo. Los efectos de la IR fueron evidentes sólo en el especialista, en variables conductuales durante la fase de reconocimiento sobre la superficie foliar del hospedero, tras experimentar un AH y ser evaluado sobre el RH. Por otro lado, el especialista mostró un mayor nivel de expresión de *MpFor* que el generalista. Además las respuestas olfativas se fueron perdiendo en la medida que se avanzó en las transferencias de hospedero, y no se observaron respuestas preferenciales en olfatometrías de exposición a dos hospederos de forma simultánea. Finalmente, el número de sensilas no difiere entre ambos taxones de áfidos; este resultado no varió tras corregir los valores por largo del segmento flagelar donde ellas se encuentran, o por tamaño corporal. Además, se registraron diferencias en la expresión relativa de genes *Obp* y diferencias en la secuencia del transcrito de *Csp3*, entre ambos taxones de áfidos.

En general, estos resultados podrían dar pie para extender los alcances de la HLN al incorporar evidencia empírica de sus efectos sobre otro rasgo cognitivo (aprendizaje). Estos resultados también confirmarían la hipótesis de un mayor impacto de la IR en el áfido especialista, para ambas fases de la búsqueda de hospedero. Por otro lado las diferencias en la expresión del gen *MpFor* podrían estar relacionadas con diferencias en adquisición de energía para el metabolismo de detoxificación de *M. p. nicotianae*, el cual le permite utilizar el tabaco como recurso; alternativamente, podría estar influenciando los resultados de desempeño de aprendizaje, posiblemente incrementando el desempeño de aprendizaje a niveles comparativamente menores de expresión de la enzima proteína kinasa G en el generalista, funcionando de forma similar a la proteína kinasa A, que maximiza la retención en memoria en individuos con sólo niveles de expresión medio de la enzima. Finalmente, los resultados permiten sugerir que las diferencias entre los quimiorreceptores de *M. persicae s. str.* y *M. p. nicotianae* pueden ser también responsables de las diferencias en amplitud de dieta entre estos áfidos y de la evolución de especialización en tabaco de *M. p. nicotianae*, si bien la influencia de otros factores no puede ser descartada.

ABSTRACT

Ecological specialization is a central topic in plant-insect relationships. The neural limitations hypothesis (NLH) proposes that specialists perceive, process and use less information and respond more efficiently to host stimuli than generalists. A limited learning capacity has been related to the occurrence of retroactive interference (RI), which is related to deffects in the retrieval of information previously learned due to a new learning event, and its occurrence may be explained mainly by a response competition rather than memory loss, and by lower levels of expression of the gene *foraging*. In addition, host-plant recognition and possibly the evolution of host-plant specialization heavily rely on chemosensory perception, involving antennal sensilla which house odourant-binding proteins (*Obps*); differences in genic expression of these proteins have been shown to be related with ecological specialization. We inquired in the generalist aphid, *Myzus persicae sensu stricto*, and the tobacco-specialised subspecies, *Myzus persicae nicotianae*: i) whether learning may be limited and, if

so, whether RI occurs in the specialist relative to the generalist, during the pre- and post-alighting phases of the host selection process, ii) whether the occurrence of RI is explained by memory loss in these aphids; iii) whether differences occur in the expression of the learning-associated orthologous gene *foraging* for the *Myzus persicae* complex (*MpFor*), and iv) whether a relationship between the number of olfactory sensilla, *Obps*-*Csps* genic expression and ecological specialization in the aphid *Myzus persicae nicotianae* exists. Differences in learning capacities between aphid taxa were assessed by olfactometric bioassays and by video recordings of their behaviour on the leaf surface, both on rearing (RH) and alternative host-plants (AH), with aphids either with or without experience on the AH; relative expression of gene *MpFor* was assessed by the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Also, hypotheses on the occurrence of RI were tested by olfactometric bioassays using aphids that had experienced several transfers to AH and subsequent returns to RH, and with aphids confronting two hosts simultaneously. Finally, the number of sensilla associated to primary rhinaria, the number of secondary rhinaria and the expression of *Obp* and *Csp* genes, were compared between these aphid taxa. I hypothesised a diminished learning capacity, a greater impact of RI, a decreased expresion of the gene *MpFor* and fewer olfactory sensilla and higher levels of *Obp* genes expression in the specialist than in the generalist taxon. Also, I hypothesised that RI is better explained by response competition than by memory loss in aphids.

After experience on AH, the specialist lost its original olfactory preference for RH, whereas such preference was conserved in the generalist; also, RI was demonstrated only in the specialist. Moreover, interference effects in the specialist aphid, but not in the generalist, were apparent in behavioural variables during the post-alighting phase of host searching, when it had experience on AH and was tested on RH. On the other hand, the specialist showed higher levels of *MpFor* expression than the generalist. In addition, the olfactory responses were lost after increasing the number of host transfers, and no olfactory response was detected in olfactometries with simultaneous presentation of two hosts. Finally, no differences were found in number of sensilla between both aphid taxa and this condition did not vary if the number of secondary rhinaria was corrected by flagellar segment length or body size. However, differences in the relative expression of *Obp* genes were detected, whereas only *Csp3* transcript sequences differed between these aphid taxa.

Overall, these results may extend the scope of the HNL to include empirical evidence of its effects on learning, a hitherto scarcely explored cognitive trait under the assumptions of the NLH. Also, they suggest a possible genetic basis to sustain the evolution of specialization in the *Myzus persicae* complex, by the possible effects of the gene *MpFor* on a potentially differential capacity for a higher energetic acquisition in *M. p. nicotianae*, which may sustain a detoxification system enabling the use of tobacco as a host. Alternatively, it may increase the learning performance in the generalist at comparatively lower levels of expression, functioning similarly as the cAMP-dependent protein kinase (PKA), which confers maximal memory retention in individuals with only intermediate levels of enzyme expression, thus potentially supporting the results on learning performance. The results also confirmed our hypothesis of a greater impact of RI on the specialist aphid, at both phases of host searching. Finally, the results suggest that chemoreceptor differences between *M. persicae s. str.* and *M. p. nicotianae* may be also responsible for their different diet breadths and the evolution of tobacco specialization in *M. p. nicotianae*, although the influence of other factors cannot be ruled out.

INTRODUCCION

Antecedentes generales

En la naturaleza, numerosas especies animales exhiben patrones de especialización en el uso de recursos. Al respecto, varias hipótesis han sido propuestas para explicar el origen de la especialización ecológica y en particular la especialización en insectos fitófagos (véase Schoonhoven y col. 2006 para revisión). Entre aquellas basadas en factores intrínsecos al individuo, la hipótesis de limitaciones neuronales o de procesamiento de la información (HLN; Bernays y Wcislo 1994; Bernays 2001; véase también Levins y MacArthur 1969) propone que individuos especialistas y generalistas difieren en la forma en que perciben, procesan y usan información relacionada con sus recursos, debido a limitaciones inherentes a sus sistemas neuronales, con diferencias en la precisión de sus decisiones durante la selección de la planta hospedera. La HLN establece que en general, las especies generalistas, dada la mayor variedad de recursos que utilizan, procesan una gran cantidad de información y poseen una gran capacidad neuronal que les permite cambios de atención a diversos estímulos y aprender características de estos estímulos para comparaciones posteriores. Por otro lado, los especialistas exhiben una alta sensibilidad hacia unos pocos estímulos relevantes, con respuestas directas y eficientes hacia estos, en línea con una baja capacidad neuronal (Bernays 2001). Además, los especialistas tendrían ventajas adaptativas en ambientes con baja variabilidad en cuanto a la oferta de especies vegetales como posibles hospederos, donde podrían reconocer su hospedero de forma más eficiente que el generalista, disminuyendo así los costos asociados al tiempo en la toma de decisiones, e invirtiendo comparativamente menos en maquinaria neuronal extra, liberando así recursos para ser invertidos en otro u otros rasgos relacionados con su adecuación biológica.

Hasta ahora la evidencia empírica que apoya la HLN muestra una mayor eficiencia en la toma de decisiones en la búsqueda y reconocimiento de hospedero, en especialistas (e.g., Janz y Nylin 1997; Bernays 1998; 1999; Bernays y Funk 1999; Bernays y Bright 2001; Oppenheim y Gould 2002; Bernays y col. 2004; Vargas y col. 2005; Egan y Funk 2006). Sin embargo, considerando que los rasgos cognitivos del individuo, entre los cuales se encuentra la toma de decisiones, están determinados por la cognición, definida ésta como los procesos neuronales responsables de la adquisición, retención y uso de la información (Dukas 2004; Pickens y Holland 2004), las limitaciones neuronales debiesen afectar a todos los rasgos cognitivos y no sólo a la toma de decisiones. En este sentido, la evidencia empírica no ha explorado cómo otro u otros rasgos cognitivos varían entre especialistas y generalistas, a la luz de la HLN. El aprendizaje es un rasgo cognitivo ampliamente documentado en insectos fitófagos (ver ejemplos en Daly y Smith 2000; Ramírez y Niemeyer 2000; Dukas y Bernays 2000; Mery y Kawecki 2004; Bernays y Bright 2005; véase también Dukas 2008). Tal como proponen Levins y MacArthur (1969), los generalistas debiesen hacer un mayor uso del aprendizaje que los especialistas. A su vez, la especialización debiese estar asociada a restricciones en la capacidad de aprendizaje (como consecuencia de limitaciones neuronales) que restringe la cantidad de información que un individuo es capaz de incorporar acerca de hospederos putativos.

La exposición al eventual aprendizaje de estímulos sucesivos puede dar origen a la interferencia retroactiva, definida como defectos en la recuperación de información previamente aprendida debido a un nuevo aprendizaje. Este es un fenómeno asociado a una menor capacidad de aprendizaje (Reaume y col. 2011), por tanto dada esta relación, diferencias en la ocurrencia de interferencia retroactiva podrían dar cuenta de diferencias en las capacidades de aprendizaje de los individuos. Además, la interferencia retroactiva está relacionada a un bajo nivel de expresión del gen *foraging* (*for*) en *Drosophila* (Reaume y col. 2011), gen también relacionado con la capacidad de aprendizaje (Mery y col. 2007). Se ha demostrado que una menor expresión de *for* está asociada a un menor desempeño del aprendizaje y con la ocurrencia de interferencia retroactiva, por tanto individuos que difieren tanto en sus capacidades de aprendizaje como en la ocurrencia de interferencia retroactiva, debiesen mostrar diferencias en los niveles de expresión del gen *for*.

Tres hipótesis han sido planteadas para tratar de explicar la ocurrencia de la interferencia retroactiva (Wixted 2004): a) pérdida de memoria, donde la información aprendida en una primera instancia es reemplazada por un nuevo aprendizaje; b) inhibición retroactiva, donde la nueva información activamente evita la recuperación de la información previa, y c) competencia entre respuestas, donde la falta de respuesta a la información previa se debe a la similitud en estimaciones cognitivas entre la información previa y la información nueva, por tanto, el organismo se muestra confundido o indeciso. Hasta ahora la evidencia no ha apoyado a la pérdida de memoria como hipótesis plausible, sustentando fundamentalmente a la competencia entre respuestas (Engelmann 2009); en insectos estas hipótesis han sido evaluadas sólo en abejas y en *Drosophila* (Cheng y Wignall 2006; Reaume y col. 2011).

En general, y dada su directa relación con las capacidades de aprendizaje en insectos, el fenómeno de interferencia retroactiva podría permitir estimar cómo las limitaciones neuronales afectan el aprendizaje, tanto en insectos especialistas como generalistas. Más importante aún, podría determinar la fidelidad hacia el hospedero en un especialista, puesto que la interferencia retroactiva limitaría la información que el insecto es capaz de recuperar al momento de buscar y reconocer su planta hospedera.

Por otra parte, el reconocimiento de la planta hospedera y posiblemente la evolución de la especialización ecológica en insectos fitófagos depende fuertemente de su capacidad de quimiorrecepción (Dicke 2000; Schoonhoven y col. 2006). La percepción incluye un primer nivel conformado por neuronas receptoras localizadas en sensilas olfativas ubicadas mayormente en las antenas (Galizia y Szyszka 2008; Galizia y Rössler 2010). Las proteínas de unión a odorantes (odourant-binding proteins, *Obps*) se ubican en estas sensilas olfativas (Nei y col. 2008; Sánchez-Gracia y col. 2009), y si bien sus funciones específicas no han sido explícitamente dilucidadas, una alta afinidad de ellas a odorantes ecológicamente relevantes para el insecto (Qiao y col. 2009; Biessmann y col. 2010; He y col. 2010; Sun y col. 2011) y una relación significativa con la especialización ecológica (Matsuo y col. 2007; Kopp y col. 2008; Dworkin y Jones 2009), han sido reportadas. En efecto, diferencias en el número de receptores antenales y en la expresión génica de *Obps* entre insectos especialistas y generalistas, han sido observadas (Bernays 2001; Dekker y col. 2006; Kopp y col. 2008; Dworkin y Jones 2009), no obstante sin una clara tendencia por un mayor o menor número de receptores y/o de niveles de expresión de *Obps*, ya sea en especialistas o generalistas.

Hipótesis

Los antecedentes descritos llevaron a la propuesta de dos hipótesis que fueron sometidas a prueba en esta tesis.

Hipótesis 1: Si, como propone la hipótesis de limitaciones neuronales, la capacidad de procesamiento de información acerca de la planta hospedera es mayor en un generalista que en un especialista, la capacidad de aprendizaje de las características de la planta, también será mayor en el generalista. Alternativamente, si la capacidad de aprendizaje del generalista es menor o igual que en el especialista, la HLN estará circunscrita exclusivamente al nivel de percepción de señales y no de procesamiento o almacenamiento de éstas.

Hipótesis nula 1: Las capacidades de aprendizaje y los sistemas receptores de especialista y generalista no muestran diferencias, por lo tanto la diferencia entre los grados de especialización de *Myzus persicae sensu stricto* y *Myzus persicae nicotianae* se explica por factores extrínsecos (por ejemplo competencia), o bien por factores intrínsecos que no involucran capacidades neuronales.

Hipótesis 2: Si, como propone la hipótesis de limitaciones neuronales, existe una mayor capacidad de aprendizaje en el generalista, esta mayor capacidad se debería reflejar en una menor incidencia de interferencia retroactiva y en mayores niveles de expresión del gen *foraging*. Por el contrario, si no hay diferencias en el aprendizaje y por tanto tampoco en la ocurrencia de interferencia retroactiva y la expresión del gen *foraging*, la diferencia en el grado de especialización entre *Myzus persicae sensu stricto* y *Myzus persicae nicotianae* estará dada por diferencias en el número de receptores antenales y/o por los niveles de expresión génica de *Obps*.

Hipótesis nula 2: Los niveles de expresión de genes de aprendizaje, tales como *MpFor*, y de quimiorrecepción, tales como las *Obps*, no difieren entre especialista y generalista, por lo que diferencias en la expresión de genes asociados a otro tipo de rasgos no relacionados con funciones neuronales, o simplemente factores extrínsecos, podrían explicar la diferencia en los grados de especialización entre *Myzus persicae sensu stricto* y *Myzus persicae nicotianae*.

Sistema de estudio

Myzus persicae sensu stricto (Sulzer) es uno de los áfidos más generalistas, capaz de alimentarse de más de 400 especies de plantas (Blackman y Eastop 2000). Por otra parte, una subespecie de *M. persicae*, *Myzus persicae nicotianae* (Blackman y Eastop) ha sido descrita como especialista en tabaco (Blackman 1987). Dadas las diferencias en el grado de especialización entre ambos áfidos, las que van de la mano de la etapa intermedia en el proceso de especiación que representa la categoría taxonómica de subespecie (por tanto ambos áfidos se encuentran potencialmente en pleno proceso de divergencia en algunos de sus rasgos), hacen de este complejo de áfidos un sistema interesante para: a) comparar las capacidades de aprendizaje entre un insecto especialista y uno generalista, b) evaluar la potencial ocurrencia de interferencia retroactiva y cómo ésta se manifiesta tanto en el especialista como el generalista, c) comparar los niveles de expresión del gen *foraging* entre un insecto especialista y uno generalista, y relacionarlos con sus respectivas capacidades de aprendizaje, d) explorar las hipótesis explicativas de interferencia retroactiva en insectos fitófagos que se alimentan del floema, y e) comparar sus receptores olfativos antenales (rinarias primarias y secundarias) y los niveles de expresión de genes *Obps*, y relacionarlos desde un punto de vista teórico con la especialización ecológica de *M. p. nicotianae*, donde la olfacción es crucial para el reconocimiento de su planta hospedera (Vargas y col. 2005).

Enfoque experimental

Las capacidades de aprendizaje en ambos áfidos fueron comparadas tanto en la fase de reconocimiento a distancia dentro el proceso de búsqueda de la planta hospedera, como en la fase sobre la superficie de ésta. Para ello se realizaron bioensayos olfatométricos (figura 1) y experimentos de registro de la conducta del áfido sobre la hoja de la planta (figura 2), utilizando insectos criados sobre hospederos de crianza (RH), con y sin experiencia sobre hospederos alternativos (AH). Durante las pruebas se podían ver enfrentados tanto a sus hospederos de crianza respectivos como al hospedero alternativo. De este modo, fue posible evaluar si ambos insectos eran capaces de aprender rasgos de las plantas y si eventos de experiencia adicional afectaban sus capacidades de recuperación de la información adquirida previamente.

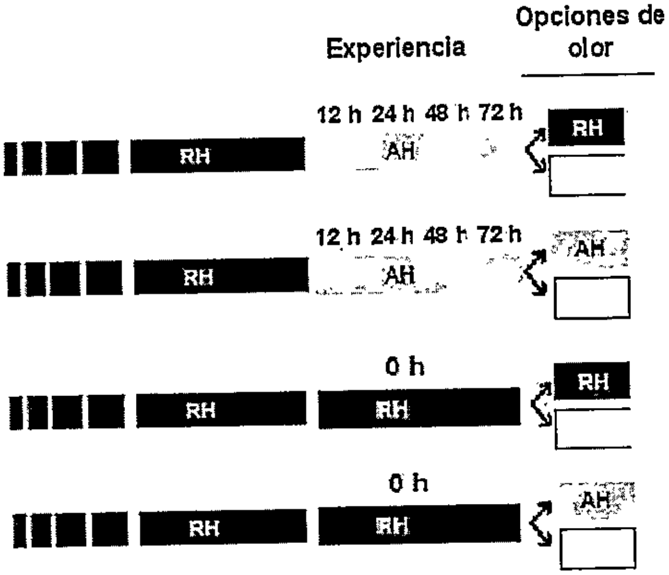

Figura 1. Diseño experimental de los bioensayos olfatométricos para experimentos de capacidad de aprendizaje en la etapa de reconocimiento a distancia. Los insectos son transferidos desde sus respectivos hospederos de crianza (RH) hasta un hospedero alternativo (AH) donde permanecen 0, 12, 24, 48 o 72 h. Los controles (0 h de experiencia en AH) corresponden a transferencias RH-RH. Cada tratamiento involucra olfatometrías donde el áfido se enfrenta a una fuente de estímulo (RH o AH) versus un blanco (aire).

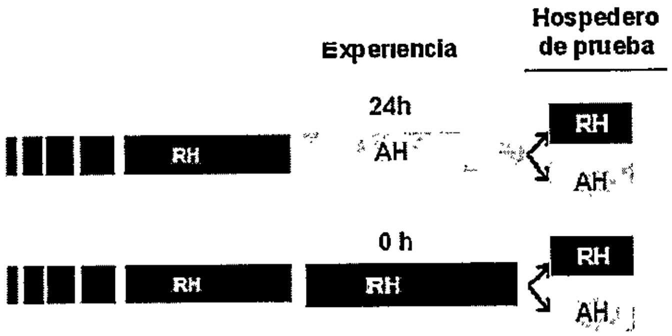

Figura 2. Diseño experimental de los bioensayos de registro de la conducta sobre la hoja, para experimentos de capacidad de aprendizaje en la etapa de reconocimiento sobre la planta. Los insectos son transferidos desde sus respectivos hospederos de crianza (RH) hasta un hospedero alternativo (AH) donde permanecen por un tiempo de 24 h. Los controles (0 h de experiencia en AH) corresponden a transferencias RH-RH. Cada tratamiento involucra el registro de la conducta de áfidos probados sobre RH y sobre AH.

Adicionalmente, y también a través de bioensayos olfatométricos, fue posible explorar la(s) hipótesis explicativa(s) de interferencia retroactiva que pudiese(n) estar operando en estos insectos fitófagos consumidores de floema. En este caso, individuos que experimentaron varios cambios de hospedero de forma sucesiva fueron evaluados (figura 3), donde la experiencia en cada evento debiese reemplazar la información previa o bien ir progresivamente reforzando las informaciones que el individuo posee.

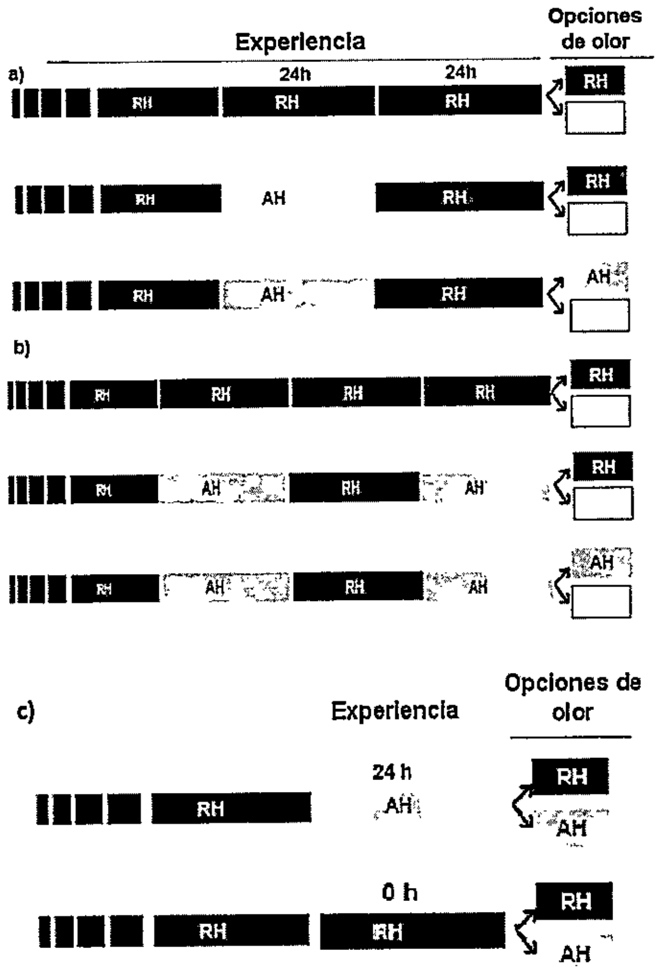

Figura 3. Diseño experimental de los bioensayos olfatométricos para experimentos de exploración de las hipótesis de interferencia retroactiva. Tres tratamientos fueron establecidos: a) transferencia desde hospederos de crianza (RH) hasta un hospedero alternativo (AH) y retorno al RH; b) transferencia RH-AH, retorno al RH y segunda transferencia a AH; c) olfatometrías con presentación simultánea de dos plantas-hospederas como fuentes de estímulo contrastantes (sin blanco), para áfidos con y sin experiencia de 24 h sobre AH. Para los experimentos de transferencias sucesivas, los áfidos permanecen por 24 h sobre cada hospedero, mientras que los controles (0 h de experiencia en AH) corresponden a transferencias RH-RH-RH y RH-RH-RH-RH. Los experimentos a) y b) involucra olfatometrías donde el áfido se enfrenta a una sola fuente de estímulo (RH o AH) versus un blanco (aire).

La expresión de los genes candidatos a comparar entre el especialista y el generalista, fue realizada mediante el método semicuantitativo de transcripción reversa de la reacción en cadena de la polimerasa (RT-PCR). De este modo, tanto el gen predicho como ortólogo en áfidos para *foraging* (este último asociado a aprendizaje e interferencia retroactiva), como los genes asociados a la percepción de señales químicas, *Obps* y *Csps*, fueron analizados en sus niveles de expresión relativa para generalista y especialista, y las secuencias de sus transcritos comparadas entre ambos taxones. También, con el fin de complementar la información acerca de los sistemas olfativos en *M. persicae s. str.* y *M. p. nicotianae*, sus receptores antenales fueron analizados mediante inspección microscópica, permitiendo el conteo de sus rinarias primarias y secundarias (figura 4) y cómo éstas se encuentran asociadas a variables morfológicas de los áfidos, para así evaluar eventuales relaciones entre los grados de especialización de cada taxón de áfido y los respectivos sistemas de recepción olfativa.

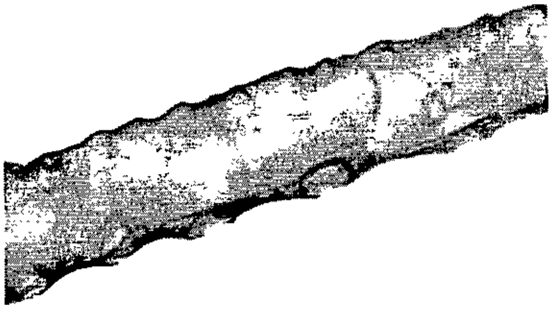

Figura 4. Sensilas olfativas localizadas en el primer segmento flagelar de la antena del áfido *M. persicae s. str.*. La fotografía fue tomada con un aumento de 40X.

Resumen de resultados

En general, si bien ambos taxones de áfidos fueron capaces de aprender señales tanto químicas como físicas de sus plantas hospederas (con la aparición de atracción olfativa hacia volátiles y mayor rapidez en las conductas asociadas al reconocimiento de la superficie foliar, del hospedero alternativo), *Myzus persicae nicotianae* manifestó interferencia retroactiva por efecto de la experiencia sobre un hospedero alternativo, tanto en bioensayos olfatométricos (por pérdida de la atracción hacia los volátiles del hospedero de crianza; véase capítulo 1) como en la exploración de la superficie de la planta (por mayor lentitud en las conductas de reconocimiento de la superficie foliar del hospedero de crianza; véase capítulo 2). Por el contrario, *M. persicae s. str.* no manifestó defectos en la recuperación de la información previa tras experimentar un hospedero alternativo, en ninguna de las fases de la búsqueda de hospedero. Así mismo, dado que los áfidos manifiestan efectos de interferencia tras sucesivas transferencias de hospederos (con pérdida de la atracción olfativa hacia volátiles de los hospederos), se descartaría la pérdida de memoria como hipótesis explicativa para la interferencia retroactiva (véase capítulo 1). Sin embargo, y contrario a lo esperado, el gen *MpFor* mostró una mayor expresión relativa en *M. p. nicotianae* (véase capítulo 1), lo cual si bien pudiese aún tener relación con las diferencias en aprendizaje entre los áfidos, también pudiese estar relacionado con diferencias en la adquisición de energía para un metabolismo detoxificador en el especialista. Por otro lado, no se registraron diferencias en el número de rinarias primarias y secundarias entre ambos áfidos, tampoco en sus relaciones con variables morfológicas de los áfidos, pero sí en sus varianzas, y, de los nueve genes putativos para quimiorrecepción analizados, se registró diferencias sólo en la secuencia del transcrito del gen *Csp3* y en los niveles de expresión relativa de los genes *Obp2*, *Obp4* y *Csp2* (véase capítulo 3), con mayores niveles de expresión de *Obp4* y *Csp2* en el generalista, y de *Obp2* en el especialista.

Estos resultados en su totalidad, fueron discutidos en los capítulos que conforman esta tesis y en del contexto de la evolución de especialización ecológica explicada por la hipótesis de limitaciones neuronales.

CAPITULO 1.

DIFFERENCES IN RETROACTIVE INTERFERENCE AND EXPRESSION OF THE GENE *FORAGING* IN TWO CLOSELY-RELATED INSECT TAXA WITH DIFFERENT DIET BREADTHS AND THEIR RELATIONSHIP WITH THE HYPOTHESIS OF NEURONAL LIMITATIONS FOR HOST SPECIALIZATION

RESUMEN

La especialización ecológica es un tema central en el studio de las relaciones insect-planta. La hipótesis de limitaciones neuronales (HLN) propone que los especialistas perciben, procesan y usan menos información y responde más eficientemente hacia los estímulos del hospedero que los generalistas. Exploramos en el áfido generalista, *Myzus persicae sensu stricto*, y en la subespecie especializada en tabaco, *Myzus persicae nicotianae*: i) si el especialista muestra una capacidad de aprendizaje limitada y, por tanto muestra una mayor ocurrencia de interferencia retroactiva comparado con el generalista, y ii) si se presentan diferencias en la expresión del gen asociado a aprendizaje, *foraging*. Adicionalmente, pusimos a prueba la pérdida de memoria como hipótesis explicativa de interferencia retroactiva. Las diferencias en capacidades de aprendizaje entre los áfidos fueron evaluadas mediante bioensayos olfatométricos, con olores provenientes del hospedero de crianza (RH) y de un hospedero alternative (AH), tras experiencia sobre este último. La expresión del gen *MpFor* fue evaluada mediante el método semicuantitativo de transcripción reversa de la reacción en cadena de la polimerasa (RT-PCR). Tras experiencia sobre el AH, el especialista pierde su preferencia por el RH, mientras

que ésta se conserva en el generalista. La ocurrencia de interferencia retroactiva fue demostrada en el especialista, la cual sería explicada mejor por una competencia entre respuestas en lugar de la pérdida de memoria. Por otro lado, el especialista mostró mayores niveles de expresión del gen *MpFor*, lo que podría relacionarse con adquisición de energía para su metabolismo de detoxificación; alternativamente, podría estar influenciando los resultados de desempeño de aprendizaje, posiblemente incrementando el desempeño de aprendizaje a niveles comparativamente menores de expresión de la enzima proteína kinasa G en el generalista, funcionando de forma similar a la proteína kinasa A, la cual maximiza la retención en memoria en individuos con niveles de expresión medio de la enzima. Estos resultados podrían ayudar a extender los alcances de la HLN al incorporar evidencia empírica relacionada con sus efectos sobre un segundo rasgo cognitivo, el aprendizaje. Además, se sugiere un potencial sustento genético para la evolución de especialización en *M. p. nicotianae* que podría hacerse extensivo a otros sistemas biológicos.

INTRODUCTION

In nature, numerous animal species exhibit specialised patterns of resource use. To this respect, disclosing the evolutionary processes underlying resourse specialization is a central topic in studies of insect-plant relationship. Several hypotheses have been proposed to explain the origin of specialization in phytophagous insects (see Schoohoven et al. 2006 for review). Among those based on factors intrinsic to the insects, the neural limitations or information-processing hypothesis (NLH; Bernays & Wcislo 1994; Bernays 2001; see also Levins & MacArthur 1969) assumes that, due to inherent limitations of their neural systems, specialists and generalists differ in the way they perceive, process and use information related to their food items, leading to disparities in decision accuracy during host selection.

In general, the NLH states that within a continuous of strategies occurring in nature, generalists, who are faced to a greater amount of information to process associated to the larger variety of resources they can exploit (in line with a high neural capacity), switch their attention to different stimuli and retain characteristics of these stimuli in memory for later comparison, whereas specialists exhibit a high sensitivity to few relevant stimuli (in line with a low neural capacity) and fixed efficient responses to them (Bernays 2001). As a consequence, specialists gain advantages under constant environmental conditions (refferred to the offer of vegetal species as posible hosts), by avoiding the high costs of investing in greater neuronal machineries (hence focusing investments in other fitness-related traits) and the time constraints involved in attention to several stimuli.

Several cases involving phytophagous insects have been reported which support the NLH, particularly in relation with decision making (e.g. Janz & Nylin 1997; Bernays 1998; 1999; Bernays & Funk 1999; Bernays & Bright 2001; Oppenheim & Gould 2002; Bernays et al. 2004; Vargas et al. 2005; Egan & Funk 2006), revealing more efficient host-searching behaviour in specialists than in generalists.

Neural constraints should affect not only decision-making, but also other cognitive traits (sensu Dukas 2004; Pickens & Holland 2004) such as learning. This assumption may be currently supported by two lines of empirical evidence: i) the mushroom bodies, the main centres of memory storage and sensory processing in insects, have shown greater surface areas, volume of calyces and number of Kenyon cells, in generalist than in specialist beetles (Farris & Roberts 2005); and ii) learning differences in terms of dynamics of consolidating memory and number of trials needed to acquire long term memory have been found between naive individuals of two related species of parasitic wasps, where the generalist one required less time and also fewer trials to consolidate memory than the specialist one, although other ecological factors may also be involved in these cognitive differences (Smid et al. 2007). Thus, learning, a cognitive trait extensively documented in phytophagous insects (e.g. Raubenheimer & Tucker 1997; Dukas 1998; Daly & Smith 2000; Ramírez & Niemeyer 2000; Dukas & Bernays 2000; Mery & Kawecki 2004; Bernays & Bright 2005; see also Dukas 2008), might exhibit differences between specialists and generalists, with generalists making a more significant use of learning (Levins & MacArthur 1969; Bernays & Wcislo 1994). Consequently, host and even ecological specialization may be the result of learning constraints (for example, as a result of neural limitations) that restrict how much information an individual is able to incorporate.

Defects in retrieving previously learned tasks due to learning of new associations, a phenomenon known as retroactive interference, have been shown to be associated with diminished learning capacities (Reaume et al. 2011). On the other hand, low levels of expression of the gene *foraging* (*for*), which has been related to differences in long term memory capacities in *Drosophila* fruit flies (Mery et al. 2007), is also related to retroactive interference (Reaume et al. 2011). Thus, differences in the occurrence of retroactive interference and in *for* levels of genic expression may account for differences in learning capacities, due to neural limitations. Three hypotheses have been proposed to explain how memory may operate in retroactive interference (Wixted 2004): (i) memory loss, when previous memory is substituted by the new information; (ii) retroactive inhibition, when new information prevents or inhibits retrieving previous information, and (iii) response competition, when previous information is not retrieved given that the individual is uncertain about which information to use. In insects, these hypotheses have been tested only in honeybees and fruit flies (Cheng & Wignall 2006; Reaume et al. 2011); the reports supported response competition in both cases and discarded memory loss.

To the best of our knowledge, no study has empirically evaluated how the NLH affects cognitive traits different to decision manking, such as learning;

also, no study has shown how retroactive interference and consequently, the expression of the *foraging* genes are related with the evolution of ecological specialization in phytophagous insects; moreover, hypotheses which may explain the occurrence of retroactive interference has not been tested in true herbivores, particularly phloem-feeder insects.

Myzus persicae (Sulzer) is one of the most generalist aphid species, able to feed on more than 400 plant species of 40 families (Blackman & Eastop 2000), whereas the subspecies *Myzus persicae nicotianae* (Blackman & Eastop) has been described as a tobacco specialist (Blackman 1987). These aphid taxa, given their close phylogenetic relationship, constitute a good system to empirically test the scope of the NLH on other cognitive traits of phytophagous insects and therefore, to compare the learning capacities between a specialist and a generalist insect. Thus, to compare the capacities of these aphids to learn host traits, and to evaluate differences in retroactive interference which may determine fidelity of the specialist to its host, the host-selection behaviour of these aphids on rearing and alternative host plants was evaluated through olfactometric bioassays. Additionally, to evaluate whether the orthologous gene of *for* in the *Myzus persicae* complex (*MpFor*) is related to differences in learning capacities between these two aphid taxa, differences in genic relative expression of *MpFor* between them, were assayed by the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Because a greater expression of the *MpFor* gene is characterised by a greater long-term memory performance (Mery et al. 2007) and lower retroactive interference (Reaume et al. 2011) in fruit flies, we predicted higher levels of expression in the predicted orthologous of *for* in aphids, in the taxon exhibiting the lowest level of retroactive interference (i.e. *M. persicae s. str.*). Finally, we conducted olfactometric bioassays with aphids subjected to concatenated experiences on rearing and alternative hosts, and olfactometries confronting aphids with and without experience to two different hosts simultaneously, in order to assess the eventual involvement of memory loss in both aphid taxa. In this case, we focused mainly on the hypothesis of memory loss given the difficulty to differentiate between the effects of retroactive inhibition and response competition on a behavioural basis only, in this particular system.

MATERIALS AND METHODS

a) Insects and plants

Individuals of both aphid taxa were obtained from monoclonal cultures (regularly purified by regenerating them from single individuals), maintained in our laboratory for several generations in isolated chambers in rooms at 21 ± 2° C and L14:D10 photoperiod. Although *M. p. nicotianae* has been found in the field in Chile as a unique clone feeding only on tobacco (Fuentes-Contreras et al. 2004), it can be reared in the laboratory on a few other plant species, mainly Solanaceae plants (Semtner et al. 1998). *Myzus persicae s. str.* was reared on sweet pepper plants, *Capsicum annuum* L. (Solanaceae), and *M. p. nicotianae* on tobacco plants, *Nicotiana tabaccum* L. cv. BY 64 (Solanaceae), both optimal host-plants for each taxon, respectively (Olivares-Donoso et al. 2007); as alternative host-plant, thorn apple, *Datura stramonium* L. (Solanaceae), was employed for both aphids. In addition, given its high performance on sweet pepper under laboratory conditions, which is comparable to its natural performance on its optimal host, tobacco (Cabrera-Brandt et al. 2010), sweet pepper was also employed as another alternative host for *M. p. nicotianae*, to explore how its potential suitability as host can affect learning in this aphid taxon.

Host transfers were performed within a period of three days after the adult alates emerge. All bioassays were carried out in a room at 21 ± 2° C; ninety-day-old plants were used for all behavioural bioassays.

b) Olfactometric bioassays

For all experiments, a four-arm Plexiglass olfactometer designed by Pettersson (1970) was used. Two adjacent arms were connected with Teflon tubing to a glass bell-jar containing a test plant (stimulus arms); the other two arms were connected to an empty bell-jar (control arms). For dispersion of volatiles from the bell-jars, air previously purified by charcoal filters was sucked through a hole in the centre of the olfactometer with a resulting flow of 200 ml min-1. Thus, the arena of the olfactometer consisted of a flat surface with four arm zones (two stimulus and two control zones) and a central zone. The olfactometer was surrounded by a cylinder of white paper (h = 15 cm) to avoid external visual stimuli. The focal individual was gently introduced into the arena through the central hole using a fine paintbrush, and its behaviour monitored for ten minutes after two minutes of acclimatization. Time spent in each arm of the olfactometer was registered using The Observer software v. 3.0 (Noldus 1995); insects were discarded if they showed no reaction within 7 min of being placed in the olfactometer. After each experiment, tubing, bell-jars and olfactometers were washed sequentially with distilled water and ethanol and then oven-dried. To avoid bias, connections between the arms of the olfactometer and stimulus sources were periodically alternated and lighting was provided from above; to avoid pseudoreplication, individuals were tested only once (Ramírez et al. 2000). For all olfactometric bioassays, total times spent in stimulus and control zones were compared using a Student's t-test for paired data.

c) Assessment of learning performances

To identify changes in the original preferences of both aphid taxa due to previous experience on an alternative host (AH), olfactometric bioassays were conducted with aphids withdrawn from their respective rearing hosts (RH) and transferred for 0 (control by transference to another RH), 12, 24, 48 and 72 hours to an AH. Thorn apple was chosen as the common AH for both aphid taxa. RH was used as stimulus in the olfactometer bioassays to assess whether the attraction to the RH mediated by its volatiles was retained after exposure for different periods to an AH. In order to assess whether olfactory learning occurs, AH was used as stimulus. Together, these two experiments assess the occurrence of retroactive interference. In all cases, treatments were replicated 10 times.

On the other hand, given that the performance of *M. p. nicotianae* on sweet pepper in laboratory studies approaches to the performance observed on tobacco (Cabrera-Brandt et al. 2010), sweet pepper was also used as AH for this aphid taxon. Olfactometric bioassays were performed to evaluate the effects of different periods of experience on sweet pepper on the original attraction to RH (using RH in olfactometric bioassays), and on learning (using this AH in olfactometric bioassays). In all cases, treatments were replicated 10 times.

d) Assesment of memory loss

Two experiments were performed to assess the eventual occurrence of memory loss in both aphid taxa. First, to assess how retroactive interference may operate after successive trainings, olfactometric bioassays were conducted with aphids subjected to concatenated experience times on AH and RH. For each aphid taxon, two treatments with their corresponding controls were established, with different periods exposed to RH and AH, as follows: (1) 24h on AH + 24h on RH (AH/RH) with its control, 24h on RH + 24h on RH (RH/RH) and (2) 24h on AH + 24h on RH + 24h on AH (AH/RH/AH) with its control, 24h on RH + 24h on RH + 24h on RH (RH/RH/RH); each treatment was replicated 10 times. By this procedure, each period of exposure to a new host may either reinforce or replace previous information acquired by the individual. In the first case, information from different hosts is progressively consolidated to a point where competition or inhibition between them occurs and information retrieval becomes difficult; this condition should lead to a loss of a significant response to volatiles after successive host transfers, in insects that otherwise should be attracted to the volatiles of their host-plants (Chittka 1998). In the second case, replacement of information should lead to a significant response only to volatiles of the last host experienced, a situation indicative of memory loss by replacement.

Also, given that all previous olfactometries may reduce potential response competition by excluding a second stimulus that may represent the competing response (using air, instead), olfactometries were performed with aphids simultaneously exposed to RH and AH. Therefore, if new information competed with and did not eliminate previous information, none of the responses that each stimuli elicited individually should be observed (Cheng & Wignall 2006). The degree of ecological specialization of the aphid and the experience on the AH were factorially combined into the following treatments: (1) *M. persicae s. str.* with experience on AH, (2) *M. persicae s. str.* without experience on AH, (3) *M. p. nicotianae* with experience on AH, and (4) *M. p. nicotianae* without experience on AH; aphids from each treatment were tested in the olfactometer using simultaneously RH and AH as stimuli. Ten replicates per treatment were performed.

e) Analysis of expression in the gen *MpFor*

Total RNA from fifty three-day-old alate individuals of each aphid taxon were separately extracted (1.3 µg/µl and 2.0 µg/µl for *M. persicae s. str.* and *M. p. nicotianae*, respectively) using the RNeasy Plant Mini kit (QIAGEN, Valencia, CA) according to the instructions of the manufacturer. Complementary DNA synthesis was performed using 2 µg of DNase-treated total RNA of *M. persicae s. str.* and *M. p. nicotianae*, respectively, using the ThermoScript™RT-PCR System (Invitrogen) in a total volumen of 22 µl. RT-PCR was performed with 1 µl of cDNA (972.96 and 974.35 µg/µl for *M. persicae s. str.* and *M. p. nicotianae*, respectively) as template for the amplification of gene *MpFor*. Specific primers (F: 5' AGTACGGACTTCGCTTTCAC 3'; R: 5' GCAAGATAGGAGGAGTTAGG 3') were designed based on the for EST sequence of the aphid *Acyrthosiphon pisum* retrieved from Aphidbase (http://www.aphidbase.com/aphidbase/) (Gbrowse accession number ACYPI008877-RA and ID = XM 001952056), using the software Primer Premiere v. 5.0 from PREMIER Biosoft International. The gene actin of *Myzus persicae* (*MpActin*) was employed as a control house-keeping gene, given that it has been used as house-keeping gene in other studies of genetic expression in *M. persicae* (e.g., Ghanim et al. 2006; Bos et al. 2010). *MpActin* was amplified from the following pair of primers: F: 5' CGGTTCAAAAACCCAAACCAG 3'; R: 5' TGGTGATGATTCCCGTGTTC 3' (Bos et al. 2010). RT-PCR for *MpFor* was performed in a total volume of 25 µl containing 10 mM dNTPs mixture, 2.0 mM Mg2+, 10 µM of each primer, 1 µl of template cDNA and 0.50 U of Pfu Ultra II Fusion HS DNA polymerase (Stratagene) in 1x polymerase chain reaction buffer. To determine the appropriate PCR cycling conditions for *MpActin* and *Mpfor*, PCR products after 20-30 cycles were visualised on an agarose gel to identify the linear range of the reaction (Ghanim et al. 2006). The selected conditions allowed for a clear visualization of PCR products in the gel and corresponded to the exponential phase of the reaction, thus avoiding saturating conditions for amplification. Therefore, amplification of *Mpfor* was performed at 95° C for two minutes, followed by 27 cycles at 95° C for 20 seconds, 56° C (annealing temperature) for 20 seconds and

72° for 1.5 min, with a final step at 72° for 10 min. On the other hand, RT-PCR for *MpActin* was performed at 94° C for 5 min, followed by 22 cycles at 94° for 30 seconds, 56° (annealing temperature) for 30 seconds and 72° for 1 min, with a final step at 72° for 10 min. RT-PCR products were visualised by loading 5 µl of each sample in a 1.5% agarose gel containing the nucleic acid gel stain GelRed™ (Phenix Research Products) and documented using an UV transilluminator and a Gel Logic 112 photographic system (Carestream Molecular Imaging). Bands were quantified from TIFF images (Ramírez et al. 2009) using the Carestream Molecular Imaging Software v. 5.0 (Carestream Molecular Imaging). Expression levels of *MpFor* transcripts were estimated as the intensity of a PCR fragment band relative to that of *MpActin* transcripts. RT-PCRs were independently replicated three times (technical replicates) and compared by a Student's t-test for independent data.

f) RT-PCR products sequencing and BLAST analysis

Purified PCR products of *M. persicae s. str.* and *M. p. nicotianae* were sequenced by Macrogen (Macrogen Inc, Seoul, Korea). Sequences were obtained from chromatograms using the software Phred (Ewing et al. 1998); sequences smaller than 100 bp were eliminated, low quality extremes cut and poly-T tails were removed. Assembly was performed with the software CAP3 (Huang & Madan 1999) using the default parameters (40 bp minimum overlap, 80% minimum identity). Multiple alignments were achieved using CLUSTALW (Thompson et al. 1994) with BLASTXs of assembled sequences. For comparisons between sequences, local alignments were performed using the Smith-Waterman algorithm (Smith & Waterman 1981), whereas for global alignments between two sequences, the Needleman-Wunsch algorithm was employed (Needleman & Wunsch 1970).

RESULTS

a) Differences in learning performances

When confronted with thorn apple as AH, attraction to RH in *M. persicae s. str.* persisted even after 72 h of experience on AH (figure 1 a), whereas in *M. p. nicotianae* attraction to RH was lost after 12 h of experience on AH (figure 1 b). On the other hand, *M. persicae s. str.* showed attraction to AH after 12 h of experience on it, whereas insects with no previous contact with this plant showed no attraction towards it (figure 1 c). Contrastingly, *M. p. nicotianae* showed no attraction to AH after experience on it, independent of experience time (figure 1 d). Finally, *M. p. nicotianae* did not exhibited attraction to RH after 12 h of experience on sweet pepper as AH, (figure 2a), but attraction to AH was found after 24 h of experience on it (figure 2 b).

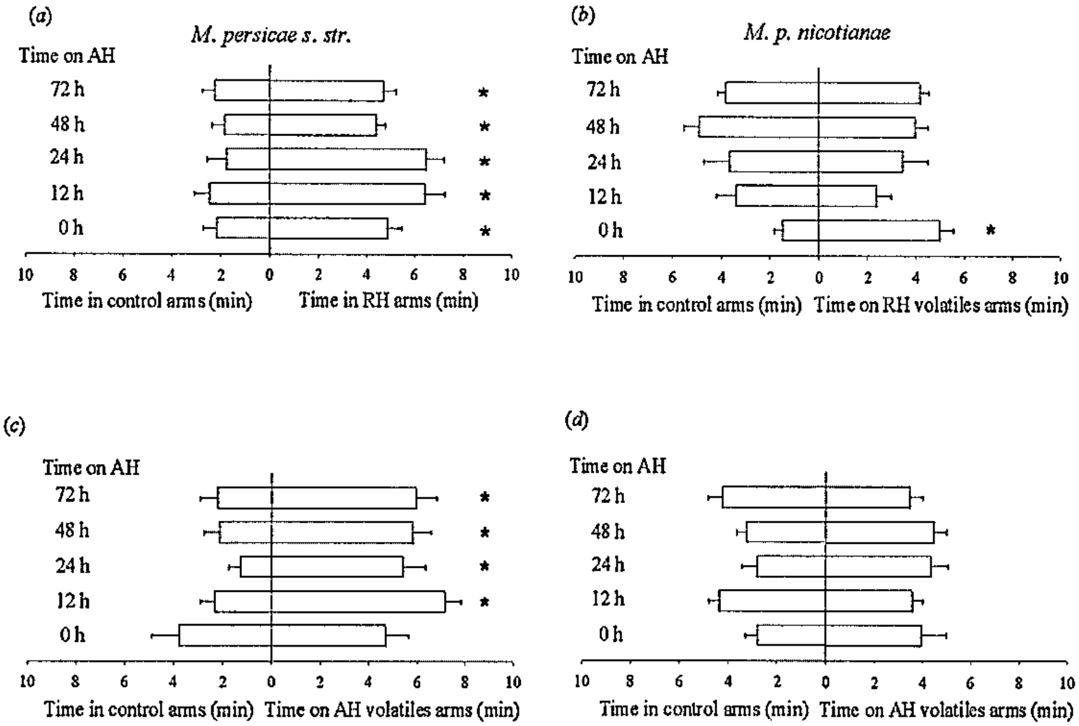

Figure 1. Olfactory preference of *Myzus persicae s. str.* (a,c) and *Myzus persicae nicotianae* (b,d) after different times of experience on an alternative host (AH). Rearing hosts (RH) were sweet pepper for *M. persicae s. str.* and tobacco for *M. persicae nicotianae;* alternative host was thorn apple for both aphid taxa in c) and d). Plants were absent in the control arms of the olfactometer. Data were compared by a Student's t-test ($n = 10$). Statistics for significant comparisons (* $p < 0.05$): (a) 0 h: $t = 2.840$, d.f. = 9, $P = 0.019$; 12 h: $t = 2.731$ d.f. = 9, $P = 0.023$; 24 h: $t = 3.361$, d.f. = 9, $P = 0.008$; 48 h: $t = 3.128$, d.f. = 9, $P = 0.012$; 72 h: $t = 2.627$, d.f. = 9, $P = 0.027$; (b) 0 h: $t = 5.464$, d.f. = 9, $P < 0.001$; (c) 12 h: $t = 3.920$, d.f. = 9, $P = 0.004$; 24 h: $t = 3.318$, d.f. = 9, $P = 0.009$; 48 h: $t = 2.709$, d.f. = 9, $P = 0.024$; 72 h: $t = 2.509$, d.f. = 9, $P = 0.033$.

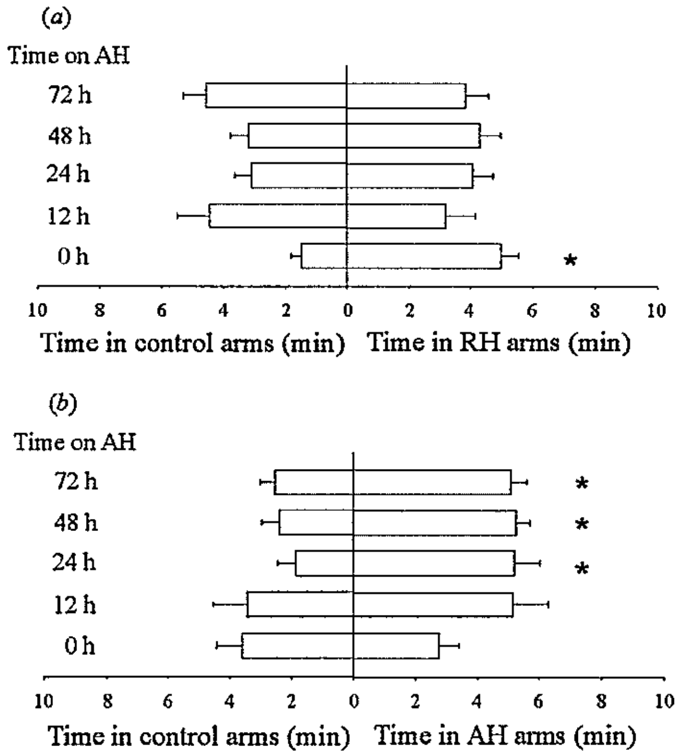

Figure 2. Olfactory preference of *Myzus persicae nicotianae* reared on tobacco (RH) after different times of experience on sweet pepper, the alternative host (AH). Plants were absent in the control arms of the olfactometer. Data were compared by a Student's t-test (n = 10). Statistics for significant comparisons (* $P < 0.05$): (a) 0 h: t = 5.464, d.f. = 9, $P < 0.001$; (b) 24 h: t = 2.525, d.f. = 9, $P = 0.032$; 48 h: t = 3.145, d.f. = 9, $P = 0.012$; 72 h: t = 2.708, d.f. = 9, $P = 0.024$.

b) Assessment of memory loss

Both *M. persicae s. str.* and *M. p. nicotianae* responded to RH only in control treatments (i.e. RH/RH and RH/RH/RH; figure 3). In treatments involving transfers to AH (AH/RH and AH/RH/AH) they did not respond to volatiles emitted either by RH or AH (figure 3). Finally, in olfactometric bioassays contrasting simultaneous response to RH and AH as stimuli, neither *M. persicae s. str.* nor *M. p. nicotianae* (both with and without experience on AH) showed attraction to any of the stimuli presented (figure 4).

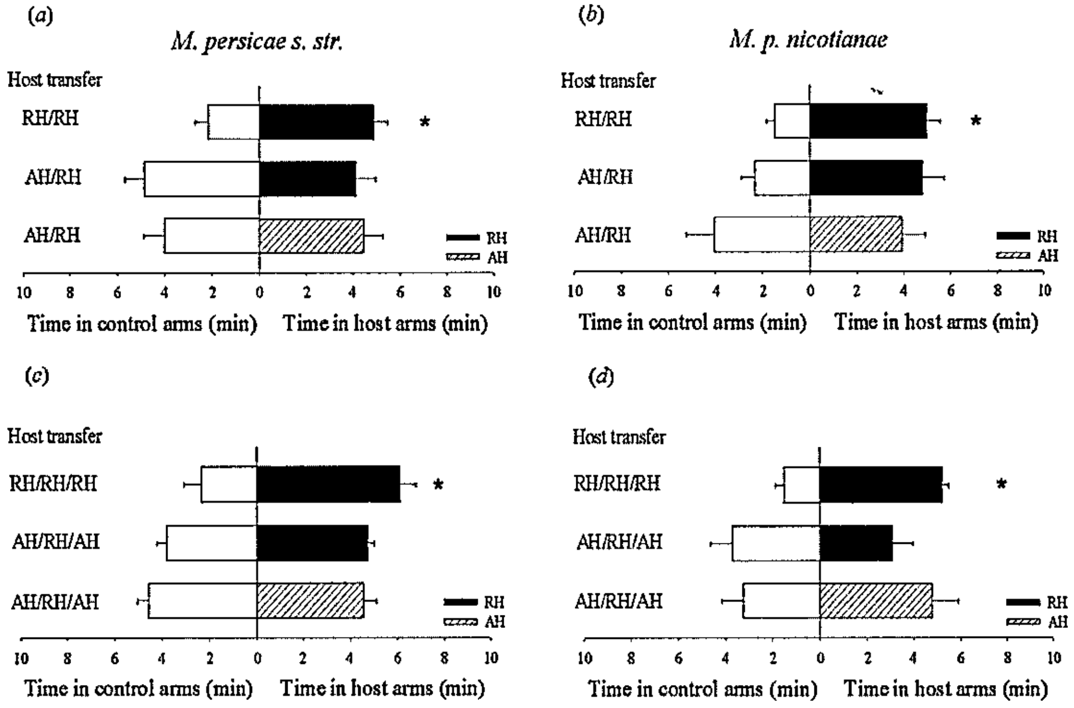

Figure 3. Olfactory preferences of *Myzus persicae s. str.* (a,c) and *Myzus persicae nicotianae* (b,d) after alternated concatenated 24 h periods in different hosts. Rearing hosts (RH) were sweet pepper for *M. persicae s. str.* and tobacco for *M. p. nicotianae*. Alternative hosts (AH) were thorn apple for *M. persicae s. str.* and sweet pepper for *M. p. nicotianae*. Plants were absent in the control arms of the olfactometer. Data were compared by a Student's t-test for paired data (n = 10). Statistics for significant comparisons (* $P < 0.05$): (a) RH/RH: t = 2.777, d.f. = 9, $P = 0.022$; (b) RH/RH: t = 5.464, d.f. = 9, $P < 0.001$; (c) RH/RH/RH: t = 2.777, d.f. = 9, $P = 0.022$; (d) RH/RH/RH: t = -7.206, d.f. = 9, $P = < 0.001$.

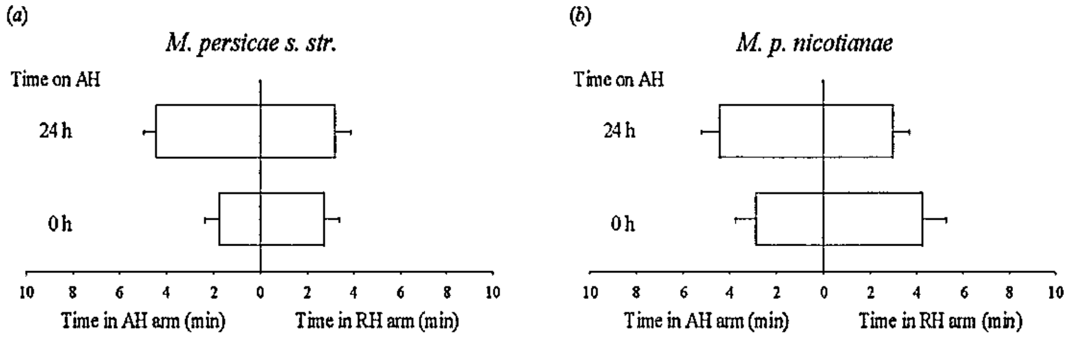

Figure 4. Olfactory preferences after 0 and 24 h of experience on an alternative host-plant (AH) by *Myzus persicae s. str.* (a) and *Myzus persicae nicotianae* (b). Rearing hosts (RH) were sweet pepper for *M. persicae s. str.* and tobacco for *M. p. nicotianae*. Alternative hosts (AH) were thorn apple for *M. persicae s. str.* and sweet pepper for *M. p. nicotianae*. Data were compared by a Student's t-test for paired data (n = 10).

c) Differences in gene *MpFor* expression

Myzus persicae nicotianae showed a significantly higher level of gene *MpFor* expression than *M. persicae s. str.* (t = 3.183, d.f. = 4, *P* = 0.033; figure 5). In addition, RT-PCR products sequencing and BLAST analysis confirmed that transcripts belonged to a *foraging* gene and were 99% and 96% identical to *A. pisum* and *D. melanogaster foraging* genes, respectively. These transcripts, although incomplete, corresponded to the coding region of the gene. In addition, they differed between the aphid taxa only in one synonymous mutation (G-A substitution), which did not modify the predicted amino acidic sequence. Both sequences (*M. persicae s. str.*: *MppFor1*, 901 bp sequenced; *M. p. nicotianae*: *MpnFor1*, 888 bp sequenced) were deposited in GenBank (Accesion numbers: *MppFor1*: JF776573, *MpnFor1*: JF776572).

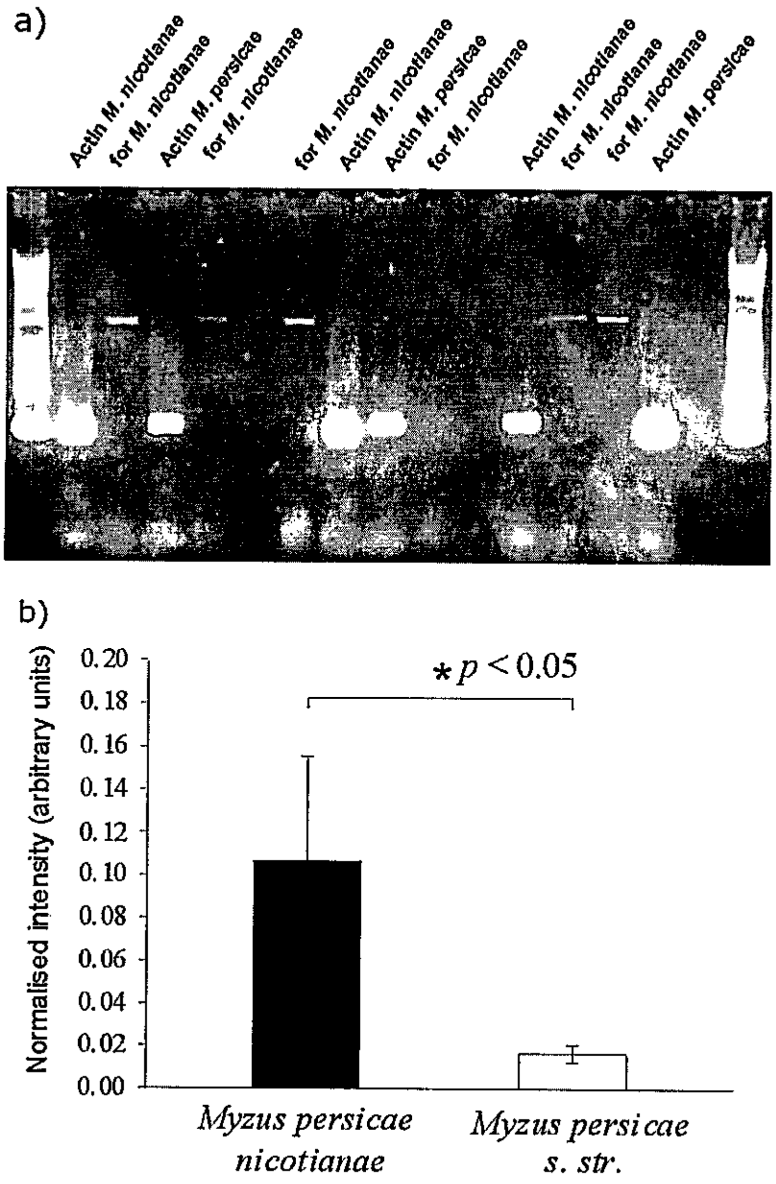

Figure 5. a) Visualization of *MpFor* PCR products of *Myzus persicae s. str.* and *Myzus persicae nicotianae* in agarose gel (three technical replicates). Products were charged in alternated order to avoid visual distortions due to position in the gel. b) Normalised abundance (mean ± SE) of *MpFor* gene transcripts in *Myzus persicae s. str.* and *Myzus persicae nicotianae*. Transcript levels were estimated based on MpActin-normalised values. Data were compared by a Student's t-test (n = 3). Significant comparisons: * $P < 0.05$.

DISCUSSION

a) Learning differences between specialist and generalist

We have demonstrated differences in learning perfoemances between a specialist and a generalist subspecies (at a single-clone level of comparison) of a phloem-feeding insect (figure 1), with the specialist exhibiting a decreased response to previous information acquired after experiencing novel information. Learning was not apparent in the specialist aphid *M. p. nicotianae* when the AH was thorn apple (figure 1 d), and also retrieving defects were observed when confronted with its RH (after experience on AH), for which previous olfactory attraction was indeed registered (figure 1 b). By contrast, the generalist aphid *M. persicae s. str.* was capable of fully retrieving information of its RH even after 72 h of experience on AH, and to acquire new information without the occurrence of retroactive interference (figure 1 a,c). These findings are in agreement with the prediction from the NLH of greater learning capacities in a generalist insect (Bernays & Wcislo 1994; Bernays 2001). Moreover, when AH was sweet pepper, *M. p. nicotianae* showed retrieval failures when the aphid was exposed to RH volatiles, in parallel with the acquisition of new information after 24 h of experience on AH (figure 2 a,b); in other words, it showed retroactive interference. In insects, this phenomenon has been demonstrated only in adult lepidopterans and hymenopterans (e.g. Chittka & Thompson 1997; Gegear & Laverty 1998; Weiss & Papaj 2003; Cheng 2005; Worden et al. 2005; Cheng & Wignall 2006; Frasnelli et al. 2010) and recently in *Drosophila* (Reaume et al. 2011); thus, this is the first report of its occurrence in a phloem-feeding insect and the first concerned with host-plant specialization. In addition, to the best of our knowledge, no studies have assessed olfactory learning in aphids and in our experiments, *M. persicae s. str.* and *M. p. nicotianae* were both able, although differently, to learn new olfactory information emanated from new putative host-plants.

Decision accuracy has been proposed as a major promoter of ecological specialization (Tosh et al. 2009). Retroactive interference may play a crucial role in decision accuracy when, for instance, some information could be ignored as a result of retrieving failures thus avoiding disruption in the focus and accuracy of host selection decisions. This proposal may represent a mechanism to reduce the sensory input and the potential occurrence of an overloading of the neural capabilities of the individual (Bernays & Wcislo 1994), whose effects may prevent efficient searching behaviours. This mechanism could be particularly relevant when an insect has had previous experience with more than one plant species during its ontogeny. When previous information can be disadvantageous, retroactive interference could be beneficial to an insect ignoring it, as a form of adaptative forgetting (Kraemer & Golding 1997); these particular conditions could extend the NLH to situations where specialization may evolve under environmental variability in terms of the potential hosts available. It should be noted that failures in retrieving acquired information and memory limitations has been proposed to promote constancy in resource exploitation by pollinators (Weiss & Papaj 2003; Ishii 2005; Worden et al. 2005).

b) Memory loss in aphids

This study provided evidence that memory loss may not be involved in retroactive interference both in the specialist and the generalist aphid here studied. Neither aphid taxon was attracted to host-plant volatiles after transfers involving an alternate host (figure 3). Furthermore, both aphid taxa were unable to discriminate between AH and RH used simultaneously as olfactory stimuli (figure 4). The predominant literature on hypotheses to explain retroactive interference currently supports response competition and is opposite to the idea of memory loss and active deletion of previously acquired information (Bouton 2002; Wixted 2004; Engelmann 2009; Frasnelli et al. 2010; Reaume et al. 2011; see also: Cheng & Wignall 2006 and references therein). Our results are consistent with this idea, and suggest that acquired information neither decayed nor was replaced, but rather was maintained in memory and possibly was progressively reinforced after each host transfer. The consequence of this process was that information from each host becomes stronger and presumably compete with each other, leading to a lack of preference for any of the hosts. Even though aphids are animals with small and comparatively simpler brains, particularly in terms of the architecture of their mushroom bodies (Kollmann et al. 2010), and with comparatively few neurons and synapses (Kristoffersen et al. 2008), our results suggest that they are capable of maintaining information without decay; this reinforces the idea that memory loss is not a likely process in learning and memory, an issue of long debate (Bouton 2002; Wixted 2004).

Although we can not rule out the idea of response inhibition, two lines of evidence provide empirical support for the hypothesis of response competition: i) the competing processes of protein syntheses that consolidate memories of distinct acquired informations (Engelmann 2009), and ii) the lateralization of distinct informations (each in one brain hemisphere) which leads to perception of each one of them by one of the sides of the neuronal machinery, leading to competition (Frasnelli et al. 2010), Hence, we propose that retroactive interference in the specialist aphid (and also in the generalist when it has been "forced" to exhibit retroactive interference after several host transfers) may be caused by response competition, as has been previously proposed for bees (Cheng & Wignall 2006; Frasnelli et al. 2010).

The non-occurrence of memory loss and the probable occurrence of response competition may also account for the lack of host-plant discrimination in bioassays contrasting RH and AH simultaneously (figure 4), since after training with information associated to each host, they may have similar reinforcements and thus similar cognitive estimation and/or predictive values.

Nevertheless, host discrimination bioassays are not conclusive, since these aphids could be affected by a phenomenon termed odour-masking (see Vargas et al. 2005). This phenomenon is frequent among phytophagous insects and occurs when an insect is exposed to a mixture of stimuli which blocks its capacity to discriminate among host-plants (see Schröder & Hilker 2008 for review). Consequently, lack of discrimination in these aphids under the more complex scenario of simultaneous availability of rearing and alternative hosts, can not be exclusively attributed to non-occurrence of memory loss (and hence to response competition), but also to this masking effect. Nevertheless, it is worth noting that both phenomena, retroactive interference and masking, may be attributed to neuronal assymetries or lateralizations of brain functions (Schröder & Hilker 2008; Frasnelli et al. 2010).

c) Differences between specialist and generalist in gene *MpFor* expression

Contrary to our expectations and to our behavioural results, the specialist aphid showed greater levels of relative expression of the gene *MpFor*. The gene *foraging* encodes a cGMP-dependent protein kinase (PKG); although the mode of action of this enzyme is not fully understood, it has been associated to memory and learning performance (Mery et al. 2007) and other neuronal functions (Hofmann et al. 2006), and is expressed in the mushroom bodies in insects. However, *foraging* is also a highly pleiotropic gene with effects not only on neuronal functions, but also on foraging activity, animal dispersal and locomotion, food consumption, nutrient absortion and stress resistance (Reaume & Sokolowsky 2009), plus several other functions in vertebrate species (Hofmann et al. 2006). In addition, it is expressed in several different tissues, both in vertebrates and invertebrates (Hofmann et al. 2006; Kaun & Sokolowsky 2009). Thus, given that our findings in learning performance did not correlate with *MpFor* expression (but taking into account that *M. persicae s. str.* and *M. p. nicotianae* did exhibit differences in *MpFor* expresion), we hypothesise that this gene is actually influencing the differences in ecological specialization between these aphid taxa, but through processes other than learning and retroactive interference. For instance, perceptual and responsiveness differences to sucrose between *Drosophila* individuals with different levels of *for* gene expression, have been reported (Scheiner et al. 2004). Additionally, *for* higher expression levels in *Drosophila* are also related to increased carbohydrate absorption (Kaun et al.

2007) and thus to a greater energy acquisition. Therefore, it is possible that higher levels of gene *MpFor* expression in *M. p. nicotianae* are related to energetic requirements for its metabolic detoxification system which enable it to survive and reproduce in tobacco (Cabrera-Brandt et al. 2010). The role of *foraging* genes in energy homeostasis has been discused (see Kaun & Sokolowski 2009) and it may be part of the evolution of specialization and adaptation of *M. p. nicotianae* to tobacco, a host-plant where the generalist *M. persicae s. str.* can not reproduce (Cabrera-Brandt et al. 2010).

Furthermore, although the higher levels of expression found in the specialist were not expected in the light of previous evidence in *Drosophila* (see Mery et al. 2007; Reaume et al. 2011), the effects of *foraging* genes can be species-dependent (Reaume & Sokolowski 2009), where contrasting results in *for* orthologous expression-behavioural response correlations between different species are not uncommon. For instance, while forager workers show higher levels of *Amfor* expression than nurse workers in *Apis mellifera* (Ben-Shahar et al. 2002), levels of expression of *Pbfor* in the ant *Pogonomirmex barbatus* shows lower levels of expression in forager workers than in nurse workers (Ingram et al.

2005). Thus, it is not strictly necessary that *Myzus persicae* responses follow the same tendencies reported in *Drosophila*, and some effects of *MpFor* may have specific outcomes for these aphid taxa.

Finally, it is worth noting that high expression levels of cAMP-dependent protein kinase (PKA), an enzyme crucial for memory formation, have been demonstrated to reduce memory in comparison with individuals expressing only the 50% of the wild type levels in *Drosophila* (Horiuchi et al. 2008). Thus, it is possible that at least in these aphids, PKG may be acting as PKA does in *Drosophila*, confering maximal memory retention to individuals with lower levels of enzyme expression, in this case, the generalist aphid. If this is true, it is plausible to suggest that PKG is indeed involved in learning differences between these aphid taxa.

d) Conclusions

We have provided evidence for differences in learning performance between a specialist and a generalist insect (of the same species but differing at the subspecies level), as proposed by the NLH. Additionally, we have identified differences in expression of the gene *MpFor*, a gene related to learning and also to energy acquisition, which could possibly be promoting ecological specialization. Thus, we have extended the scope of the NLH by empirical testing of its effects on an additional cognitive trait (learning) and by proposing theoretical conditions for the NHL to work under environmental variability. Finally, we have provided one potential genetical basis that may introduce the necessary variation to explain the evolution of ecological specialization in *M. p. nicotianae*, and that could be acting in other systems with contrasting degrees of ecological specialization. Thus, it is crucial to expand this comparison to other taxa for more general evidence that supports our findings.

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CAPITULO 2.

DIFFERENCES IN RETROACTIVE INTERFERENCE BETWEEN A SPECIALIST AND A GENERALIST INSECT: EFFECTS ON THE POST-ALIGHTING PHASE OF HOST-SEARCHING BEHAVIOUR

RESUMEN

La interferencia retroactiva (IR) hace referencia a defectos en la recuperación de información previamente aprendida debido a un nuevo evento de aprendizaje, y ha sido relacionada con un menor desempeño del aprendizaje. Dada la existencia diferencial de limitaciones neuronales que podrían restringir la cantidad de información que un insecto especialista puede incorporar acerca de la planta hospedera, hipotetizamos un mayor impacto de la IR en los especialistas que en los generalistas. En insectos fitófagos y en particular en áfidos, la búsqueda de hospedero involucra una fase de reconocimiento a distancia y una de reconocimiento sobre la superficie de la planta. Durante la fase de reconocimiento a distancia, el áfido especialista, *Myzus persicae nicotianae*, mostró una mayor incidencia de IR que el áfido generalista, *M. persicae sensu stricto*. En este trabajo, evaluamos esta vez la ocurrencia de IR en la fase de reconocimiento sobre la planta, en estos taxones de áfidos, mediante videograbaciones de sus conductas sobre la superficie foliar de hospederos de crianza (RH) y de un hospedero alternativo (AH), utilizando áfidos con o sin experiencia sobre el AH; esta experiencia podría alterar la recuperación de información previamente incorporada.

Efectos de interferencia en el áfido especialista, que no fueron detectados en el generalista, fueron evidentes en el tiempo a la primera prueba, el tiempo que permanece probando antes de una prueba de larga duración (> 5 min), el número de pruebas antes de una prueba de larga duración, y la proporción de individuos que realizan una prueba de larga duración, cuando el insecto ha tenido experiencia sobre el AH y es probado en el RH. Los resultados confirmarían a nivel intraespecífico nuestra hipótesis de un mayor impacto de la IR en el especialista, para la fase de reconocimiento sobre la superficie de la planta. En este sentido, el presente trabajo presenta evidencia adicional en relación con diferencias en la capacidad de aprendizaje entre un áfido especialista y uno generalista (a nivel sub-específico), que podrían influir en el proceso de selección de hospedero en su totalidad y, en consecuencia, en la evolución de especialización ecológica.

INTRODUCTION

Retroactive interference (RI) involves defects in retrieving previously learned information due to the learning of new associations. RI has been related to diminished learning capacities (Reaume et al. 2011), proposed as a major cause of forgetting (Kraemer & Golding 1997; Bouton 2002; Anderson 2003; Wixted 2004, Engelman 2009), and verified in different taxa of the animal kingdom (Bouton 2002; Anderson 2003; Wixted 2004, Engelman 2009). In insects, RI has been shown to occur in adult lepidopterans and hymenopterans (e.g. Chittka & Thompson 1997; Gegear & Laverty 1998; Weiss & Papaj 2003; Cheng 2005; Worden et al. 2005; Cheng & Wignall 2006; Frasnelli et al. 2010), and more recently in Drosophila (Reaume et al. 2011) and aphids (see chapter 1).

Host searching in aphids involves pre- and post-alighting phases, in which different combinations of sensory modalities are used to assess plant suitability (Powell et al. 2006). Thus, during the pre-alighting phase, plant suitability is assessed mainly through the olfaction of plant volatiles (Niemeyer 1990; Pickett et al. 1992); during the post-alightening phase, mainly tactile and gustatory modalities are used and involve a wider range of stimuli, e.g. plant surface structures such as trichomes, epicuticular waxes and long-chain alkanes and other compounds they contain, plus internal plant metabolites (Powell et al. 2006). On the other hand, the degree of specialization in aphids may influence learning, decision-making and cognitive traits involved in host-plant selection (see chapter 1). Specialist aphids are restricted in the amount of information they can incorporate about different host-plants and hence are limited in their diet breadth, but they show improved accuracy in decisions related to host searching as compared with generalists (Bernays & Funk 1999; Vargas et al. 2005; see also chapter 1). In studies involving the specialist aphid, *Myzus persicae nicotianae*, and the generalist aphid, *Myzus persicae sensu stricto*, olfactometric bioassays during the pre-alighting phase of host searching showed higher incidence of RI in the specialist aphid (see chapter 1). Therefore, given that the range of host-searching stimuli for aphids is greater during the post-alighting phase and that specialist aphids are more restricted in their neural capacity than generalists, we hypothesise that RI will have a greater impact on specialist than on generalist aphids, at the post-alighting phase.

In the present work, we evaluated RI during the post-alighting phase of host-searching in the aphids *M.*

persicae s. str. and M. p. nicotianae. We performed video recordings of the behaviour of aphids on the leaf surface of rearing and alternative host-plants, with aphids either with or without experience on the alternative hosts. This experience might alter the retrieval of information previously experienced, especially in the specialist taxon.

MATERIALS AND METHODS

a) Insects and plants

Aphids were obtained from monoclonal cultures (regularly purified by regenerating it from a single individual) maintained in the laboratory for several generations, in isolated chambers at rooms at 21 ± 2° C and L14:D10 photoperiod. *Myzus persicae s. str.* colonies were reared on sweet pepper plants, *Capsicum annuum* L. (Solanaceae), and *M. p. nicotianae* colonies on tobacco plants, *Nicotiana tabaccum* L. cv. BY 64 (Solanaceae). These hosts have been described as optimal hosts for these aphid taxa (Olivares-Donoso et al. 2007) and were designated as the rearing host (RH) for each taxon respectively. On the other hand, in order to be able to compare results from bioassays performed during pre-alighting (see chapter 1) with post-alighting, thorn apple, *Datura stramonium* L.

was chosen as a common alternative host (AH) for both aphid taxa, introduced as an interference effect on the probing behaviour. Host transfers were performed within a period of three days after the adult alates emerge. All bioassays were carried out in a room at 21 ± 2° C; ninety-day-old plants were used for all behavioural bioassays.

b) Learning capacities during post-alighting behaviour

The effects of the transfer to AH on searching behaviour once the insect had landed on a host-plant (either RH or AH) were studied through video-recordings made with a digital video camera (Sony DCR-HC62); the video recordings were later analysed using The Observer software v. 3.0 (Noldus 1995). In our experiments, whole plants attached to a stand were employed. Recordings started when the aphid was gently placed with a fine paintbrush on the first mature leaf counted from the top to the bottom of the plant, and lasted until either the aphid flew away from the plant, remained inactive for more than 15 minutes, started a long duration probe (probe lasting more than five minutes), or until a pre-set observation period (30 min) was achieved. Position of the rostrum, antennae and body were used as indicators of probing by the aphid on the plant (Troncoso et al. 2005;

Vargas et al. 2005). The following behavioural patterns could be discerned: antennal and rostrum movements, wing displays, displacement on the leaf, and abandoning of the plant. In relation with these patterns, the variables evaluated were: (i) time before the first probe, (ii) time spent probing before a long duration probe, (iii) time to long duration probe, (iv) number of probes before the aphid performed a long duration probe, (v) proportion of individuals that performed a long duration probe, (vi) proportion of individuals that took-off from the plant, and (vii) time to take-off from the plant.

The degree of ecological specialization of the aphid taxon, the experience on the AH, and the test host were combined into the following treatments: (1) specialist with experience on AH, tested on RH; (2) specialist with experience on AH, tested on AH; (3) specialist without experience on AH, tested on RH; (4) specialist without experience on AH, tested on AH; (5) generalist with experience on AH, tested on RH; (6) generalist with experience on AH, tested on AH (7) generalist without experience on AH, tested on RH, and (8) generalist without experience on AH, tested on AH (n = 30 for each treatment). Given that behavioural changes due to learning of a new host during the pre-alighting phase of host searching were apparent after 24 h of experience on it and did not change dramatically afterwards (Tapia et al., submitted), this was the time chosen for experience treatments in the present study.

c) Statistical analysis

Behavioural variables were analysed using a three-way MANOVA on ranked data, since data was not normally distributed (Conover & Iman 1981); this was followed by an LSD post-hoc analysis to characterise specific differences. Comparisons between proportions were performed with a Chi-square contingency test for several proportions, followed by a Z-test for proportions with the Yates correction for continuity (Zar 1996). As mentioned above, factors were specialization, experience and host-plant.

RESULTS

Three-way MANOVA demonstrated that both degree of ecological specialization and experience on AH affected post-alighting behaviour of aphids, with also significant experience on AH × host-plant and specialization × experience on AH × host-plant interactions, and only marginally non-significant specialization × host-plant interaction (Table 1).

Table 1. Three-way MANOVA on ranked data of behaviours during the post-alighting phase of host-searching by the generalist *Myzus persicae s. str.* and the tobacco specialist *Myzus persicae nicotianae* (n = 30). Factors were degree of specialization (two levels: specialist-generalist), time of experience (two levels: with and without experience on the alternative host) and test-plant (two levels: rearing-host, RH; and alternative host, AH).

	Wilks' λ	F	Effect	Error	*P* level
Specialization	0.777	15.11	4	211	<<0.0001
Experience on AH	0.951	2.73	4	211	0.0302
Host	0.973	1.48	4	211	0.2081
Specialization ×Experience	0.969	1.65	4	211	0.1634
Specialization × Host	0.958	2.33	4	211	0.0572
Experience × Host	0.603	34.68	4	211	<<0.0001
Specialization × Experience × Host	0.834	10.47	4	211	<<0.0001

Post-hoc comparisons revealed that *M. p. nicotianae* without experience on AH initiated probing sooner on RH than when it had experience on AH; however, when it was tested on AH and had experience on AH, it performed a first probe sooner than when it did not have experience on AH (Table 2). Also, *M. persicae s. str.* with experience on AH and tested on RH performed a first probe sooner than *M. p. nicotianae* under the same treatment, and did not differ from *M. persicae s. str.* tested on RH but with no experience on AH. Additionally, when the test-plant was AH *M. persicae s. str.* with experience on AH performed a first probe sooner than when it had no experience on AH and also sooner than *M. p. nicotianae* with experience on AH.

Table 2. Behavioural variables (mean ± SE, and proportions) during the post-alighting phase of host-searching by the generalist *Myzus persicae s. str.* and the tobacco specialist *Myzus persicae nicotianae*. Variables were compared across aphid/experience/test-plant combinations. Within a row, different letters denote significant differences accross aphid/experience/test-plant combinations (see text for details of the statistical tests performed). Rearing hosts (RH) were sweet pepper for *Myzus persicae s. str.* and tobacco for *Myzus persicae nicotianae*. Alternative host (AH) was thorn apple for both taxa.

Variables	*M. persicae s. str.*				*M. p. nicotianae*			
	Without experience on AH		With experience on AH		Without experience on AH		With experience on AH	
	Test on RH	Test on AH	Test on RH	Test on AH	Test on RH	Test on AH	Test on RH	Test on AH
Time to first probe (min)	0.78 ± 0.12 af	1.34 ± 0.13 b	1.01 ± 0.16 ah	0.84 ± 0.16 a	1.84 ± 0.36 ce	2.69 ± 0.53 de	6.65 ± 1.66 d	1.32 ± 0.26 bcfh
Time probing before a long duration probe (min)	0.62 ± 0.16 a	2.03 ± 0.34 b	1.1 ± 0.29 A	0.6 ± 0.18 a	0.51 ± 0.15 a	2.09 ± 0.34 b	3.81 ± 0.58 b	0.69 ± 0.21 a
Time to long duration probe (min)	1.58 ± 0.28 a	4.77 ± 0.74 b	2.81 ± 0.44 c	2.16 ± 0.42 ac	2.03 ± 0.3 ac	9.11 ± 0.92 d	16.48 ± 1.64 d	2.36 ± 0.48 ac
Number of probes before a long duration probe	0.53 ± 0.12 a	2.03 ± 0.33 b	0.9 ± 0.15 A	0.7 ± 0.17 a	0.73 ± 0.15 a	2.63 ± 0.28 b	3.3 ± 0.54 b	1.27 ± 0.32 a
Proportion of individuals performing a long duration probe	1 a	0.97 ab	1 A	1 a	1 a	0.70 cc	0.77 b	0.73 cc
Proportion of individuals that took-off from the plant*	0 a	0 a	0 A	0 a	0 a	0.27 c	0.03 a	0.27 c
Time to taking-off (min)						10.15 ± 2.18	27.11	4.32 ± 1.26

* ab vs. c: Z = 2.170, P = 0.03; a vs. c: Z = 2.659, P = 0.008

Time spent probing before a long duration probe and number of probes before a long duration probe showed the same pattern. *M. p. nicotianae* experienced on AH and tested on RH probed longer and required more probes to achieve a long-duration probe than all other treatments (excepting *M. persicae s. str.* and *M. p. nicotianae* both without experience on AH and tested on AH). By contrast, *M. persicae s. str.* experienced on AH and tested on RH did not show differences compared to M. persicae s. str. without experience and tested on RH. Also, *M. p. nicotianae* without experience on AH spent more time probing and performed more probes before a long-duration probe than when it was experienced on AH, when both were tested on AH.

Post-hoc comparisons also revealed differences in the time to perform a long-duration probe among treatments. In general, *M. p. nicotianae* with experience on AH and tested on RH took more time to achieve a long-duration probe than the rest of the treatments excepting when it had no experience on AH and was tested on AH. *M. p. nicotianae* with no experience on AH and tested on AH took more time than all *M. persicae s. str.* treatments and also than *M. p. nicotianae* with experience on AH and tested on AH. Likewise, *M. persicae s. str.* without experience on AH and tested on AH took more time than the other treatments with *M. persicae s. str.*. *M. persicae s. str.* experienced on AH took more time than *M. persicae s. str.* with no experience on AH, both tested on RH

The proportion of aphids that performed a long-duration probe and the proportion that took-off from the plant, showed statistically significant differences between treatments ($\chi 2$ = 41.749, d.f. = 7, $P < 0.001$ and $\chi 2$ = 43.003, d.f. = 7, $P < 0.001$, respectively). In particular, *M. p. nicotianae* with experience on AH showed a higher proportion of individuals that did not perform a long-duration probe than when it had no experience, both tested on RH, and than all treatments with *M. persicae s. str.* (all cases: $Z = 2.388$, $P = 0.017$), excepting when the latter had no experience on AH and was tested on AH. However, comparing *M. p. nicotianae* with and without experience on AH tested on AH no differences in the proportion of individuals that did not perform a long duration probe were apparent. A single individual of *M. persicae s. str.*, with no experience on AH and tested on AH, did not perform a long-duration probe. Thus, *M. persicae s. str.* treatments did not exhibit statistical differences among them.

Finally, the time for the single individual of *M. p. nicotianae* with experience on AH which took-off from RH was 27.11 min, while time to take-off from AH it ranged from 0.95 to 10.84 min; the time for non-experienced *M. p. nicotianae* to take-off from AH ranged from 6.42 to 22.43 min. By contrast, generalist individuals did not take-off from the plant except for a single individual with no experience on AH which took-off from AH after 21.22 min. When the proportion of individuals that took-off from the plant was analysed, *M. p. nicotianae* with experience on AH and tested on RH differed from *M. p. nicotianae* with experience on AH was tested on AH (with experience on AH: *Z* = 2.169, *P* = 0.03; without experience on AH: *Z* = 2.170, *P* = 0.03). Also, when *M. p. nicotianae* with experience on AH was tested on AH, it showed a greater proportion of individuals that took-off from the plant that all *M. persicae s. str.* treatments. Finally, no individuals of *M. persicae s. str.* took-off from the plant.

DISCUSSION

Differences between *M. persicae s. str.* and *M. p. nicotianae* in the occurrence of RI were observed during the post-alighting phase of host-searching behaviour, as were earlier observed for the pre-alighting phase (see chapter 1). Interference effects on the specialist aphid were apparent when it had experience on AH and afterwards was tested on RH. Thus, time to the first probe, time spent probing before a long-duration probe and time to achieve a long-duration probe were largely longer than when the specialist did not have previous experience on AH, and than in the generalist (Table 2); also, more probes before a long-duration probe were required, and less individuals achieved a long-duration probe (therefore, less individuals showed host acceptance). A relationship between the difficulty of the task and the speed of the final decision has been established (Chittka et al. 2009); thus, longer times taken by the specialist under those experimental conditions may reflect difficulties in the process of host recognition as a result of retrieving failures of previous information, due to the experience on the AH. Taking into account that these failures are concomitant with learning of new information of a novel host, this response may reflect the occurrence of RI. By contrast, the generalist aphid with experience on AH tested on RH did not show significant differences in comparison with the treatment without experience on AH tested on RH (excepting for the time to achieve a long-duration probe); thus, retrieving failures of previous information were not the general tendency in the generalist. The results confirm our hypothesis of a greater relative impact of RI on the specialist aphid at the post-alighting phase of host searching.

The hypothesis of neural limitations or information processes for the evolution of ecological specialization (Bernays & Wcislo, 1994; Bernays, 2001) states that there are differences between specialists and generalists in the way they perceive, process and use information related to their resources, with specialists using a few stimuli in line with a limited neuronal capacity, and generalists with a greater amount of stimuli processed by a comparatively greater neuronal capacity (with trade-offs involved in each strategy; see chapter 1). Therefore, these differences can result in differences in learning capacities between specialists and generalists (Farris & Roberts 2005; Smid et al. 2007; see also chapter 1). Hence, differences in learning performance and in RI in post-alighting behaviours between the specialist and the generalist aphids were expected and confirm findings when pre-alighting behaviour was analysed (see chapter 1).

Also, learning improved recognition in both aphids since they exhibited longer times and higher frequencies in the behavioural variables evaluated when they did not have previous experience on the AH and were tested on the AH, than when they did have experience on AH. In this sense, insects and in particular generalists can benefit from learning, with more efficient feeding as a result of experience (Dukas & Bernays 2000; Bernays & Bright 2005). In summary, our results indicate that in these aphids, pre- and post-alighting phases of host-searching behaviours are influenced by learning and its associated processes. Nevertheless, it is important to note that recognition failures in the specialist aphid may be enhanced by the traits of its RH. Vargas et al. (2005) and Troncoso et al. (2005) reported that tobacco is a particularly complex host to cope with, which may explain a host-recognition process particularly difficult. On the other hand, an opportunistic behaviour in the generalist aphid may improve the speed in its behavioural responses and that no individuals abandoned the plant (Table 2).

Overall, the present work presents further evidence to suggest that learning differences between a specialist and a generalist aphid as a result of differences in neural limitations between them, may influence the whole process of host selection. This represent a necessary complement to the scope of the neural limitations hypothesis and consequently, for explanations on the evolution of ecological specialization.

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Capitulo 3.

Comparison of olfactory sensilla and genic expression of odourant-binding and chemosensory proteins between the generalist *Myzus persicae sensu stricto* and its specialised subspecies *Myzus persicae nicotianae*

RESUMEN

El reconocimiento de la planta-hospedera y posiblemente la evolución de la especialización sobre un hospedero, pueden depender fuertemente de la recepción quimiosensorial. Esta involucra neuronas receptoras olfativas localizadas en sensilas antenales, las que albergan proteínas de unión a odorantes (*Obps*), así como también a proteínas quimiorreceptoras (*Csps*). Se ha demostrado que diferencias en la expresión génica de estas proteínas están relacionadas con la especialización ecológica. Con el fin de evaluar la relación entre cantidad de sensilas olfativas, expresión génica de *Obps-Csps* y especialización ecológica en *Myzus persicae nicotianae*, el número de sensilas asociadas a las rhinarias primarias y el de rhinarias secundarias, y la expresión relativa de genes *Obp* y *Csp* fueron comparadas entre este áfido especialista y el generalista *M. persicae s str.*. En general no fueron detectadas diferencias en el número de sensilas, pero sí en la varianza en el número de rinarias secundarias entre ambos taxones de áfidos. Estos resultados no variaron si el número de rinarias secundarias era corregido por el largo del primer segmento flagelar o por el largo corporal; solamente fueron detectadas diferencias en tamaño corporal y en largo y asimetría del primer segmento

flagelar. Adicionalmente, se identificaron diferencias entre *M. persicae s. str.* y *M. p. nicotianae*, en la expresión relativa de *Obp2*, *Obp4* y *Csp2*, y también entre las secuencias de *Csp3*, con una sustitución T-G que modifica la secuencia aminoacídica de la proteína predicha. En resumen, nuestros resultados sugieren que el sistema olfativo y las potenciales diferencias en quimiorrecepción entre *M. persicae s. str.* y *M. p. nicotianae*, contribuirían a sus diferentes grados de especialización y a la evolución de especialización en tabaco de *M. p. nicotianae*.

INTRODUCTION

Ecological specialization is a widespread phenomenon among phytophagous insects, in which selection is limited to a small number of host-plant species from the variety available in the environment. Host-plant recognition and possibly evolution of host-plant specialization, heavily rely on chemosensory perception (i.e. olfactory and gustatory) by the insect (Dicke 2000; Schoonhoven et al. 2006), from reception and decodification of the chemical message to storage and integration of the information in higher centres of the brain. The first line of perception includes olfactory receptor neurons in olfactory sensilla located in the antennae and in some cases in maxillary palps and in legs (Galizia & Szyszka 2008; Galizia & Rössler 2010). A large family of multigene chemoreceptors, the odourant-binding proteins (*Obps*) are housed in these olfactory sensilla (Nei et al. 2008; Sánchez-Gracia et al. 2009). Although the specific functions of *Obps* have not been explicitly revealed, a high affinity to odourants ecologically pertinent for the insect (e.g. Qiao et al. 2009; Biessmann et al. 2010; He et al. 2010; Sun et al. 2011) and a significant relationship with ecological specialization (Matsuo et al. 2007; Kopp et al. 2008; Dworkin & Jones 2009) have been demonstrated. Chemosensory proteins (*Csps*) may correspond to a second class of *Obps* with similar but possibly more diverse functions than the latter and expressed in several different tissues (Monteforti et al. 2002; Gong et al. 2007). *Csps* differ in aminoacidic sequence and three-dimensional structure from *Obps*, and they are more conserved and are codified by fewer genetic sequences than *Obps* (Wanner et al. 2004; Sánchez-Gracia et al. 2009). However, similar evolutionary patterns between *Obps* and *Csps* have been proposed (Sánchez-Gracia et al. 2009; Zhou et al. 2010).

Differences in chemoreceptors might be expected between specialist and generalist insects. For instance, specialised insects may need more receptors for reception of specific volatiles than generalists, which by contrast may require the reception of a number of rather generic odours (Bernays 2001). However, dissimilar results have been reported when olfactory receptors are compared between specialists and generalists. Bernays et al. (2000) found a higher abundance of secondary rhinaria in specialist individuals of the aphid *Uroleucon ambrosiae* in comparison with generalist individuals; in contrast, fewer and shorter receptors were found in the specialist *Drosophila sechellia* compared with the generalist *D. melanogaster* (Dekker et al. 2006). On the other hand, *Obps* did not show specific tendencies in gene expression:

both increased and decreased expression of particular genes has been reported when specialist and generalist species were compared (Kopp et al. 2008; Dworkin & Jones 2009).

Host-plant volatiles constitute important signals for host-plant recognition by aphids (e.g. Quiroz & Niemeyer 1998; Vargas et al. 2005; Powell et al. 2006; Glinwood et al. 2007; Amarawardana et al. 2007; Webster et al. 2008). Two types of antennal sensilla are present in aphids: a) primary rhinaria, consisting in two large placoid sensilla located on the third and fourth flagellar segments, and two smaller placoid sensilla - which may be fused into one in some species- surrounded by three coeloconic pegs, next to the distal placoid sensillum; these sensilla occur in all morphs and developmental stages; and b) secondary rhinaria located on the first flagellar segment of adults and particularly, in winged morphs (Anderson & Bromley 1987). Primary rhinaria are the main receptors for the alarm pheromone and have been suggested to play a role in the detection of host-plant volatiles (Park & Hardie 2004), while secondary rhinaria are responsible for detection of plant volatiles in females and the sex pheromone in males (Pettersson, 1970). *Myzus persicae* (Sulzer) is one of the most generalist aphid species, able to feed on more than

400 plant species of 40 families (Blackman & Eastop 2000); the specialist subspecies *Myzus persicae nicotianae* (Blackman & Eastop) has tobacco as optimal host. We hypothesise that differences in the diet breath of these taxa may be partly explained by differences in their capacities to perceive signals from host-plants, through differences in the number of primary and secondary rhinaria and/or *Obps* and *Csps* expression. We tested this hypothesis by determining the mean number of primary and secondary rhinaria by microscopic inspection, and *Obps* and *Csps* expression by the semiquantitative reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

a) Aphids

Aphids were obtained from monoclonal cultures 8regularly purified from a single individual) maintained in our laboratory for several generations, in isolated chambers in rooms at 21 ± 2° C and L14:D10 photoperiod. *Myzus persicae s. str.* colonies were reared on sweet pepper plants, *Capsicum annuum* L. (Solanaceae), and *M. p. nicotianae* colonies on tobacco plants, *Nicotiana tabaccum* L. cv. BY 64 (Solanaceae), both optimal host-plants for each taxon, respectively (Olivares-Donoso et al. 2007).

All assays were carried out using three-day-old alate individuals, whose emergence was stimulated by a increased density of the colony on the host-plant, since crowding is the main triggering factor for emergence of winged morphs (Müller et al. 2001).

b) Comparison of primary and secondary rhinaria

Thirty individuals of *M. persicae s. str.* and 34 of *M. p. nicotianae* were separately stored in 70% ethanol and mounted on slides as described by Blackman & Eastop (2000). Preparations were recorded at X10 in an Olympus BX-51 microscope coupled with a CoolSnap-Pro digital camera (Photometrics, Tucson, AZ). Camera operation and image measures were performed using the software Image-Pro Express (Media Cybernetics, Silver Spring, MD). Analyses of differences in secondary rhinaria between the aphid taxa followed the procedures described Bernays et al. (2000) which included determination of the number of sensilla and the length of the first flagellar segment in the left and right antennae, body size (estimated as the length of the right prothoraxic femur), and the absolute asymmetry (Palmer 1994) in number of sensilla and in length of the first flagellar segment. A comparison of the variances in number of primary and secondary sensilla between the aphid taxa, and the number and the absolute asymmetry of sensilla associated to primary rhinaria (i.e. placoid sensilla and coeloconic pegs), were also analysed. As suggested by Bernays et al. (2000), measurements considered the right side of the aphid only except for the assessment of asymmetry.

c) Analysis of *Obp* and *Csp* genes expression

Total RNA from 50 three-day-old alate individuals of each aphid taxon were separately extracted using the RNeasy Plant Mini kit (QIAGEN, Valencia, CA) according to the instructions of the manufacturer. Complementary DNA synthesis was performed from 2 µg of DNase-treated total RNA of *M. persicae s. str.* (1.3 µg/µl) and *M. p. nicotianae* (2.0 µg/µl), respectively, using the ThermoScript™ RT-PCR System (Invitrogen) in a total volume of 22 µl. RT-PCRs were performed with 1 µl of cDNA (972.96 and 974.35 µg/µl for *M. persicae s. str.* and *M. p. nicotianae*, respectively) as template for the amplification of candidate genes. Specific primers were obtained from *Obp* and *Csp* primer sequences designed by Xu et al. (2009) for *Myzus persicae* and by Zhou et al. (2010) for *M. persicae Obp* orthologous from *Acyrthosiphon pisum* (Table 1). The gene actin of *Myzus persicae*

(*MpActin*), was employed as a control house-keeping gene, given that it has been used as house-keeping gene in other studies of genetic expression in *M. persicae* (e.g., Ghanim et al. 2006; Bos et al. 2010). *MpActin* was amplified from the following pair of primers: F: 5' CGGTTCAAAAACCCAAACCAG 3'; R: 5' TGGTGATGATTCCCGTGTTC 3' (Bos et al. 2010). RT-PCR for *Obp* and *Csp* genes were performed in a total volume of 25 µl containing 10 mM of dNTPs mixture, 20 mM Mg2+, 10 µM of each primer, 1 µl of cDNA template and 5 U/µl of RBC Taq DNA polymerase (RBC Biosciences Corp) in 1 x polymerase chain reaction buffer. To determine the appropriate PCR cycling conditions for *MpActin* and *Obp*s, PCR products after 20-30 cycles were visualised on an agarose gel to identify the linear range of the reaction (Ghanim et al. 2006). The selected conditions allowed for a clear visualization of PCR products in the gel, and were included in the exponential phase of the reaction and thus, avoiding saturating conditions for amplification. Hence, amplification for *Obp* genes were performed at 94° C for three minutes, followed by 20 cycles at 94° C for 50 seconds, 65° C (annealing temperature; decreasing in 0.5° at each cycle) for 1 minute and 72° for 50 sec, and by 7 cycles at 94° C for 50 seconds, 55° C for 1 min and 72° for 50 sec, with a final step at 72° for 10 min. On the other hand, RT-PCR for *MpActin* was performed at 94° C for 5 min, followed by 22 cycles at 94° for 30 seconds, 56° (annealing temperature) for 30 seconds and 72° for 1 min, with a final step at 72° for 10 min. PCR products were visualised by loading 5 µl of each sample in a 1.5% agarose gel containing a nucleic acid gel stain GelRed™ (Phenix Research Products); gel separations were recorded under UV transillumination. PCRs were independently replicated three times (technical replicates) and RT-PCR products were visualized by loading 5 µl of each sample in a 1.5% agarose gel containing the nucleic acid gel stain GelRed™ (Phenix Research Products), and documented using an UV transilluminator and a Gel Logic 112 photographic system (Carestream Molecular Imaging). Bands were estimated from TIFF images (Ramírez et al. 2009) using the Carestream Molecular Imaging Software v. 5.0 (Carestream Molecular Imaging). Expression levels of *Obps* transcripts were calculated as the intensity of a PCR fragment band relative to that of *MpActin* transcripts.

d) Validation of RT-PCR products by sequencing and BLAST analysis

Purified PCR products of *M. persicae s. str.* and *M. p. nicotianae* were sequenced by Macrogen (Macrogen Inc, Seoul, Korea). Sequences were obtained from chromatograms using the software Phred (Ewing et al. 1998); sequences shorter than 100 bp were eliminated, low quality extremes cut and poly-T tails removed. Assembly was performed with the software CAP3 (Huang & Madan 1999) using the default parameters (40 bp minimum overlap, 80% minimum identity). Multiple alignments were achieved using CLUSTALW (Thompson et al. 1994) with BLASTXs of assembled sequences. For comparisons between sequences, local alignments were performed using the Smith-Waterman algorithm (Smith & Waterman 1981), whereas for global alignments between two sequences, the Needleman-Wunsch algorithm was employed (Needleman & Wunsch 1970).

e) Statistical analyses

Comparisons in number of rhinaria and in flagellar length between aphid taxa were first performed with a generalised linear model using a Poisson distribution, since data were not normally distributed. These comparisons considered the taxon as factor (with two levels: specialist and generalist); body size and antennal length were considered as covariates only for secondary rhinaria, since the latter were distributed along the first flagellar segment whereas primary rhinaria were grouped in two specific sites of the antenna. In addition, variance in number of primary and secondary rhinaria were separately compared between aphid taxa, by a Levene test, while absolute asymmetry in number of sensilla and in length of the first flagellar segment were separately compared also with generalised linear models; body size (femur length) was compared with a Student's t-test for independent data. Finally, technical replicates of RT-PCR of each aphid taxon were compared by a Student's t-test for independent data, for each candidate gene separately.

RESULTS AND DISCUSSION

The length of the first thoracic femur (as an estimation of body size) of *M. persicae s. str.* (952 ± 10 µm) and *M. p. nicotianae* (1139 ± 13 µm) were significantly different ($t = 10.826$; d.f. = 60; $P < 0.001$). Additionally, the number of sensilla associated to primary rhinaria was 7.07 (± 0.15) and 6.81 (± 0.18) for *M. persicae s. str.* and *M. p. nicotianae*, respectively. The number of secondary rhinaria was 11.13 (± 0.18) and 11.06 (± 0.28) for *M. persicae s. str.* and *M. p. nicotianae*, respectively, with first flagellar segment lengths of 964 ± 9 µm and 1078 ± 15 µm, for *M. persicae s. str.* and *M. p. nicotianae*, respectively. Although significant differences in first flagellar segment length were found

($\chi 2$ = 4.22; P = 0.04), no statistical differences in number of both types of rhinaria (corrected and uncorrected by body size and first flagellar segment length in the case of secondary rhinaria). Interestingly, *M. p. nicotianae* showed a significantly greater variance than *M. persicae s. str.* in secondary rhinaria (Mpn s^2 = 2.602, Mpp s^2 = 1.016; $F_{1,62}$ = 5.805, P = 0.02; figure 1), but not in sensilla associated to primary rhinaria (Mpn s^2 = 1.061, Mpp s^2 = 0.61; $F_{1,57}$ = 3.304, P = 0.07). Absolute asymmetry values of primary rhinaria were 0.84 (± 0.12) and 0.73 (± 0.12), for *M. persicae s. str.* and *M. p. nicotianae*, respectively; of secondary rhinaria were 1.27 (± 0.21) and 1.85 (± 0.22) for *M. persicae s. str.* and *M. p. nicotianae*, respectively, and of first flagellar segment length were 27.24 (± 3.96) and 28.4 (± 5.18), for *M. persicae s. str.* and *M. p. nicotianae*, respectively; significant differences between aphid taxa were detected only in asymmetry in the length of the first flagellar segment ($\chi 2$ = 5.21; P = 0.02).

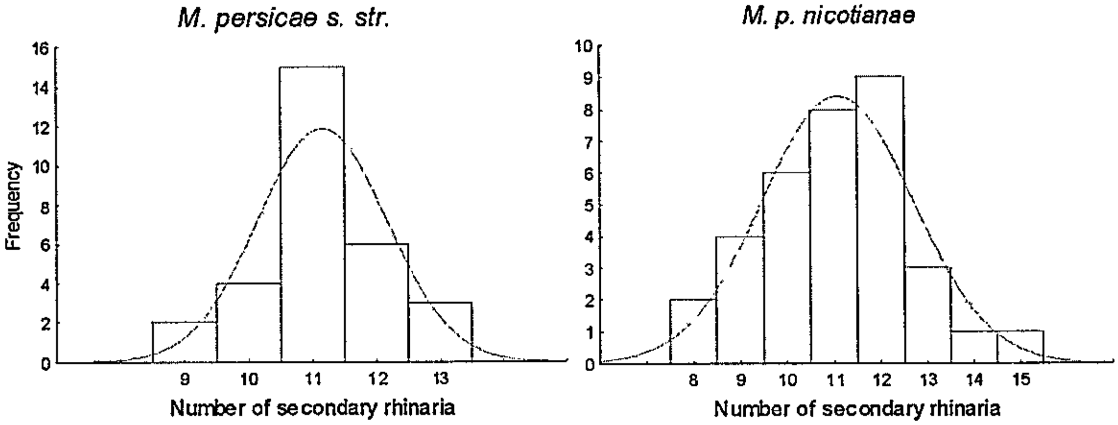

Figure 1. Histograms of frequencies in number of secondary rhinaria for the aphids *M. persicae s. str.* and *M. p. nicotianae*. Variances of these aphid taxa were compared by a Levene test for homogeneity of variances.

In general, when comparing number of receptors between specialist and generalist insects, the evidence is variable with no apparent tendency (Bernays et al. 2000; Dekker et al. 2006; see also references in Bernays 2001). Bernays et al. (2000) noted that variation in number of sensilla may depend on the general tendency observed at a coarse-grained comparison, i.e., at the phylogenetic level; in other words, whereas in an insect order where generalization is dominant a tendency for fewer receptors in specialist species may be observed, in one where specialization is dominant more receptors should be found in the specialists; this latter pattern may apply to aphids where specialization is predominant (Hille Ris Lambers 1979). However, our results showed only a greater variance in number of secondary rhinaria in *M. p. nicotianae*, a greater asymmetry in first flagellar segment length in the generalist *M. persicae s. str.*, and longer first flagellar segment in the specialist *M. p. nicotianae*. This data is not conclusive enough to establish a relationship between rhinaria and diet breath in these aphid taxa and the evolution of specialization in *M. p. nicotianae*, given that differences in first flagellar segment length did not imply differences in the number of secondary rhinaria. On the other hand, the differences detected in the variance in the number of secondary rhinaria could indicate the occurrence of stabilizing selection on the number of receptors in the generalist *M. persicae s. str.*, in contrast with the specialist *M. p. nicotianae*. Although the effect of using a single clone per each taxa in the analysis can not be neglected (therefore other patterns in rhinaria variance may be excluded), it is interesting to note that the sole clone of *M. p. nicotianae* present in Chile, may possess a high phenotypic plasticity in this trait and could represent one of many factors that explain its success, despite its monoclonal status (Fuentes-Contreras et al. 2004). On the other hand, the permanent purification of our colonies through several generations in order to maintain them as monoclonal cultures, as an artificial selection, may have reduced the variance in the number of rhinaria in the generalist; without the possibility to isolate this effect, we can only speculate about the explanations behind the difference in variance in the number of secondary rhinaria between these aphid taxa.

RT-PCR products sequences and BLAST analysis confirmed that transcripts belonged to six *Obp* genes and three *Csp* genes for the *Myzus persicae* complex (Table 1). Among them, differences in the nucleotide sequences between *M. persicae s. str.* and *M. p. nicotianae* were observed only in *Csp3*, with a T-G substitution that changes lysine for asparagine in the predicted amino acid sequence for *M. p. nicotianae* (figure 2). On the other hand, significant differences in the levels of relative genic expression between *M. persicae s. str.* and *M. p. nicotianae* were detected, in *Obp2*, *Obp 4* and *Csp2* (t = 7.442, d.f. = 4, *P* = 0.002; t = -4.818, d.f. = 1, *P* = 0.009; t = -8.596, d.f. = 4, *P* = 0.001) and only a statistically marginal in *Obp1* (t = 2.752, d.f. = 4, *P* = 0.051) (figure 3).

Table 1. Primers used for amplification of *Obp* and *Csp* cDNA by RT-PCR.

cDNA Primers				Length of PCR product	
Gene names	Forward primer 5' 3'	Reverse primer 5' 3'	Genbank accesión code	*M. persicae s. str.*	*M. p. nicotianae*
OBP1	TTCAACTGACAGAACACCGAA	TGGATGGAATCAAAACTGTATCGTT	FJ215308	496	499
OBP2	ACTCAGTTTTATTCTCAATCCACGAG	TTCCTTTGTACCCAGGATCTATCAG	FJ215309	609	604
OBP3	TATACACTTCGATCTTAAACGCAAAA	TTAATAGTAATTTAAAGGCGTACAGCG	FJ215310	556	539
OBP4	ATTCTACAGTTCACAATCAAAGTGCG	CTGGTTCGCTTGACTCTCGTTTC	FJ387486	487	499
OBP5	GCCAAGAAGAAATCAAATCC	ATAGAAATACAATGCCTAACTG	FM242544	277	224
OBP6	ATGACCGAAGGAATAAACA	TGCGGATTGGAATACTGG	FM242545	249	236
CSP1	CGCTTTTGAGTACAATACATTAAATCA	TTTGTAGAGTCCTTGGGGGTCAT	FJ387489	378	623
CSP2	GGCTTACCAAATATCGTGTCTTCTT	CATGCAACTTTGACGTTTGAGAG	FJ387490	629	628
CSP3	ACACAGGTCACCCACAACAACAC	TAGACGATTTTCACATTGCGACA	FJ387491	636	653

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                         1                  20                  40
M. p. nicotianae CSP3    HNNTRAVAAMNCKVLIALCCVAVYAAHASPAGAATAAAASADEEIKDFPAYM

M. persicae s. str. CSP3 HNNTRAVAAMNCKVLIALCCVAVYAAHASPAGAATAAAASADEEIKDFPAYM

                         54              70                  90
M. p. nicotianae CSP3    KRFDKLNVEQVLNNDRVLASHLKCFLNEGPCVQQSRDLKRVIPVIANNGCNG

M. persicae s. str. CSP3 KRFDKLNVEQVLNNDRVLASHLKCFLNEGPCVQQSRDLKRVIPVIANNGCNG

                         106           120                 140
M. p. nicotianae CSP3    CTERQMTTIKKSLNFLRTKKPVEWARLVNIYDPSGTKLNKFLDA-TT-YCDN

M. persicae s. str. CSP3 CTERQMTTIKKSLNFLRTKKPVEWARLVKIYDPSGTKLNKFLDA-TT-YCDN

                         158                   180                 200
M. p. nicotianae CSP3    TIT--YIYIIYYTARVRRLTIIRRSCR-YYVSHARLLVYTCIPTQDFARCRN

M. persicae s. str. CSP3 TIT--YIYIIYYTARVRRLTIIRRSCR-YYVSHARLLVYTCIPTQDFARCRN

                         217
M. p. nicotianae CSP3    VKIV

M. persicae s. str. CSP3 VNIV
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Figure 2. Amino acid sequences for predicted *Csp3* proteins of *M. persicae s. str.* and *M. p. nicotianae*, under 5'-3' reading frame 1. Alignments were performed with CLUSTALW; difference in amino acids is shown in a black box.

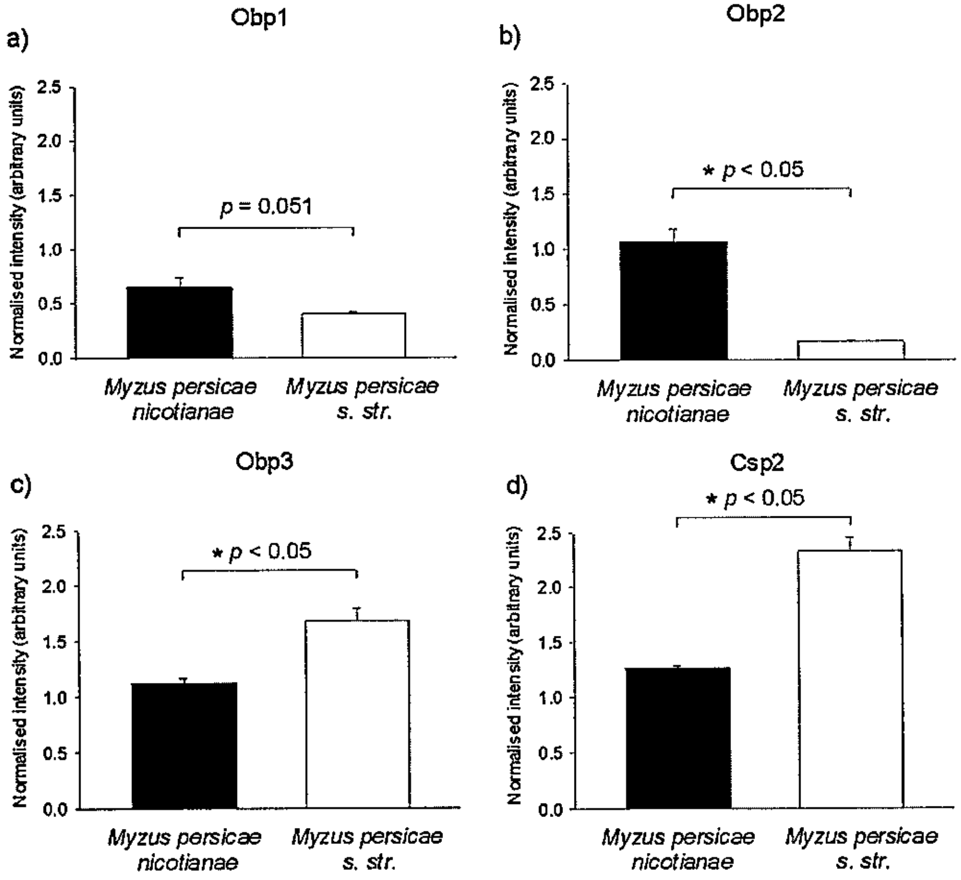

Figure 3. Normalised abundance (mean ± SE) of a) *Obp1*, b) *Obp2*, c) *Obp4* and d) *Csp2* gene transcripts in *Myzus persicae s. str.* and *Myzus persicae nicotianae*. Transcript levels were estimated based on *MpActin*-normalised values. Data were compared by a Student's t-test (n = 3). Significant comparisons: * $P < 0.05$.

In the light of these results, we suggest that *Obps* and *Csps* may play a role in the differences in ecological specialization between *M. persicae s. str.* and *M. p. nicotianae*. In particular, the relative down-regulation of *Obp4* and *Csp2* in *M. p. nicotianae* may be related to the loss of perception of possible repellent volatiles or deterrent secondary internal metabolites of the tobacco plant, thus enabling *M. p. nicotianae* to exploit it as a host; alternatively they may restrict the capacity to perceive chemical cues from other potential host-plants and to expand its host-breadth. It is worth noting that down-regulation of *Obp* genes has been reported in the specialist *Drosophila sechellia*, where *Obp56e* shows a reduced level of expression compared to other *Drosophila* species, and this difference is correlated with specific olfactory preferences in *D. sechellia* for odorants from its host-plant (Dworkin & Jones 2009). Likewise, *Obp99c* also showed decreased expression in this specialist species (Kopp et al. 2008).

Nevertheless, up-regulation in *Obp2* and marginally in *Obp1*, was also observed in the specialist *M. p. nicotianae*, relative to genic expression in the generalist *M. persicae s. str.*. These differences could be related to perception of specific cues from tobacco that may be involved in specific host-recognition by *M. p. nicotianae*. Similarly, the specialist *D. sechellia* also showed up-regulation of *Obp* genes compared to other *Drosophila* species (Kopp et al. 2008). More specifically, *Obp50a* and *Obp83ef* showed increased levels of expression in *D. sechellia*, and suggested that, in general, olfactory genes (including olfactory receptor genes, *Ors*) express as a dynamic mosaic in specialist insects, where some genes are up- and other down-regulated; this seems to be also the case for *M. p. nicotianae*.

Even though *Csps* are proteins mainly expressed in sensory organs (i.e. antennae, palps), they are also expressed in several other tissues such as wings, thorax, legs, pheromone glands and ejaculatory ducts; moreover, functions other than chemosensorial have been suggested (Gong et al. 2007; Pelletier & Leal 2009). Hence, *Csp3* could be either associated to the olfactory attraction of *M. p. nicotianae* to tobacco volatiles (see Vargas et al. 2005) and/or to metabolic functions related to its specialization on tobacco (see Cabrera-Brandt et al. 2010). In addition, consequences on the structure and/or function of the predicted protein encoded by *Csp3* are plausible, due to the differences in charge at physiological pH between asparagine and lysine (neutral and positively charged side chain, respectively). Furthermore, it is interesting to note that our evidence points to a divergence in the sequence of a candidate gene between two non-fully differentiated aphid taxa with different degrees of specialization. Nevertheless, the effects of the change in the aminoacidic sequence are currently speculative, since the mutation did not occurr in an identified conserved region of the protein, such as the insect pheromone-binding family OS-D, present in all insect *Csps* (McKenna et al. 1994). Also, our RNA was obtained from the whole individual (not only from heads or antennas), thus more specific studies of *Obps* expression using only heads are necessary to complement our findings. Finally, specific physiological studies are crucial to elucidate the specific relationship of these proteins with *M. p. nicotianae* specialization to tobacco.

Although several non mutually exclusive mechanisms could account for the evolution of specialization in *M. p. nicotianae*, e.g. differences in neural capacity (see chapter 1), in detoxification metabolism (Cabrera-Brandt et al. 2010) or even intertaxa competition (Tapia et al. 2008), our results indicate that differences in host-plant specialization between *M. persicae s. str.* and *M. p. nicotianae* may be related to their receptor systems. Therefore, it is crucial to complement the current knowledge on *Obp* and *Csp* genes present in *Myzus* with the actual function of each of these proteins and with exploration of olfactory receptor genes (*Or*), and to include a polyclonal approach in the exploration of antennal receptors, for a more complete view of their role in the evolution of ecological specialization in these aphids.

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DISCUSION GENERAL

La búsqueda del recurso, particularmente de la planta-hospedero, puede ser menos eficiente en la medida que aumenta la cantidad de estímulos sensoriales y en consecuencia, pueden ser necesarios mecanismos para reducirla y así alcanzar una mayor eficiencia en el proceso de búsqueda y reconocimiento de hospedero (Bernays y Wcislo 1994). A la luz de nuestros resultados, diferencias en las capacidades neuronales pudiesen dar pie a diferencias en las capacidades de aprendizaje entre generalistas y especialistas, y la interferencia retroactiva asociada a limitaciones en las capacidades de aprendizaje, pudiese representar un mecanismo para reducir el "input" sensorial en los especialistas, resultando en una toma de decisiones más eficiente, en comparación con los generalistas. Es importante considerar que la hipótesis de limitaciones neuronales (HLN) ha sido planteada para explicar la ocurrencia de especialización ecológica, enfocada fundamentalmente en los potenciales beneficios de ser especialista en un ambiente con condiciones relativamente constantes o predecibles en cuanto a la oferta de recurso (Bernays 2001). En este sentido, se plantea la existencia de compromisos donde el especialista ganaría, por un lado, eficiencia en la búsqueda de su recurso y en el ahorro de maquinaria neuronal extra (pudiendo así invertir esos recursos en otro(s) rasgo(s) relacionado(s) con su adecuación biológica). Sin embargo, a su vez perdería flexibilidad para afrontar condiciones ambientales variables; estos compromisos explican por qué los especialistas se verían favorecidos en estos ambientes menos variables en cuanto a la oferta de recursos y, en el caso particular de los insectos fitófagos, de plantas potencialmente hospederas. No obstante, la ocurrencia de interferencia retroactiva podría "liberar" de este requisito a la evolución de especialización por limitaciones neuronales, por cuanto ante variabilidad ambiental (por ejemplo, cambios en la oferta y disponibilidad de especies vegetales como potenciales hospederos), sólo la información incorporada recientemente sería considerada por el insecto, respondiendo exclusivamente a ésta y evitando así que información anterior afectase la eficiencia de búsqueda.

También, la ocurrencia de interferencia retroactiva preferentemente en el especialista, demostraría que el aprendizaje en el especialista, si bien se restringiría a unas pocas señales, no sería tan profundo.

Por otro lado, según sugieren nuestros resultados, la evolución de especialización ecológica en *M. p. nicotianae* tendría una base genética otorgada, por un lado, a través del gen *MpFor* (véase capítulo 1) y por otra parte, por genes *Obp* (véase capítulo 2). *MpFor* podría estar relacionado con una mayor adquisición de energía para facilitar el metabolismo de detoxificación de *M. p. nicotianae*, el cual le permite usar tabaco como planta-hospedera; alternativamente, podría estar actuando como la proteína kinasa A que confiere mayor retención en memoria a individuos con niveles medios de expresión de la enzima y, por tanto, en parte determinaría limitaciones neuronales diferenciales entre especialista y generlista al incrementar las capacidades de aprendizaje en este último (Horiuchi y col. 2008). Por otra parte, los genes *Obp* podrían otorgar al especialista pérdida de recepción de señales repelentes o ganancia de señales atractivas específicas (véase Kopp y col. 2008; Dworkin y Jones 2009). Sin embargo, muchos otros genes pudiesen estar involucrados en las diferencias en grados de especialización entre *M. persicae s. str.* y *M. p. nicotianae*. Por ejemplo, puesto que el gen *MpFor* podría no estar correlacionado con las diferencias en capacidades de aprendizaje y ocurrencia de interferencia retroactiva entre ambos taxones de áfidos, otro(s) gen(es) asociados a capacidades de aprendizaje, tales como *dunce*, pudiesen estar determinando esta variación. Del mismo modo, otros genes *Obps* aún no descritos para el complejo *Myzus persicae*, así como también genes de receptores olfativos (*Or*), también pudiesen variar entre especialistas y generalistas en sus niveles de expresión relativa (véase Kopp y col. 2008 para ejemplo).

Cabe señalar también que nuestros resultados en ningún caso pueden ser interpretados como explicaciones exclusivas para la evolución de especialización en el complejo *Myzus persicae*. Otros factores tales como las diferencias en metabolismos de detoxificación (Cabrera-Brandt et al. 2010) o la competencia intertaxones (Tapia et al. 2008) descritas para estos áfidos, perfectamente pueden estar actuando de forma simultanea con las diferencias en limitaciones neuronales (véase capítulo 1), o en la percepción específica de estímulos (véase capítulo 3) para promover la especialización ecológica en este complejo de áfidos.

Finalmente, es importante tomar en consideración que nuestros resultados se basan en una comparación a nivel intraespecífica que involucra sólo dos taxones y cada uno en condición monoclonal. Por lo tanto, es importante complementar este estudio con otros clones de *M. persicae s. str.* y de *M. p. nicotianae*, además de otros taxones de áfidos de tal modo de poner a prueba la generalidad de nuestra propuesta.

CONCLUSIONES

La presente tesis entrega evidencia que demuestra que: i) los áfidos son capaces de aprender señales olfativas emitidas por sus plantas hospederas, y ii) que existen diferencias en la capacidad de aprendizaje entre un insecto especialista, *Myzus persicae nicotianae*, y uno generalista, *M. persicae sensu stricto*, tal como propone la hipótesis de limitaciones neuronales (HLN); éstas fueron observadas tanto en la fase de reconocimiento a distancia de la planta (a través de bioensayos olfatométricos) como en la fase de reconocimiento sobre la superficie de la planta, del proceso de búsqueda de planta hospedera en áfidos (mediante la observación de la conducta del áfido sobre la planta). En el primer caso (olfatometrías), la interferencia retroactiva fue evidente en *M. p. nicotianae* después de experimentar pimentón como hospedero alternativo, en tanto que para *M. persicae s. str.* no se observaron defectos en la recuperación de información. En el segundo caso (conducta sobre la hoja), la experiencia en el hospedero alternativo también causó interferencia retroactiva en la recuperación de información acerca de su hospedero de crianza, observada en variables conductuales relacionadas con el reconocimiento de hospedero una vez que el individuo se ha posado sobre la planta, las que en general requirieron más tiempo o más intentos para llevarlas a cabo. También se pudo constatar a través de bioensayos olfatométricos, que la hipótesis de pérdida de memoria en áfidos no sería sustentable como explicación a la ocurrencia de interferencia retroactiva, y que eventualmente la competencia entre respuestas sería el fenómeno predominante. Adicionalmente, fueron identificadas diferencias en los niveles de expresión del gen *MpFor* entre ambos áfidos, un gen relacionado con aprendizaje e interferencia retroactiva, pero también con la adquisición de energía, con mayores niveles de expresión en el especialista *M. p. nicotianae*; dados estos resultados, el gen *MpFor* posiblemente sea promotor de especialización ecológica al permitirle a *M. p. nicotianae*, una mayor adquisición de energía lo que facilitaría el funcionamiento de su metabolismo de detoxificación, el cual le permite utilizar el tabaco como recurso. Alternativamente, *MpFor* pudiese ejercer máximos efectos en las capacidades de aprendizaje del individuo a niveles medios de expresión, tal como sucede con la proteína kinasa A en *Drosophila,* determinando así diferencias en sus capacidades neuronales. Con todo esto hemos sido capaces de proponer una extención de los alcances de la HLN incluyendo evidencia empírica relativa a sus efectos sobre otro rasgo cognitivo (aprendizaje), además de proponer una potencial base genética para sustentar la evolución de especialización ecológica en *M. p. nicotianae*. Junto a esto, esta tesis propone un mecanismo por el cual la interferencia retroactiva podría promover la especialización ecológica en insectos fitófagos, al ignorar información sobre otras plantas hospederas experimentadas previamente, a través de defectos al momento de recuperarla, optimizándose así la precisión en la toma de decisiones al tomar en cuenta una sola información. Esta situación proveería las condiciones para que la HLN pueda operar en ambientes (tanto ecosistemas silvestres como agroecosistemas con oferta de malezas) con una alta variabilidad en la oferta de especies vegetales como posibles hospederos, y no sólo bajo condiciones de baja variabilidad en la oferta de recursos. Esta tesis también demuestra que tanto las diferencias en capacidad de aprendizaje como de ocurrencia de interferencia retroactiva afectan el proceso de selección de planta hospedera en todas sus fases, dada la coherencia entre los resultados de reconocimiento a distancia y de reconocimiento sobre la planta.

También, los resultados reportados en esta tesis sugieren que las diferencias en grado de especialización entre *M. persicae s. str.* y *M. p. nicotianae* estarían relacionadas con diferencias en sus sistemas de recepción de señales olfativas, dada sus diferencias en expresión relativa y secuencia de los transcritos de genes *Obp*. Sugieren también que un conocimiento completo acerca de genes *Obp* en el complejo *Myzus persicae* así como también el desarrollo de experimentos de carácter fisiológicos para identificar funciones específicas, son cruciales para establecer una relación completa entre los genes de *Obp* y la especialización en este sistema de estudio.

Finalmente, es posible afirmar que las diferencias en los rasgos cognitivos como resultado de diferencias en las capacidades neuronales, así como también en la quimiorrecepción de señales, son hipótesis para la explicación de evolución de especialización en el complejo *Myzus persicae*, que no son excluyentes de otras hipótesis que también pudiesen representar explicaciones plausibles para este fenómeno. A su vez, es necesario incrementar la diversidad de clones estudiados así como el número de taxones, para establecer conclusiones aun más generales para nuestras propuestas.

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