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ESCUELA INTERNACIONAL DE DOCTORADO

Seasonal Biology and Reproductive Strategy of the Predatory Mirid *Pilophorus gallicus* (Hemiptera: Miridae) in the Pear Crop in the Mediterranean Area

Biología Estacional y Estrategia Reproductiva del Mírido Depredador *Pilophorus gallicus* (Hemiptera: Miridae) en el Cultivo del Peral en el Área Mediterránea

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Biología estacional y estrategia reproductiva del mírido depredador *Pilophorus gallicus* (Hemiptera: Miridae) en el cultivo del peral en el área mediterránea

TESIS DOCTORAL
Dña. Mª José Ramírez Soria
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A mis padres y a mi abuelita Teresa,

por todo y por todos

“I have no special talent.
I am only passionately curious.”

Albert Einstein

“Rien dans la vie n’est à craindre,
tout doit être compris.
C’est maintenant le moment
de comprendre davantage,
afin de craindre moins.”

Marie Curie

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PRELIMINARES Y ESTRUCTURA DE LA TESIS DOCTORAL

La presente tesis doctoral, titulada “Biología estacional y estrategia reproductiva del mírido depredador *Pilophorus gallicus* (Hemiptera: Miridae) en el cultivo del peral en el área mediterránea” y realizada por Maria José Ramírez Soria en el marco del programa de doctorado "Biodiversidad y Gestión Ambiental" de la Universidad de Murcia, surgió del interés de la empresa Biobest N.V. por el estudio del mírido *Pilophorus gallicus* como potencial depredador de plagas. Dicha empresa se dedica a la investigación y comercialización de enemigos naturales y polinizadores empleados en la producción agrícola. Los trabajos de investigación han sido realizados en un marco de colaboración entre Biobest N.V. y el Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA). El desarrollo de este estudio ha sido codirigido por el Dr. Juan Antonio Sánchez Sánchez, coordinador del equipo de Control Biológico y Servicios Ecosistémicos del IMIDA, y el Dr. Felix Wäckers, director del departamento I+D de Biobest N.V. Es por ello que, de acuerdo con lo establecido en la normativa de la Universidad de Murcia, la doctoranda opta a la Mención Industrial en el título de Doctor.

La estructura de la memoria presentada puede considerarse una variación del documento clásico estipulado para la defensa de una tesis doctoral. Con vistas a la publicación de los trabajos en revistas científicas, la tesis se ha organizado en cuatro capítulos homogéneos con estructura de artículos científicos, todos ellos complementarios con la idea de abordar desde una amplia perspectiva el estudio de la biología estacional y la estrategia reproductiva del mírido *P. gallicus*. Esta estructura hace que en ocasiones se incurra en la repetición de ciertos contenidos comunes, fundamentalmente en las secciones de introducción y metodología de cada capítulo. La elección de esta modalidad de redacción implica que la lengua empleada para la mayor parte de la tesis haya sido el inglés, a excepción de la introducción y la discusión general que han sido escritas en castellano. El resumen y las conclusiones generales figuran en ambos idiomas conforme a lo exigido en la normativa de la Universidad de Murcia que regula la obtención de la Mención

Internacional en el título de Doctor. Hasta la fecha, parte de los resultados han sido comunicados en congresos nacionales e internacionales, y los dos primeros capítulos han sido publicados en revistas incluidas en el *Journal Citation Report* (JCR):

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INTRODUCCIÓN GENERAL Y OBJETIVOS

Foto: Juan Antonio Sánchez Sánchez

INTRODUCCIÓN GENERAL Y OBJETIVOS

1. ANTECEDENTES

1.1. El cultivo del peral en Jumilla

Jumilla es un municipio situado en la Comarca del Altiplano, al noreste de la Región de Murcia, en el sureste de España (Figura 1). Es una zona tradicional de cultivo de peral (*Pyrus communis* L., Rosaceae), datando las primeras referencias sobre este cultivo del siglo XVI, durante el reinado de Felipe II. Parajes como la 'Casa del Peral' en Las Puntillas testifican su existencia desde tiempos muy lejanos. En las publicaciones "Datos históricos sobre el cultivo de la pera en la comarca de Jumilla", de Emiliano Hernández Carrión, y "El Altiplano de Jumilla-Yecla", de Alfredo Morales Gil, se relata como el cultivo del peral en los años 70 en esta región consistía en plantaciones con escasa extensión o en orillas de bancales y caminos. Las producciones se dedicaban al autoconsumo, al intercambio por otras mercancías, a ventas en los mercados o en las puertas de las casas. Debido a la elevada producción por árbol, su buena aceptación en el mercado y un precio superior al de otras especies frutales, el cultivo del peral se arraigó fuertemente en las tierras de Jumilla, fundamentalmente con la variedad Ercolini. Por otro lado, el perfeccionamiento y generalización de los medios de transporte facilitando la comercialización de productos perecederos, así como la mejora de regadíos con la apertura de acuíferos permitiendo nuevas áreas cultivables, desencadenaron la intensificación del cultivo de la pera Ercolini en Jumilla (http://www.regmurcia.com). Amplios valles destinados al secano con viña, olivar, cereales o almendros fueron transformados en campos de frutales que proporcionaban un mayor potencial productivo. Así, la superficie destinada a peral pasó de unas 120 ha en 1970 a 953 ha en la actualidad, cultivándose íntegramente en regadío, y representando el 18% de la superficie dedicada a frutales no cítricos en el municipio (Estadística Agraria de la Consejería de Agricultura y Agua, 2015, http://www.carm.es/) (Figura 2).

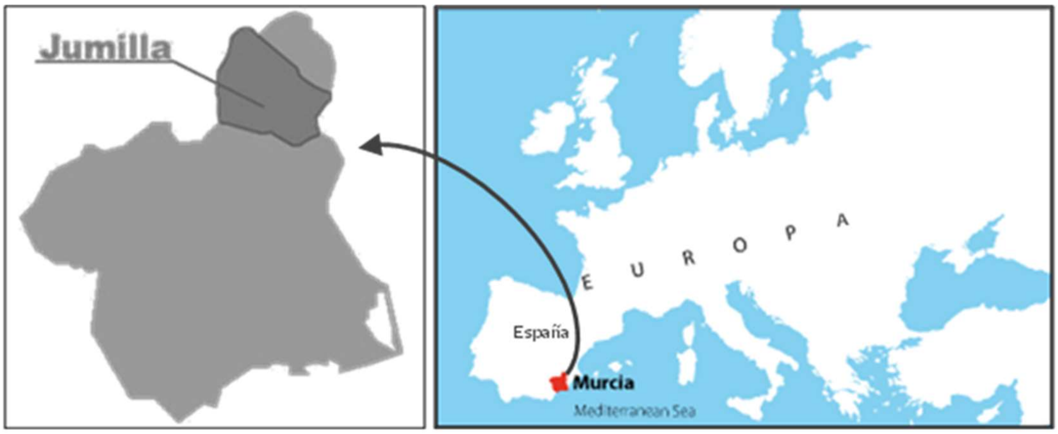

Figura 1. Localización del área de estudio, municipio de Jumilla en la Comarca del Altiplano, al noreste de la Región de Murcia, en el sureste de España.

Figura 2. Campos de peral en la Comarca del Altiplano de la Región de Murcia.

Actualmente Jumilla es la mayor productora de pera Ercolini en España y Europa, con un volumen anual cercano a las 20.000 toneladas, representando un 50 y un 24% de la producción nacional y europea respectivamente (Superficies y Producciones Anuales de Cultivo (2016), http://www.mapama.gob.es; http://www.peradejumilla.com). La variedad de pera Ercolini producida en este municipio goza de la etiqueta "Denominación de Origen" en reconocimiento a su calidad intrínseca. Cultivada en un área intermedia entre la meseta Castellano-

Manchega y el litoral Mediterráneo, goza de un microclima singular, cálido pero con suficiente altitud para que las temperaturas invernales bajas puedan satisfacer los requerimientos de frío del cultivo. La floración tiene lugar en el mes de marzo; a finales de primavera, las cálidas temperaturas medias (18°C, http://siam.imida.es/) y la ausencia de heladas favorecen el crecimiento rápido de los frutos (Figura 3). Durante la maduración, el elevado número de horas de sol y la alta radiación solar logrados por la orientación de los valles y cañadas al mediodía, permiten que las "Peras de Jumilla" presenten características diferenciadoras con respecto a otras regiones: la chapa rojiza en su cara soleada y un grado de azúcar superior son dos de sus principales marcas distintivas. La época de recolección es otra ventaja comparativa del cultivo de la pera Ercolini en Jumilla, debido a su precocidad, pudiendo comenzar a recolectarsea partir del 24 de junio y extendiéndose hasta el mes de julio.

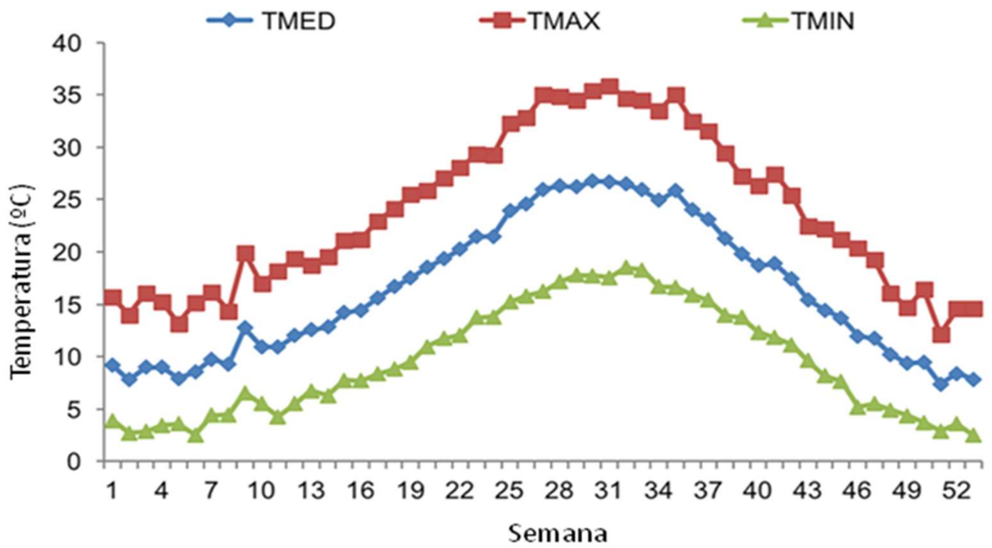

Figura 3. Temperaturas medias semanales registradas en Jumilla (Murcia, España) estación climática JU71, Las Encebras (38°23'40.01"N, 1°14'21.58"W) desde 2007 hasta 2011 (http://siam.imida.es/).

1.2. El control de plagas en el cultivo del peral

Las principales plagas que afectan al cultivo del peral en el noreste de Murcia son la psila (*Cacopsylla pyri L.*) (Hemiptera: Psyllidae), el pulgón (*Aphis gossypii*

Glover y *Aphis spiraecola* Patch) (Hemiptera: Aphididae), el piojo de San José (*Quadraspidiotus perniciosus* Comstock) (Hemiptera: Diaspididae), el agusanado de los frutos o Carpocapsa (*Cydia pomonella L.*) (Lepidoptera: Tortricidae) y Hoplocampa (*Hoplocampa brevis* Klug) (Hymenoptera: Tenthredinidae) (Ministerio de Agricultura, Pesca y Alimentación, 2011). La psila del peral *C. pyri* (Figura 4) es con diferencia la plaga que más daños causa en el cultivo de la pera en esta región, así como de otras zonas productoras de España (Artigues *et al.*, 1996; Vilajeliu, 1989; Vilajeliu *et al.*, 1998) y de Europa (Erler, 2004; Hodkinson, 1984; Rieux *et al.*, 1992; Silva *et al.*, 2005; Stamenkovic *et al.*, 2001; Nin *et al.*, 2012). Este homóptero provoca daños directos al debilitar el árbol succionando su savia, e indirectos por la transmisión de enfermedades (micoplasmas) y la excreción del exceso de azúcares en forma de melaza (Figura 5) (Avinent *et al.*, 1997; Erler, 2004). La melaza, a su vez, es un medio adecuado para la colonización de hongos saprófitos y la consiguiente formación de negrilla que acaba recubriendo gran parte de los frutos y de la superficie foliar cuando la abundancia de psila es alta, dificultando la actividad fotosintética del árbol, y manchando y deformando el fruto por lo que se reduce su valor comercial (Figura 6).

Figura 4. Adulto de *Cacopsylla pyri*.

Figura 5. Melaza producida por *Cacopsylla pyri*.

Figura 6. Frutos manchados con negrilla.

El control de *C. pyri* en los perales del noreste de Murcia se realiza de manera convencional mediante la aplicación sistemática de insecticidas, pero pese al elevado número de intervenciones, no se logra reducir las poblaciones de plaga satisfactoriamente. La inefectividad del control químico puede deberse al desarrollo de resistencias por el uso reiterado de materias activas, como ya se ha observado previamente en otras zonas productoras de peral frente a compuestos de diferentes familias (e.g. organofosforados y piretroides) (Berrada *et al*., 1994; Buès *et al*., 1994, 2003). Por otro lado, la contaminación asociada al uso de estos productos, especialmente de los acuíferos subterráneos, así como el riesgo para la salud humana y otros organismos no-objetivo han llevado a la prohibición de numerosas materias activas por parte de la Unión Europea en estos últimos años (Usta, 2013). Dicha problemática ha puesto de manifiesto la necesidad de adoptar medidas alternativas para el control de plagas en peral, dando prioridad al control biológico. Según la FAO, el Manejo Integrado de Plagas (IPM, por sus siglas en inglés) consiste en la implementación de los métodos disponibles para el control de poblaciones plaga que minimicen los impactos negativos en la salud humana, incrementen la biodiversidad y mantengan una elevada productividad en el cultivo. El control biológico de plagas es uno de los métodos contemplados en IPM; consiste en el empleo de organismos vivos con objeto de controlar las poblaciones de otros organismos (Flint y Dreistadt, 1998; Jacas y Urbaneja, 2008). Su idoneidad ha sido demostrada con éxito frente a un control químico insuficiente que comprometía la viabilidad de algunos cultivos, como por ejemplo el pimiento (Sanchez y Lacasa, 2006). En el caso del peral, trabajos previos demuestran la existencia de una diversa comunidad de artrópodos asociada a este cultivo capaz de contribuir a la regulación de las poblaciones de insectos fitófagos (Bogya *et al*., 1999; Erler, 2004; Rieux *et al*., 1999; Sanchez *et al*., 2010, 2011; Sanchez y Ortín-Angulo, 2012; Solomon *et al*., 2000). El actual control biológico de la psila del peral en Europa está basado principalmente en la liberación y conservación de especialistas, como *Anthocoris nemoralis* (F) (Hemiptera: Anthocoridae) (Erler, 2004; Scutareanu *et al*., 1999; Sigsgaard *et al*., 2006; Vilajeliu *et al*., 1998). Los estudios realizados en España revelan la presencia de hormigas, arañas y hemípteros depredadores (e.g.

Anthocoris nemoralis F. (Hemiptera: Anthocoridae) y *Pilophorus* spp. (Hemiptera: Miridae)) especialmente abundantes en campos no sometidos a tratamientos químicos (Artigues *et al*., 1996; Avilla *et al*., 1992; Sanchez *et al*., 2010; Sanchez y Ortín-Angulo, 2012; Vilajeliu *et al*., 1998). Sanchez y Ortín-Angulo (2012) señalan la importancia de *Pilophorus gallicus* Remane como depredador generalista que puede incidir en la regulación de las poblaciones de áfidos y psila en el cultivo del peral del área Mediterránea. Dado su potencial como depredador de artrópodos fitófagos plaga del peral, el estudio de los parámetros biológicos de este mírido y de su dinámica poblacional se hace necesario para su manejo dentro de programas de control biológico, y en última instancia poder establecer un método de cría eficiente.

1.3. Pilophorus gallicus: un potencial controlador biológico

Pilophorus gallicus es un mírido perteneciente a la tribu monofilética Pilophorini (Hemiptera: Miridae: Phylinae) con distribución cosmopolita en la actualidad (Schuh, 1991; Wheeler, 2001) (Figura 7). *Pilophorus* es el género más abundante de esta tribu, y entre sus características destaca la existencia de numerosas especies mirmecomórficas (Yasunaga y Schuh, 2013). Muchas de las especies del género presentan hábitos zoofitófagos, depredando principalmente sobre pequeños hemípteros (e.g. áfidos y psílidos) pero también sobre larvas de díptero y ácaros (Duwal *et al*., 2014; Schuh, 1974; Wagner y Weber, 1964; Wheeler, 2001). *Pilophorus gallicus* fue descrito sobre hospedantes del género Salicaceae (*Populus alba* y *Salix* spp.) en el sur de Francia (Remane, 1954), y desde entonces ha sido citado en el ámbito Mediterráneo: en diversas zonas de la Península Ibérica (Cataluña, Baleares, Murcia) (Ribes, 1965; Ribes *et al*., 2008; Sanchez *et al*., 2010, 2011; Sanchez y Ortín-Angulo, 2012) y Francia (Córcega) (Pericart, 1965). Sanchez y Ortín-Angulo (2012) ponen de manifiesto altas densidades poblacionales de *P. gallicus* en los cultivos de peral del sureste peninsular y su importancia en el control de la psila del peral *C. pyri*, lo cual despierta el interés por el conocimiento de la biología de este mírido.

Figura 7. Diferentes estadíos de desarrollo de *Pilophorus gallicus*. De izquierda a derecha: huevo, huevo insertado en tallo, ninfa de IV estadío, adulto.

Sanchez y Ortín-Angulo (2012) apuntan también la presencia de los estadíos móviles de *P. gallicus* (ninfas y adultos) en el cultivo únicamente entre principios de marzo y finales de octubre. Su ausencia invernal y la variabilidad climática existente en el área de estudio (Figura 3) inducen a pensar en la existencia de estrategias o mecanismos para superar las condiciones adversas del invierno (Musolin y Saulich, 1999). Las adaptaciones de los insectos frente a climas variables tienen ciertos puntos en común pero no obedecen un patrón general, cada especie desarrolla de forma muy específica su estrategia buscando sincronizar sus necesidades con su hábitat, dando lugar a una biología estacional propia. Estas estrategias pueden ser muy complejas e incluso en ocasiones se combinan entre ellas (Musolin, 1996; Musolin y Saulich, 2000; Perez y Taylor, 2004); dado que están directamente relacionadas con el periodo de actividad de los organismos y la adaptación a su hábitat, es conveniente su consideración en el diseño de programas de control biológico, ya que puede resultar fundamental para una implementación exitosa o una mejora de su eficiencia.

2. LA BIOLOGÍA ESTACIONAL DE LOS INSECTOS

En general, todos los hábitats terrestres están sujetos a ciclos de alternancia entre periodos favorables y adversos, debido a la rotación de la Tierra alrededor del Sol y a su eje de inclinación (Koštál, 2011). La clase Insecta representa el grupo más numeroso dentro del reino animal, con aproximadamente un millón de especies descritas (el 80% de las especies de la fauna animal mundial). Esta diversidad, así como su antigüedad (los primeros insectos se originaron hace unos 480 millones de años) y la gran variedad de ecosistemas que habitan, ponen en evidencia la capacidad de adaptación de estos artrópodos a las variaciones ambientales (Misof *et al*., 2014). A lo largo de la historia, las especies han generado cambios graduales para su supervivencia, siendo las características actuales el resultado de los éxitos y los fracasos de sus antecesores (Begon *et al*., 2006). La mayoría de insectos tienen ciclos de vida complejos "diseñados" para hacer frente a fluctuaciones diarias o estacionales de las condiciones ambientales (e.g. temperatura, humedad, fotoperiodo, depredación, competencia inter o intra-específica) (Danks, 2002; Tauber *et al*., 1986). El estudio de las adaptaciones estacionales es por tanto abordado mediante la relación de los organismos con los factores bióticos y abióticos de su entorno. En general, la temperatura y el fotoperiodo son los factores principales involucrados en el desarrollo de estos procesos en las regiones templadas (Nishizuka *et al*., 1998). La temperatura es uno de los factores abióticos que más influyen en el ciclo biológico de los insectos, permitiendo o impidiendo su desarrollo y/o reproducción; por tanto, suele tener un papel causal en la adopción de los mecanismos de adaptación. El fotoperiodo, sin embargo, actúa más bien como desencadenante, siendo un indicador de la llegada de las condiciones adversas. El estudio en detalle de estas interacciones permite descifrar las adaptaciones empleadas por los insectos para hacer frente a las variaciones climáticas y su influencia en las dinámicas poblacionales. Estos conocimientos representan una valiosa herramienta a incorporar en los programas de control biológico.

2.1. Estrategias de adaptación en climas variables

Las adaptaciones estacionales se definen como la habilidad de los organismos (tanto a nivel fisiológico como bioquímico o comportamental) de sobrevivir a las variaciones ambientales y sincronizar su actividad con la disponibilidad de recursos y las condiciones favorables para su desarrollo y reproducción (Musolin y Saulich, 1999; Tauber *et al.*, 1986). La capacidad de responder mediante mecanismos adaptativos ha permitido a numerosas especies habitar lugares inviables para la actividad permanente (Saunders, 2011). En las regiones con climas templados, la estación desfavorable suele venir asociada a temperaturas extremas, bien invernales o bien estivales. Migrar a lugares más adecuados, o entrar en un estado de latencia (quiescencia o diapausa invernal/estival) son las estrategias más empleadas por los insectos para sobrevivir a temperaturas que exceden su rango de tolerancia (Dingle, 1978; Saunders, 2010; Tauber *et al.*, 1986; Taylor y Karban, 1986). La migración implica forzosamente movimiento, en busca de lugares con condiciones ambientales más favorables o de huéspedes más apropiados para pasar el periodo desfavorable. Así pues las distancias pueden ser variables, pero el estadío involucrado suele ser el adulto alado; por el contrario, la adopción de un estado latente permite a los individuos permanecer in-situ y en cualquier estadío del desarrollo, desde las primeras fases de la embriogénesis hasta el adulto (Dingle, 1978; Kennedy, 1961; Solbreck, 1978). Los estados de latencia (i.e. quiescencia y diapausa) difieren a su vez en sus características intrínsecas y en la manera de relacionarse con las condiciones ambientales. La quiescencia es una respuesta directa a las condiciones adversas, cuya duración suele ser similar a la del periodo desfavorable, sin embargo la diapausa es una respuesta genéticamente determinada, una secuencia de etapas bien definidas (inducción, mantenimiento y terminación) que requieren unos estímulos muy concretos para su progresión y, generalmente, se prolonga en el tiempo más allá de las condiciones adversas (Bale y Hayward, 2010; Beck, 1980; Dolling, 1991). El fotoperiodo y la temperatura suelen ser los factores clave para la estimulación de la diapausa, influyendo en el desencadenamiento de este mecanismo, su mantenimiento y su terminación (Denlinger, 2002; Koštál, 2011; Saunders, 2002; Saunders y Bertossa, 2011). Por tanto la migración, la quiescencia y la diapausa tendrán repercusiones diferentes a nivel de dinámica de poblaciones. La primera representa una evasión en el espacio, mientras que la segunda y la tercera son una evasión en el tiempo para sobrevivir a condiciones ambientales desfavorables (Danks, 1987). El estudio de estas fluctuaciones en espacio y tiempo merecen pues especial consideración como partes integrantes del ciclo biológico de los insectos, especialmente en el caso de especies plaga o depredador, dada su importancia a la hora de predecir sus dinámicas poblacionales, entender su ecología y tomar decisiones (Nylin, 2001). Para ello, es necesario conocer cómo interactúan dichos mecanismos con los factores ambientales; estas relaciones pueden llegar a ser complejas y han sido objeto de numerosos estudios.

2.2. *Factores ambientales reguladores de los ciclos de vida de los insectos*

2.2.1. *La temperatura: un factor limitante para la vida de los insectos*

Los insectos son organismos ectotermos; consecuentemente, su temperatura corporal no es constante y depende de las condiciones ambientales (Addo-Bediako *et al*., 2000; Denlinger, 2009; Deutsch *et al*., 2008). El umbral y la constante térmicos, parámetros característicos de cada especie, son indicadores de las condiciones favorables para su existencia (Messenger, 1970). La Figura 8 ilustra como la relación entre la temperatura y el rendimiento de un organismo ectotermo está acotada por sus umbrales térmicos; entre ambos puntos se sitúa la temperatura óptima correspondiente al máximo rendimiento. La temperatura influye directamente en el desarrollo y reproducción de los insectos, y determina su rango de distribución así como su abundancia estacional (Deutsch *et al*., 2008; Régnière *et al*., 2012a, 2012b). Un insecto necesita temperaturas superiores a su umbral mínimo y satisfacer su constante térmica para completar su desarrollo y reproducirse. A mayor temperatura disminuye el tiempo de desarrollo y aumenta la fecundidad, hasta alcanzar la temperatura óptima; a temperaturas superiores estos parámetros biológicos se ven negativamente afectados, siendo la temperatura letal el umbral superior de tolerancia (Bursell, 1964; Frazier *et al*., 2006; Logan *et al*., 1976). El conocimiento de los indicadores térmicos de cada especie (umbral y constante térmica) son de gran utilidad para conocer su zona de tolerancia y prever su periodo de actividad a lo largo del año (Beck, 1980). Asimismo, el estudio del efecto de la temperatura sobre los parámetros biológicos (e.g. tiempo de desarrollo, fecundidad, longevidad) completa la comprensión de la dinámica poblacional de la especie y permite deducir su temperatura óptima. En última instancia, este tipo de estudios puede aplicarse a la cría masiva de insectos para mejorar la eficiencia de la producción.

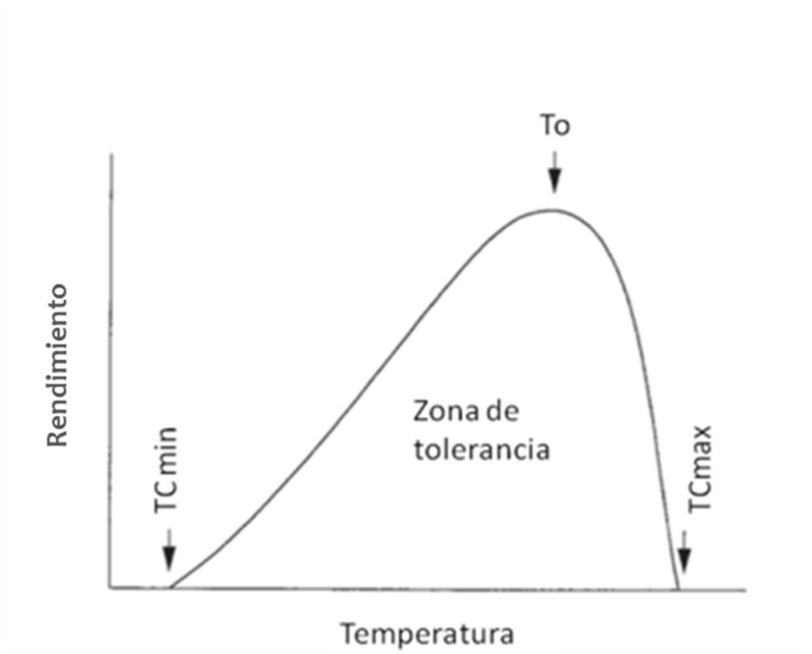

Figura 8. Curva hipotética de rendimiento de un insecto en función de la temperatura (CTmin: temperatura crítica mínima; CTmax: temperatura crítica máxima; To: temperatura óptima) (adaptado de Denlinger, 2009).

La variación térmica estacional propia de los climas templados se caracteriza por veranos calurosos e inviernos fríos en los cuales la temperatura puede disminuir muy por debajo del umbral mínimo para el desarrollo de los insectos. Esto supone un obstáculo para el desarrollo y la reproducción continua de los insectos, lo cual les obliga a desarrollar adaptaciones estacionales en su comportamiento y/o metabolismo para superar dichas adversidades. Un periodo de bajas temperaturas puede desencadenar la manifestación de quiescencia – o estado de latencia transitoria - reanudándose la actividad tras el restablecimiento de condiciones favorables (Chang *et al.*, 1996; Denlinger, 1991). Sin embargo, en las regiones templadas la temperatura no suele tener un rol principal en la inducción de la diapausa, pero puede modular el efecto inductor del fotoperiodo (Fantinou y Kagkou, 2000; Musolin y Saulich, 1999; Saulich y Musolin, 2007; Tauber *et al.*, 1986). Pese a la aparente ausencia de influencia térmica en el proceso de inducción, la necesidad de bajas temperaturas para el mantenimiento y terminación de la diapausa invernal ha sido ampliamente demostrada en la clase Insecta (Fisher, 1997; Higaki y Ando, 2005; Moribe *et al.*, 2010; Yamaguchi y Nakamura, 2015; Zhou *et al.*, 2016). La persistencia de las bajas temperaturas tras la terminación de la diapausa puede también impedir la reanudación de la actividad por parte del organismo, sometiéndolo a una quiescencia post-diapausa a la espera del restablecimiento de las condiciones favorables (Koštál, 2006). En cuanto a la migración, esta suele producirse como respuesta a un deterioro ambiental frecuentemente relacionado con la sobrepoblación, la escasez de alimento o el impedimento de la reproducción, por tanto, una combinación de factores en la que la temperatura también puede tener un efecto (Dingle, 2009; Solbreck, 1978). Así pues, la temperatura puede tener un papel muy polivalente en los ciclos estacionales de los insectos. Cada especie se adapta de manera muy específica a su ambiente, por ello es difícil establecer normas generales. El conocimiento de la influencia de la temperatura proporciona pues una base esencial para la comprensión del la biología de la especie, tanto a nivel de regulación del desarrollo como de su función en las adaptaciones estacionales.

2.2.2. *El fotoperiodo: un indicador fidedigno*

La mayoría de los factores ambientales que gobiernan la biología estacional de los insectos suponen una amenaza o una oportunidad para la supervivencia de las poblaciones, debido a su acción directa sobre su metabolismo (e.g. temperaturas extremas, sequías, escasez de alimento). No obstante, el fotoperiodo no ejerce directamente ninguna función beneficiosa o dañina sobre los organismos; se considera más bien una fuente de información. En contraposición a otros factores cuya variación puede ser más impredecible, el fotoperiodo representa una señal fiable dada su regularidad y predictibilidad a lo largo del año (Bale y Hayward, 2010). Los insectos han desarrollado el fotoperiodismo, i.e. la habilidad de percibir e interpretar los cambios estacionales en las horas de luz para prever la llegada de condiciones desfavorables (Saunders, 2011). Numerosos estudios han demostrado la relación del fotoperiodismo con la modificación de procesos comportamentales (e.g. actividad locomotora, alimentación), ligados al desarrollo (e.g. eclosión, ecdisis), fisiológicos (e.g. consumo de oxígeno, formación de la cutícula), y estacionales (e.g. polimorfismo, migraciones, latencia) (Beck, 1980; Bradshaw y Holzapfel, 2007; Dingle, 2009; Kogure, 1933; Sabrosky *et al*., 1933; Saunders, 2013); el objetivo común es siempre sincronizar sus funciones biológicas con las condiciones ambientales propicias.

Por no ser un factor deletéreo *per se*, el fotoperiodo normalmente no tiene influencia en la quiescencia. No obstante, el fotoperiodo es el principal estímulo ambiental para la inducción de la diapausa en una amplia variedad de insectos, especialmente en las regiones templadas (Denlinger, 1991). Esto se explica porque suele estar estrechamente correlacionado con otros cambios estacionales como la variación de temperatura, humedad o disposición de alimento. La *curva de respuesta fotoperiódica* de cada insecto representa el porcentaje de incidencia de la diapausa en función de las horas de luz a las que ha estado sometido durante su fase fotosensible. De ahí se deduce el *fotoperiodo crítico*, definido como el fotoperiodo que induce la diapausa en el 50% de la población (Tauber *et al*., 1986). Ambos son útiles para describir la respuesta de una especie frente a diferentes fotoperiodos. Asimismo, la respuesta fotoperiódica puede verse modificada por la acción de la temperatura (Fantinou y Kagkou, 2000; Musolin y Saulich, 1999; Saulich y Musolin, 2007; Tauber *et al*., 1986). Por otro lado, el rol del fotoperiodo durante el mantenimiento y la terminación de la diapausa no es tan evidente, siendo en ocasiones nulo (Tauber *et al*., 1986). Así pues, el estudio de la influencia del fotoperiodo con los ciclos estacionales de los insectos debe abordarse desde un punto de vista predictivo, teniendo en cuenta los periodos fotosensibles y su eventual interacción con la temperatura, lo cual puede llegar a resultar, en ocasiones, complejo de interpretar.

2.3. La biología estacional en el control biológico de plagas

Las estrategias de adaptación a ambientes desfavorables tienen en común que conllevan una interrupción del ciclo biológico (Kennedy, 1961; Solbreck, 1978).

Sin embargo, la interacción de los organismos con las condiciones ambientales difiere según la estrategia, por tanto la dinámica poblacional de las especies puede verse condicionada en este sentido. La diapausa es un mecanismo predecible siempre y cuando se conozcan los estímulos de la inducción y la terminación, así pues, puede resultar útil el conocimiento de dichos factores en la toma de decisiones. Sin embargo, la quiescencia puede dar lugar a una variabilidad impredecible en el ciclo biológico de las especies. Hultine *et al*. (2015) y Bean *et al*. (2012) demostraron la importancia del fotoperiodo crítico en un caso de control biológico clásico de las especies invasoras *Tamarix* spp. (Tamaricaceae) en Norte América. El fitófago *Diorhabda carinulata* Desbrochers (Coleoptera: Chrysomelidae) se establecía satisfactoriamente en aquellas regiones de latitud similar a la de su país de origen, sin embargo fracasaba en latitudes inferiores, ya que entraba en diapausa prematuramente debido a su largo fotoperiodo crítico. Consecuentemente, *Tamarix* spp. eran capaces de recuperarse gracias al cese de la herbivoría bajo condiciones aún favorables para su desarrollo. Por otro lado, la diapausa en las especies plaga puede ser aprovechada para reducir las poblaciones, ya que en estado diapausante su movilidad es nula o reducida y conociendo su localización pueden ser más fácilmente atacadas. Sappington *et al*. (2006) y Gray *et al*. (2009) reportaron como, por un periodo de tiempo, las plagas del maíz *Diabrotica* spp. (Coleoptera: Chrysomelidae) fueron controladas gracias a la rotación de cultivos: tras la diapausa invernal enterradas en el suelo, encontraban cultivos de soja donde el año anterior hubo maíz, y su especificidad en la alimentación les impedía completar su ciclo. En cuanto a las poblaciones de enemigos naturales, la elección de ciertas actividades culturales puede condicionar el establecimiento de poblaciones permanentes. Por ejemplo, en el caso de quiescencia o diapausa invernales, las poblaciones permanecen *in situ* durante las condiciones adversas, por tanto, habría que tener en cuenta su presencia en los tratamientos invernales. Aunque hay autores que demuestran la mayor resistencia frente a pesticidas en estado latente (Buès *et al*., 1999; Denlinger, 2008; Van de Baan and Croft, 1991), sería aconsejable emplear productos respetuosos con la fauna auxiliar. En caso de migración a otro huésped, el control primaveral de poblaciones plaga incipientes puede verse por un retorno tardío al cultivo (Erler, 2004). El establecimiento de poblaciones permanentes se vería beneficiado por la presencia de dichas especies huésped en los márgenes del cultivo, para así reducir las distancias de los desplazamientos. La mayoría de los míridos de la zona climática templada hibernan como huevos diapausantes (Schuh and Slater, 1995). Sin embargo, algunas especies no entran en diapausa y sobreviven exitosamente al invierno mediterráneo (e.g. *Nesidiocoris tenuis* Reuter, *Macrolophus caliginosus* Wagner, *Macrolophus pygmaeus* Rambur (Heteroptera: Miridae)) (Backer *et al*., 2014; Hatherly *et al*., 2005; Hughes *et al*., 2009). Comparativamente, otros míridos de distribución mediterránea que hibernan en estadío diapausante tienen mayor tolerancia a las bajas temperaturas y son capaces de establecerse en regiones septentrionales con inviernos más severos (e.g. *Dicyphus hesperus* Knight) (Hatherly *et al*., 2008). Los ejemplos presentados anteriormente ilustran cómo el conocimiento de la(s) estrategia(s) empleada(s) por las especies plaga o depredador presentes en el cultivo puede condicionar el éxito o el fracaso de un programa de control biológico de plagas. Por ello, este trabajo se centra en el estudio de los mecanismos de adaptación del mírido *P. gallicus* frente a la variabilidad climática, concretamente a través de la influencia de la temperatura en su ciclo de vida, y el papel del fotoperiodo en la regulación de su biología estacional.

3. OBJETIVOS

El objetivo general de esta tesis es estudiar los factores que influyen sobre la dinámica poblacional del mírido depredador *Pilophorus gallicus* en el cultivo del peral en el sureste de la Península Ibérica. Los objetivos más concretos se centran en estudiar el efecto de la temperatura sobre sus parámetros biológicos, determinar los mecanismos adoptados por la especie para sobrevivir a periodos desfavorables y los factores que regulan dichos procesos.

Para responder a estos objetivos, se llevaron a cabo trabajos con poblaciones en condiciones de campo así como ensayos bajo condiciones controladas con colonias de *P. gallicus* mantenidas en el laboratorio. Los resultados de la tesis están estructurados en cuatro capítulos, los cuales se corresponden a un artículo publicado en *Entomological Science*, otro en *el Journal of Agriculture and Forest Entomology* y otros dos que se enviarán a revistas incluidas en el *Journal Citation Report* (JCR). Los objetivos de cada capítulo se detallan a continuación.

En el primer capítulo se estudia la influencia de la temperatura en los parámetros biológicos de *P. gallicus*. Los objetivos específicos fueron: (a) determinar los tiempos de desarrollo y la reproducción (fecundidad y fertilidad) a diferentes temperaturas, (b) estimar los umbrales y las constantes térmicos para cada estadío del desarrollo, y (c) obtener los parámetros demográficos y el rango óptimo de temperaturas para *P. gallicus.* Estos resultados sirvieron de base para el segundo capítulo, en el que se estudió la dinámica poblacional de *P. gallicus* y su sincronización con la variación estacional mediante el trabajo con poblaciones silvestres. El objetivo fue identificar la estrategia (migración, diapausa, quiescencia) empleada por *P. gallicus* para sobrevivir a periodos de condiciones desfavorables en los cultivos de peral del sureste de la Península Ibérica. Dado que este trabajo puso en evidencia el empleo de la diapausa embrionaria como mecanismo de supervivencia invernal, el tercer capítulo se centró en el estudio de la expresión de la diapausa. Los objetivos específicos fueron: (a) investigar la influencia del fotoperiodo y la temperatura en la inducción de la diapausa, y (b) determinar la influencia de la temperatura, el fotoperiodo y tiempo de exposición en la terminación de la diapausa. Para terminar, en el cuarto capítulo se profundizó en el estudio del fotoperiodismo en *P. gallicus.* El objetivo fue determinar el efecto del fotoperiodo desde tres puntos de vista: (1) estudiar la respuesta diapausante de *P. gallicus* bajo diferentes niveles de fotoperiodo constante, obtener la curva de respuesta fotoperiódica y determinar el fotoperiodo crítico; (2) investigar el efecto de la dirección del fotoperiodo (creciente o decreciente) en la inducción de la diapausa en *P. gallicus*, y (3) estudiar si la inducción de la diapausa embrionaria podía revertirse en las hembras progenitoras cambiando la longitud del fotoperiodo.

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CHAPTER 1

Thermal effects on the biological parameters of the predatory mirid *Pilophorus gallicus* (Hemiptera: Miridae)

Photo: Elena López Gallego

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ABSTRACT

Pilophorus gallicus Remane (Hemiptera: Miridae) is a predatory mirid reported for deciduous trees in western Mediterranean area. This work aimed to determine the biological and demographic parameters for this species at different temperatures (15, 20, 25 and 30°C). Egg hatching times shortened from 57.8 days at 15°C to 9.2 days at 30°C, and nymphal development times declined from 62.8 days at 15°C to 11.1 days at 30°C. The hatching and nymphal survival rates were low at 15 and 30°C. The lower thermal thresholds for the egg and nymphal development were 12.4 and 12.0°C, respectively. These high thermal thresholds could be a safety mechanism to avoid the emergence of nymphs in the unfavourable winter period. Female weight increased between 15 and 25°C and decreased at 30°C. The fecundity increased from 70.2 eggs per female at 15°C to 212.4 eggs per female at 25°C, and decreased to 88.5 eggs per female at 30°C. Fertility ranged from 9.4% at 15°C to 40.9% at 25°C, being 24.9% at 30°C.The intrinsic rate of natural increase (r_m) rose from 0.001 to 0.081 between 15 and 25°C and decreased to 0.05 at 30°C. In summary, this species performs poorly at low temperatures and has a relative tolerance of high temperatures (30°C); its performance was best at 25°C. Knowledge of the variation in the biological parameters with temperature may be very useful for the understanding of its ecology and population dynamics.

Key words: demographic parameters, development, mirids, population dynamics, predators, reproduction, thermal thresholds.

1. INTRODUCTION

Pilophorus gallicus Remane is a generalist predator within the Pilophorini tribe (Hemiptera: Miridae: Phylinae). This monophyletic tribe of tropical Gondwanian origin nowadays has a cosmopolitan distribution (Schuh, 1991; Wheeler, 2001). The genus *Pilophorus* is the largest of the tribe and includes many species that are strongly ant-mimetic (Schuh, 1991; Yasunaga and Schuh, 2013). Some of the species within this genus show zoophytophagous habits, preying mostly on small hemipterans such as aphids and psyllids, but also on dipteran larvae or mites (Wagner and Weber, 1964; Schuh, 1974; Wheeler, 2001; Duwal *et al.,* 2014). *Pilophorus gallicus* was described on trees of the Salicaceae (*Populus alba* and *Salix* spp.) (Remane, 1954) in southern France, and has since been reported from several localities in Spain (Ribes, 1965, *et al*., 2008; Sanchez *et al*., 2010, *et al*., 2011) and France (Corsica) (Pericart 1965). Sanchez and Ortín-Angulo (2012) found *P. gallicus* in great numbers in pear orchards in southeastern Spain, where it may contribute to the control of aphids and the pear psyllid *Cacopsylla pyri* L. (Hemiptera: Psyllidae), a principal pest of European orchards (Stamenkovic *et al*., 2001; Erler, 2004; Garcia-Chapa *et al*., 2005).

Insects are highly dependent on the thermal conditions of the surrounding environment. As they are poikilothermic and ectothermic organisms, their body temperature is not constant and it is regulated by behavioural strategies or physiological adaptations (Addo-Bediako *et al*., 2000; Deutsch *et al.,* 2008; Denlinger, 2009). Temperature has a strong effect on the development and reproduction of insects, and it determines their geographical distribution and seasonal abundances (Deutsch *et al*., 2008; Régnière *et al.,* 2010; Régnière *et al.,* 2012). The thermal threshold and constant may be useful indicators to predict the potential distribution and abundance of insects (Messenger, 1970). Generally, fecundity and development rate rise as temperature increases from the lower thermal threshold to the optimum temperature; at higher temperatures, the values of these biological parameters decline progressively until the lethal temperature is reached (Bursell, 1964; Logan *et al*., 1976; Frazier *et al.,* 2006). This pattern has been observed for several predatory mirids (Hemiptera: Miridae) such as *Macrolophus caliginosus* Wagner [Syn. *Macrolophus melanotoma* (Costa)], *Macrolophus pygmaeus* Rambur, *Nesidiocoris tenuis* Reuter and *Pilophorus typicus* Distant. These species have differing levels of thermophilia but all of them are able to develop and reproduce between 15°C and 30°C (Fauvel *et al.,* 1987; Perdikis and Lykouressis, 2002a, 2002b; Sanchez *et al.,* 2009; Nishikawa *et al.,* 2010). Knowledge of the performance of natural enemies as a function of temperature may help to predict their population dynamics and periods of arrested development and reproduction (Van der Meiracker, 1994; Hart *et al.,* 2002; Sanchez and Lacasa, 2002; Bahşi and Tunç, 2008).

In southern Spain, *P. gallicus* appears generally between the end of March and the beginning of April, and nymphs and adults decline in number in the autumn, disappearing from pear orchards in winter (Sanchez and Ortín-Angulo, 2012). Large numbers of individuals are generally registered in warm summer conditions; in contrast, low winter temperatures may be unfavourable for the development and reproduction of *P. gallicus*, which quite likely overcomes the adverse period by overwintering as eggs (Sanchez and Ortín-Angulo, 2012). Little is known about the overwintering biology of *P. gallicus*; low temperatures may prevent or delay the hatching of *P. gallicus* eggs in winter and early spring, and high summer temperatures may reduce population growth. Hence, the *P. gallicus* abundance is expected to vary according to seasonal temperature fluctuations. Insights into the influence of temperature on *P. gallicus* biological parameters will be useful to explain the abundance and population dynamics of the species in its natural habitat. The aim of this work was to determine the variation in the development times of the pre-imaginal instars and in the reproduction of *P. gallicus* as a function of temperature, and to estimate the thermal thresholds, demographic parameters and optimum temperature for the species.

2. MATERIALS AND METHODS

2.1. Insect rearing

A laboratory colony was established with *P. gallicus* collected in pear orchards from the northeast of Murcia (Spain) several months before the start of the assay. The insects were reared on *Pyrus communis* L. (Rosaceae) seedlings in mesh-walled, wooden-framed cages (40 x 40 x 40 cm) at 25 ± 1°C and 70 ± 10% relative humidity under long-day conditions (16L:8D) (Photo 1 Annex of photos). *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs provided by Biobest N.V were used as factitious prey supplied on sticky paper (Post-it® note; 3M, St Paul, MN, USA).

2.2. Development of the pre-imaginal instars of P. gallicus at different temperatures

The development of *P. gallicus* eggs and nymphs was studied at four different temperatures: 15, 20, 25 and 30°C. To determine hatching times, a uniform cohort of eggs was obtained for each temperature. Four hundred young adults (1 to 7 days-old) were divided into four groups and each one was placed in a mesh-walled, wooden-framed cage (40 x 40 x 40 cm) with 20 *P. communis* seedlings as egg-laying substrate and *E. kuehniella* eggs as food. Each cage was placed in a plant growth chamber (Binder KBWF-750, Tuttlingen, Germany) at the corresponding temperature, 70 ± 10% RH and 16L:8D photoperiod. Because of the low oviposition of *P. gallicus* females at 15°C, eggs laid at 20°C were used to determine the hatching time at 15°C. After 24 hours the adults were removed and the pear saplings were maintained at the corresponding temperature until the emergence of the nymphs. The plants were inspected every day to register the hatching time and the emerged nymphs were removed.

To determine the development of the nymphs at the different temperatures, eggs were obtained by placing 200 young adults (1 to 7 days-old) in a mesh-walled, wooden-framed cage (80 x 40 x 40 cm) with 40 *P. communis* seedlings as egg-laying substrate and *E. kuehniella* eggs as food at 25°C, 70 ± 10% RH and 16L:8D photoperiod. After 24 hours the adults were removed and the pear saplings were kept under the same conditions and inspected every day to observe the emergence of the nymphs. Just-emerged nymphs were placed at 15, 20, 25 and 30 ± 1°C, 60 ± 10% RH and 16L:8D in individual 100-ml, translucent cups with a ventilation window in the lid covered with fine-mesh nylon muslin. Each cup contained a pear leaf, placed with the petiole inserted in a 5-mm agar (1%) layer, and a small piece of cotton soaked in water. The agar surface was covered with a plastic disc to avoid nymphs getting stuck. *Ephestia kuehniella* eggs were provided on a 1-cm^2 sticky paper (Photo 2 Annex of photos). The pear leaf, the *E. kuehniella* egg paper strips and the cotton were changed every 2 or 3 days. The nymphs were checked daily until they completed development. The adults were left for 1 day before being sexed and weighed.

2.3. Reproduction of *P. gallicus* at different temperatures

The oviposition of *P. gallicus* was assayed at 15, 20, 25 and 30 ± 1°C, 70 ± 10% RH and 16L:8D. To obtain females and males for this trial, first-instar nymphs were reared in plastic containers (4700 ml capacity) with meshed windows, placed in growth chambers (Binder KBWF-750, Tuttlingen, Germany) at the different temperatures assayed. Water was provided in soaked cotton and *E. kuehniella* eggs were given *ad libitum* on sticky papers; pear leaves and crumpled pieces of absorbent paper were put in the cages to reduce cannibalism. The pear leaves, cotton and sticky paper with eggs were replaced three times a week. When the nymphs reached the fifth instar, they were transferred individually to 100-ml translucent cups with a pear leaf on a 5-mm agar (1%) layer, a piece of water-soaked cotton and *E. kuehniella* eggs as food. Thereafter, the nymphs were checked every day until they completed development. Newly-emerged adults were kept together for mating for 24h in 4700-ml plastic containers, as described above. Then, pairs of females and males were formed and placed in a 2000-ml translucent plastic cylinder with a 9-cm-diameter mesh on the top and *P. communis* seedlings (approximately 20 cm high) as laying substrate (Photo 3 Annex of photos). A minimum of 26 couples were assayed for each temperature. *Ephestia kuehniella* eggs offered on a 2-cm^2 sticky paper were used as food and were changed every 2 days. Dead males were replaced. The seedlings were changed daily and observed under a stereomicroscope for egg counting. Thereafter, the saplings were kept at their respective temperatures and the emergence of the nymphs was checked daily.

2.4. *Statistical analyses*

The lower threshold temperature and the thermal constant for eggs and every nymphal instar were estimated by linear regression using the model proposed by Ikemoto and Takai (2000), DT = k + tD, where DT is the product of development time in days (D) and temperature in degrees Celsius (T). The intercept, k, is the thermal constant in degree days (DD) and the slope, t, the lower development threshold in °C. ANOVA was used to analyse the influence of temperature on egg hatching time, nymphal development time and adult weight. The Tukey test (α = 0.05) was used for the separation of the mean development times for every pre-imaginal instar and adult weight, among the temperatures and between the sexes. Development data for eggs and pre-imaginal instars were log10-transformed to account for heteroscedasticity. The emergence rates for eggs at each temperature were calculated by dividing the total number of eggs laid by each female by the number of eggs emerged. The binomial proportion test was used to compare the survivorship of nymphs and emergence rate of eggs among the temperatures. This test was performed using the function 'prop.test' ('stats' package) in R statistical software (R Development Core Team, 2008).

The effect of temperature on fecundity, fertility, oviposition period, pre-oviposition period and longevity was tested using ANOVA and the Tukey test was used for the separation of the means. These parameters were calculated taking into account only ovipositing females. The following demographic parameters were calculated: the intrinsic rate of natural increase (rm), calculated with the equation $\sum l_x m_x e^{-r_m x} = 1$ derived by Lotka (1907, 1913 in Krebs 1985), which is defined as the rate of population increase per head under specific physical conditions, considering the age-specific fecundity and the survival rates, in an unlimited environment where the effects of increasing density are not considered (Birch, 1948); the net reproduction on rate ($R_0 = \sum l_x m_x$), which represents the number of female births per female throughout her lifetime; the mean generation time ($G = \sum_0^{max} x l_x m_x / R_0$); the population doubling time ($D = ln2/r_m$) and the finite rate of increase ($\lambda = e^{r_m}$). All the analyses were performed using R statistical software (R Development Core Team, 2008).

3. RESULTS

3.1. *Egg and nymphal development*

The egg hatching and nymphal development times decreased with increasing temperature. Incubation time ranged from 57.8 ± 1.0 days at 15°C to 9.2 ± 0.1 days at 30°C, and nymphal development declined from 62.8 ± 0.8 days at 15°C to 11.1 ± 0.1 days at 30°C (Table 1). In all the pre-imaginal instars of *P. gallicus*, the development times were significantly influenced by temperature (Eggs: $F = 6015$; df= 3, 239; $P < 0.001$. N1: $F = 743.2$; df = 3, 380; $P < 0.001$. N2: $F = 846.8$; df = 3, 337; $P < 0.001$. N3: $F = 493.6$; df = 3, 305; $P < 0.001$. N4: $F = 756.8$; df = 3, 265; $P < 0.001$. N5: $F = 2302$; df = 3, 215; $P < 0.001$. N1 to Adult: $F = 4171$; df = 3, 215; $P < 0.001$). Females and males did not differ significantly in their development times (N1 to Adult) at any of the temperatures assayed ($F = 1.83$; df = 1, 210; $P > 0.05$). Regardless of the temperature, the development time for N1 was always longer than for N2 and N3; egg and N5 were the stages with the longest duration. The rate of egg emergence increased from 0.094 at 15°C to 0.409 at 25°C and declined at 30°C. Significantly lower hatching rates were registered at 15 and 30°C in comparison with those obtained at 20 and 25°C ($P < 0.05$). The nymphal survivorship increased between 15 and 20°C and decreased at higher temperatures; the highest survival rate for N1 to

Adult (0.703) was registered at 20°C, differing significantly from that at the rest of the temperatures (Table 1). Generally, N1 and N5 were the nymphal instars with greatest mortality. The eggs had the highest thermal threshold (12.43 ± 0.05°C) and N1 the lowest (11.94 ± 0.15°C) (Table 2). The eggs (152.70 ± 1.26 DD) had the highest thermal constant, followed by N5 (53.52 ± 0.63 DD); for N1 to N4 the cumulative thermal requirements were similar and lower than for the rest of the instars (range: 30.86 ± 0.61 - 39.50 ± 0.83 DD). There was a very good fit of the models to the experimental data with the R^2 values ranging from 0.939 to 0.996 and high significant P-values (Table 2).

Adult weight was significantly affected by temperature, with a significant interaction temperature:sex (Temperature: $F = 9.47$; df = 3, 193; $P < 0.001$; Temperature:Sex: $F = 2.77$; df = 3, 193; $P < 0.05$) (Table 3). The weight of the females increased between 15 and 25°C and decreased at 30°C. In contrast, the weight of the males increased between 15 and 20°C and decreased at higher temperatures. Differences in weight between the sexes were only significant at 25°C (Tukey test, $P < 0.001$) (Table 3).

Table 1. Development times (days) (mean ± S.E.) and survivorship of the pre-imaginal instars of *Pilophorus gallicus* at 15, 20, 25 and 30°C.

Temperature (°C)	Instar	Development time	Survivorship
15	Egg (n)	57.8 ± 1.0 a (68)	0.094 A
	n	90	
	N1	10.7 ± 0.3 a	0.833 A
	N2	10.2 ± 0.2 a	0.773 A
	N3	10.0 ± 0.2 a	0.845 A
	N4	13.2 ± 0.3 a	0.918 AB
	N5	20.0 ± 0.2 a	0.622 AB
	N1 to Adult	62.8 ± 0.8 a	0.311 A
20	Egg (n)	20.1 ± 0.1 b (35)	0.390 B
	n	64	
	N1	5.5 ± 0.2b	0.844 A
	N2	4.6 ± 0.1 b	0.907 AB
	N3	4.6 ± 0.2 b	0.980 A
	N4	5.2 ± 0.1 b	1.000 A
	N5	7.4 ± 0.1 b	0.938 B
	N1 to Adult	27.2 ± 0.3 b	0.703 B
25	Egg (n)	11.9 ± 0.1 c (144)	0.409 B
	n	144	
	N1	3.1 ± 0.1c	0.854 A
	N2	2.3 ± 0.1 c	0.911 B
	N3	2.5 ± 0.1 c	0.920 A
	N4	3.0 ± 0.1 c	0.854 B
	N5	4.2 ± 0.1 c	0.864 B
	N1 to Adult	14.8 ± 0.1 c	0.528 C
30	Egg (n)	9.2 ± 0.1 d (96)	0.249 C
	n	149	
	N1	2.3 ± 0.1d	0.886 A
	N2	1.8 ± 0.0 d	0.924 B
	N3	1.9 ± 0.1 d	0.893 A
	N4	2.3 ± 0.1 d	0.807 B
	N5	3.1 ± 0.1 d	0.795 A
	N1 to Adult	11.1 ± 0.1 d	0.470 C

n is sample size; N is nymphal instar. Statistical comparison among temperatures for the same instar. Mean values followed by the same letter are not significantly different (Tukey test, $P > 0.05$). Survivorship values for the same instar at the different temperatures followed by the same capital letter are not significantly different (Binomial test, $P > 0.05$).

Table 2. Thermal equation, development threshold temperature (t) (°C) (mean ± S.E.) and thermal constant (k) (DD) (mean ± S.E.) of the pre-imaginal instars and N1 to adult of *Pilophorus gallicus*. The R^2 and *P*-values are given for every equation.

Instar	Equation	t (°C)	k (DD)	R^2	*P*
Egg	152.7030+12.4277D	12.43± 0.05	152.70±1.26	0.9956	< 0.001
N1	39.5003+11.9436D	11.94±0.15	39.50±0.83	0.9468	< 0.001
N2	30.8617+12.3471D	12.35±0.12	30.86±0.61	0.9674	< 0.001
N3	31.8164+12.4183D	12.42±0.18	31.82±0.87	0.9392	< 0.001
N4	38.1939+12.3586D	12.36±0.12	38.19±0.77	0.9746	< 0.001
N5	53.5174+12.4189D	12.42±0.075	53.52±0.63	0.9923	< 0.001
N1 to Adult	198.9392+12.0140D	12.01±0.074	198.94±2.07	0.9918	< 0.001

Table 3. Sex ratio and weights (mean ± standard error, in mg) of *Pilophorus gallicus* males and females at different temperatures.

		Females		Males	
Temperature (°C)	Sex Ratio	N	Weight (mg)	N	Weight (mg)
15	0.462	12	2.142± 0.119 d	14	2.146 ± 0.078 d
20	0.556	24	2.822 ± 0.092 ab	19	2.473 ± 0.060 bd
25	0.579	44	2.919 ± 0.088 a	31	2.317 ± 0.075 cd
30	0.588	36	2.635 ± 0.096 abc	21	2.253 ± 0.071 cd

N is sample size. Mean values followed by the same letter are not significantly different (Tukey test, $P > 0.05$).

3.2. *Reproduction and demographic parameters*

The total fecundity was significantly influenced by temperature ($F = 37.95$; df = 3, 107; $P < 0.001$). It increased from 70.23 ± 6.26 eggs per female at 15°C to 212.39 ± 13.21 eggs per female at 25°C, and decreased to 88.50 ± 11.76 eggs per female at 30°C (Table 4). The fertility ranged from 9.4% at 15°C to 40.9% at 25°C, decreasing to 24.9% at 30°C (Table 4). The oviposition period shortened as the temperature increased, from 65.06 ± 4.62 days at 15°C to 18.46 ± 1.89 days at 30°C (Figure 1). In contrast, the pre-oviposition period, as well as the time of the oviposition peak, shortened with rising temperature (Figure 1, Table 4), ranging from 11.55 ± 0.35 days at 15°C to 3.92 ± 0.13 days at 30°C. The oviposition peak was very conspicuous at 15, 25 and 30°C, but the daily oviposition was spread uniformly over a long period at 20°C (Figure 1). At 15, 25 and 30°C the maximum oviposition was observed at day 14, day 5 and day 5, corresponding to 2.6, 10.2 and 6.9 eggs per female per day, respectively (Figure 1). At 20°C the daily oviposition per female oscillated around 3 eggs (range: 1.4 to 3.9) from day 10 to day 40. The percentage of ovipositing females ranged from 53.1% at 20°C to 100% at 15°C, without any apparent temperature-dependent trend. The longevity of females was also affected by temperature, decreasing from 86.65 ± 4.76 days at 15°C to 22.42 ± 1.96 at 30°C (Table 4).

Table 4. Average ± standard error of fecundity, fertility, pre-oviposition and oviposition period, and female longevity of *Pilophorus gallicus* at four temperatures.

	15°C	20°C	25°C	30°C
N	31	49	30	27
% Ovipositing females	100	53.1	93.3	96.3
Fecundity	70.23 ± 6.29 b	82.00 ± 11.95 b	212.39 ± 13.21 a	88.50 ± 11.76 b
Fertility (%)	9.4 a	39.0 bc	40.9 b	24.9 c
Pre-oviposition period	11.55±0.35 a	9.58±0.56 b	4.86±0.18 c	3.92±0.13 c
Oviposition period	65.06±4.62 a	24.77±2.99 bc	31.00±2.04 b	18.46±1.89 c
Longevity females	86.65±4.76 a	38.42±3.62 b	39.04±1.76 b	22.42±1.96 c
Oviposition period/longevity	0.75	0.64	0.79	0.82

N is sample size. The same letters for each parameter denote no significant differences between temperatures (Tukey test, *P* > 0.05).

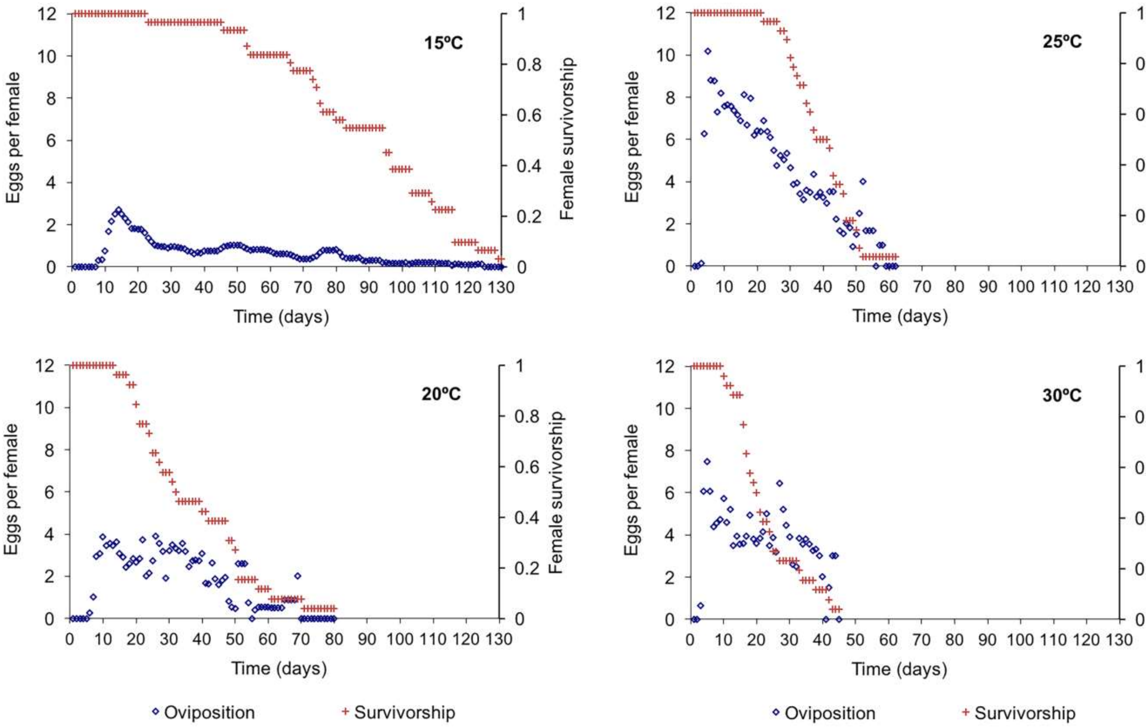

Figure 1. Average daily oviposition (blue open diamonds) and survivorship (red crosses) of *Pilophorus gallicus* females at 15, 20, 25 and 30°C.

Table 5 shows demographic parameters of *P. gallicus*. All of them were greatly influenced by temperature. The mean generation time (G) decreased from 157.38 days at 15°C to 32.11 at 30°C. The net reproduction rate (Ro) increased between 15 and 25°C and decreased at 30°C. The intrinsic rate of natural increase (r_m) increased from 0.001 to 0.081 between 15 and 25°C and decreased to 0.05 at 30°C. In agreement with r_m, the population doubling time (*D*) was very long at 15°C (990.62 days) and short at 25°C (8.56 days). The population doubling time (D) and the finite rate of increase (λ) showed the same trend as the r_m.

Table 5. Demographic parameters of *Pilophorus gallicus* at 15, 20, 25 and 30°C. *n*, number of females; *Ro*, net reproduction rate; *G*, generation time (days); *D*, population doubling time (days); r_m, intrinsic rate of natural increase/day; λ, finite rate of increase.

T (°C)	*n*	*Ro*	*G*	*D*	r_m	λ
15	31	1.12	157.38	990.62	0.001	1.001
20	49	14.7	67.39	17.38	0.040	1.041
25	30	29.17	41.67	8.56	0.081	1.084
30	27	4.95	32.11	13.91	0.050	1.051

4. DISCUSSION

Temperature has a strong effect on the development and reproduction of *P. gallicus*. This species performs poorly at low temperatures and has a relative tolerance of high temperatures (30°C); its performance was best at 25°C. Low temperature (15°C) reduced development rates, increased mortality in the pre-imaginal instars and lowered daily oviposition rates. Nishikawa *et al.* (2010) reported high nymphal mortality for *P. typicus* at 17.5 and 30°C, but similar hatching rates for all the temperatures assayed. The increase in temperature from 15 to 30°C shortened by almost six times the egg hatching and nymphal development times, but at 30°C the egg hatching rates, nymphal survival, adult weight, fecundity and fertility declined. This reduction in performance at high temperatures was also reported for other predatory mirids such as *Dicyphus hesperus* Knight (Hemiptera: Miridae)*, M. caliginosus, N. tenuis*, *P. typicus* and the anthocorids *Orius niger* Wolff (Hemiptera: Anthocoridae) and *Orius laevigatus* Fieber (Hemiptera: Anthocoridae) (Fauvel *et al*., 1987; Sanchez and Lacasa, 2002; Bahşi and Tunç, 2008; Sanchez *et al*., 2009; Nishikawa *et al*., 2010).

In comparison to other mirid species, the thermal threshold for *P. gallicus* eggs (12.43°C) was higher than for *N. tenuis* (10.3°C), *M. pygmaeus* (6.92–7.61°C), *M. caliginosus* (7.9–9.5°C) and *D. hesperus* (7.3°C) (Hart *et al*., 2002; Perdikis and Lykouressis, 2002b; Gillespie *et al.,* 2004; Sanchez *et al.,* 2009), similar to that of *O. laevigatus* (12.8°C) (Sanchez and Lacasa, 2002) and lower than for *Orius albidipennis* Reuter (Hemiptera: Anthocoridae) (14.4°C) (Sanchez and Lacasa, 2002). In the same way, the thermal threshold for nymphal development (N1 to Adult) for *P. gallicus* (12.01°C) was higher than for *M. pygmaeus* (8.23–9.19°C), *M. caliginosus* (7.2–8.1°C) and *D. hesperus* (7.8–8.01°C) (Hart *et al*., 2002; Perdikis and Lykouressis, 2002b; Gillespie *et al*., 2004), similar to that of *P. typicus* (egg to adult: 12.0–12.1°C), *N. tenuis* (11.7°C) and *O. laevigatus* (egg to adult: 11.3°C) and lower than for *O. albidipennis* (egg to adult: 14.2°C) (Sanchez and Lacasa, 2002; Sanchez *et al.,* 2009; Nishikawa *et al*., 2010). The thermal requirements (k) for *P. gallicus* eggs (152.70 DD) and the nymphal period (198.94 DD) were higher than those of *N. tenuis* (eggs: 148 DD; nymphs: 182.3 DD), *O. albidipennis* (egg to adult: 212.8 DD) and *O. laevigatus* (egg to adult: 285.7) (Sanchez and Lacasa, 2002; Sanchez *et al.,* 2009), but lower than those of *M. pygmaeus* (eggs: 182 DD; nymphs: 253 DD), *M. caliginosus* (eggs: 184.4 DD; nymphs: 270.3 DD) and *D. hesperus* (eggs: 227.2–231.9 DD; nymphs: 274.4–301.9 DD) (Hart *et al*., 2002; Perdikis and Lykouressis, 2002b; Gillespie *et al.,* 2004). *Pilophorus typicus* has thermal requirements (egg to adult: 357.1 DD) similar to those of *P. gallicus* (Nishikawa *et al*., 2010).

The variation in fecundity and daily oviposition rates in relation to temperature in *P. gallicus* was similar to that observed in other predatory hemipterans, with a reduction in fecundity at 15°C, a maximum at about 20–25°C and a decline at 30°C and higher temperatures. For *M. caliginosus, M. pygmaeus* and *O. laevigatus*, maximum fecundity was reached at 20°C, while *P. gallicus* peaked at 25°C, like *O. albidipennis* and *P. typicus* (Fauvel *et al.,* 1987; Perdikis and Lykouressis, 2002a; Sanchez and Lacasa, 2002; Nishikawa *et al*., 2010). Temperature had a strong effect on *P. gallicus* fertility; although the fecundity at 15°C was close to that at 20°C, the fertility at 15°C was greatly reduced due to the low hatching rate (8%). Strong reductions in fecundity and fertility at low temperatures have been reported for other mirid species such as *P. typicus* and *N. tenuis* (Sanchez *et al.,* 2009; Nishikawa *et al*., 2010), while others (e.g. *M. caliginosus* and *M. pygmaeus*) showed relatively high oviposition and hatching rates (Fauvel *et al.,* 1987; Perdikis and Lykouressis, 2002a). The r_m of *P. gallicus* increased with temperature, reaching its maximum at 25°C (0.081) and declining at 30°C; comparable r_m values and a similar trend were reported for *P. typicus* (Nishikawa *et al*., 2010). The r_m values of *O. laevigatus* and *O. albidipennis* were found to be much higher, peaking at 30 and 35°C, respectively (Sanchez and Lacasa, 2002). The weight of *P. gallicus* females increased between 15 and 25°C and decreased at 30°C. A variation in weight in relation to temperature has been reported for other predatory mirids such as *N. tenuis* and *D. hesperus* (Gillespie *et al.,* 2004; Sanchez *et al.,* 2009). The variation in the fecundity of the *P. gallicus* females followed the same trend as their weight. Although this does not necessarily mean that the two parameters are correlated (Klingenberg and Spence, 1997), the issue merits further exploration because of the great impact it may have on the population dynamics and mass rearing of the species. A correlation between body mass and fecundity was found in *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae), but the effect disappeared through the generations (Mohaghegh *et al.*, 1999).

Insects are poikilothermic organisms and, therefore, their geographical distribution is tied closely to their tolerance of and adaptation to the range of temperatures in the areas they inhabit (Régnière *et al.,* 2009; Bentz *et al.,* 2010; Safranyik *et al.,* 2010). *Pilophorus gallicus* is a Mediterranean species, living on deciduous trees (e.g. poplar, willow, pear trees), that has been reported - to date - in southern France and the northeast and southeast of Spain (Remane, 1954; Pericart, 1965; Ribes *et al.,* 2008; Sanchez and Ortín-Ángulo, 2012). The geographical distribution of *P. gallicus* is similar to that of other thermophilous hemipterans such as *O. albidipennis* and *N. tenuis*, and it seems to have a distribution that is more restricted than those of other Mediterranean predatory hemipterans (e.g. *M. pygmaeus, M. melanotoma* and *O. laevigatus*) (Wagner and Weber, 1964; Pericart, 1972; Aukema and Rieger, 1999; Sanchez *et al*., 2012)*. Pilophorus gallicus* shares with *O. albidipennis* and *N. tenuis* high development thermal thresholds, but its performance at high temperature is clearly inferior to those of the more thermophilous species (e.g. *O. albidipennis*), being closer to those of species with a wider geographical distribution (e.g. *O. laevigatus*) (Sanchez and Lacasa, 2002; Sanchez *et al*., 2009). The explanation for the high development thermal threshold of *P. gallicus* may lie in its overwintering strategy. *Pilophorus gallicus* overwinters as eggs on trees and the first nymphs start to appear at the end of March – beginning of April (Sanchez and Ortin-Angulo, 2012). It seems to resume its activity in coincidence with the sprouting of some tree species (e.g. pear trees) and when prey (e.g. pear psyllids) start to be available; before that, the environment is not favourable for the development of the species. In the area of southern Spain where *P. gallicus* was collected in pear orchards for this study, average winter temperatures are generally below 10°C and temperatures are below the thermal threshold during more than half of the day (Supp. material, S1 and S2). When pear trees sprout and blossom, around weeks 13-14 (early spring), the average temperature is above the thermal threshold for egg hatching (12.4°C) and the number of hours per day under the threshold is rapidly decreasing. According to these considerations, the high development thermal threshold in *P. gallicus* is likely a safety mechanism to avoid the emergence of nymphs in a period that would be unfavourable for the life-cycle of the species. In the summer months (July-August: weeks 27 to 35), average temperatures are around 25°C, matching the optimum temperature for the species. Temperatures are above 30°C during part of the day in a few weeks in summer (Supp. material, S2), but this short-term exposure to high temperatures should not have strong deleterious effects. Insects are known to withstand exposure to extreme temperatures during short periods of time and to resume their normal activity when the negative factor recedes (Liu *et al.,* 1995; Petavy *et al*., 2001; Saleh and Sengonca, 2003; Régnière *et al.,* 2012; Wilches *et al.,* 2016). With the arrival of autumn, trees start to lose their leaves and the environment again becomes adverse with regard to the life-cycle of *P. gallicus*. The average temperature in October (weeks 43-44) is below 15°C and values under the thermal threshold in the following weeks will prevent egg hatching during late autumn and winter.

Knowledge of the variation of *P. gallicus* biological parameters in relation to temperature may be very useful to understand its ecology and to predict the population dynamics of the species (Hart *et al*., 2002; Gillespie *et al.,* 2004; Kontodimas *et al.,* 2007; Sanchez *et al*., 2009). The biological potential of this species and, therefore, its impact on prey populations will be greatly modified by the variation in temperature through the seasons. The results of the present work show that the species is well adapted to the annual temperature variations of southern Europe.

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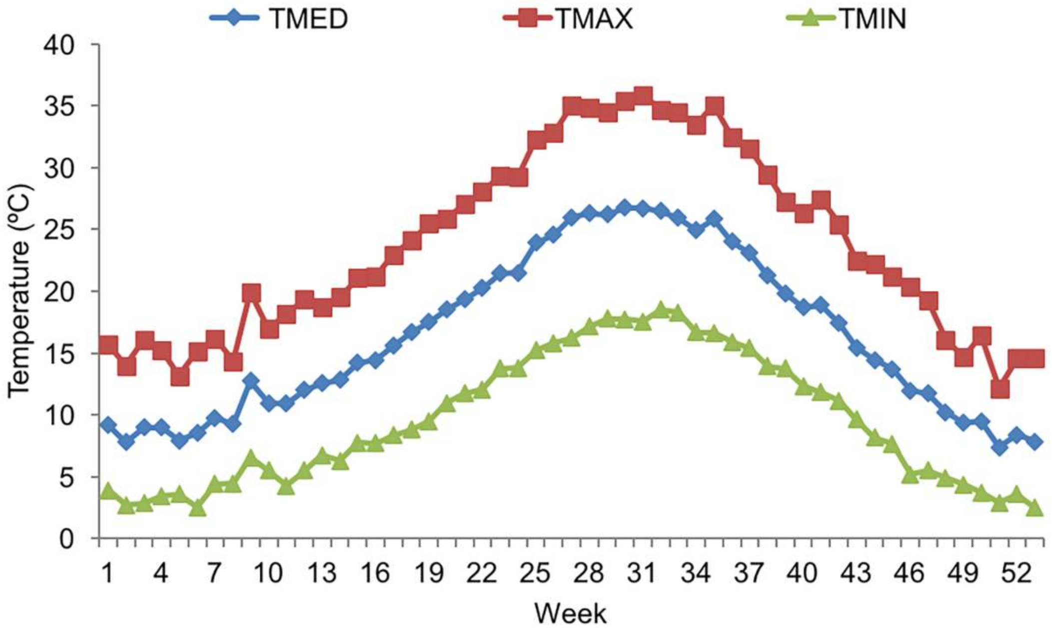
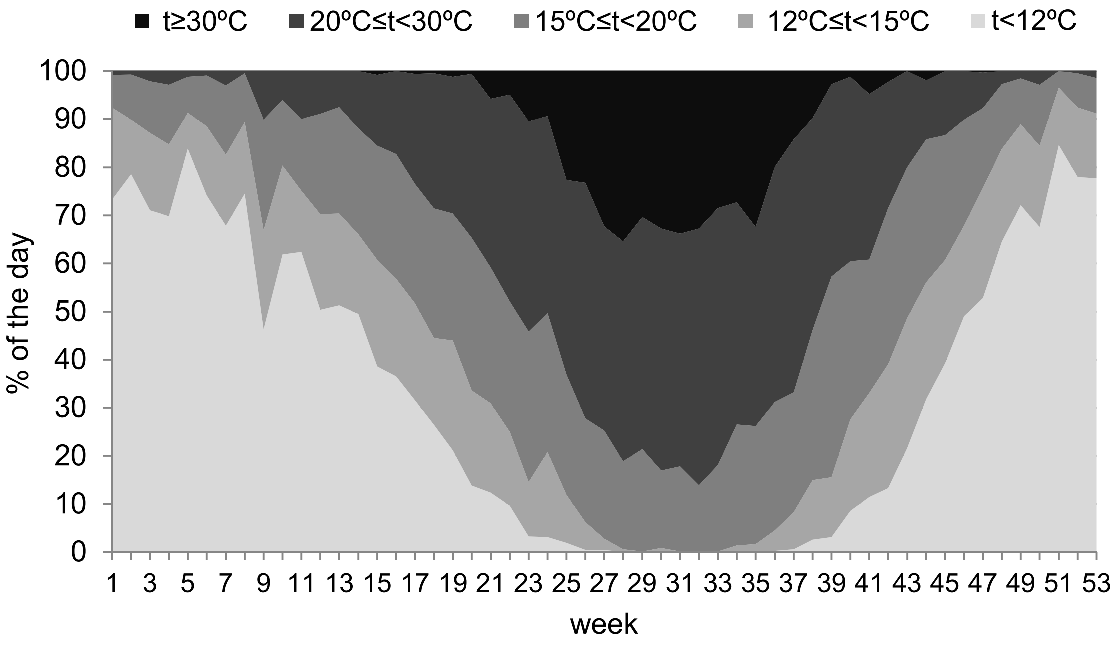
SUPPLEMENTARY MATERIAL



Supplementary material S1. Weekly averages of the temperature registered at the Jumilla (Murcia, Spain) climatic station (JU71, Las Encebras) (Lat: 38° 23' 40.01" N, Lon: 1° 14' 21.58" W) from 2007 to 2011 (http://siam.imida.es/).



Supplementary material S2. Weekly averages of the daily range of temperatures, from 2007 to 2011. Records from the Jumilla (Murcia, Spain) climatic station (JU71, Las Encebras) (Lat: 38° 23' 40.01" N, Lon: 1° 14' 21.58" W) (http://siam.imida.es/).

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CHAPTER 2

Population dynamics and seasonal variation in the embryonic dormancy of *Pilophorus gallicus* (Hemiptera: Miridae): "Don’t put all your eggs in one basket"

Photo: Elena López Gallego

CHAPTER 2: Population dynamics and seasonal variation in the embryonic dormancy of *Pilophorus gallicus* (Hemiptera: Miridae): "Don't put all your eggs in one basket"

ABSTRACT

1. *Pilophorus gallicus* Remane is a generalist predator in southern European pear orchards. Nymphs and adults are present in orchards from March to November; their winter absence suggests either migration to other hosts or embryonic dormancy on pear trees to overcome the adverse period. Besides, it has been hypothesised that aestivation takes place to cope with extreme summer conditions.

2. The objective was to investigate the reproductive strategy of *P. gallicus* to overcome unfavourable periods. For this, (1) its population dynamics were followed during several years; (2) females were sampled in three different seasons to study the condition (diapausing or non-diapausing) of the laying.

3. The results obtained show that nymphs were always the first mobile instar to show up in pear orchards and the presence of adults was delayed, indicating egg overwintering. Non-diapausing eggs prevailed in spring and summer, while the autumn eggs were mainly diapausing. Aestivation was rejected.

4. Worthy of note are: (a) the presence of diapausing eggs under favourable conditions; (b) the existence of females laying both diapausing and non-diapausing eggs - thus, "not laying all the eggs in one basket". The reproductive strategy of *P. gallicus* is considered as *bet-hedging* for the short- and long-term survival of the species.

Key words: bet-hedging, biological control, embryonic diapause, migration, *Pilophorus gallicus*, population dynamics, variable climates.

1. INTRODUCTION

Pilophorus gallicus Remane (Hemiptera: Miridae) is a generalist predator that may play an important role in the regulation of populations of the pear psyllid *Cacopsylla pyri* L. (Hemiptera: Psyllidae) and other small arthropod pests in pear (*Pyrus communis* L., Rosaceae) orchards in the Mediterranean area (Sanchez and Ortín-Angulo, 2012). *Cacopsylla pyri* is one of the main pests of pear trees, causing important economic damage in European production areas (Hodkinson, 1984; Vilajeliu, 1989; Rieux *et al.*, 1992; Artigues *et al.*, 1996; Vilajeliu *et al.*, 1998; Stamenkovic *et al.*, 2001; Erler, 2004; Silva *et al.*, 2005). The control of this pest has usually involved insecticides, but the development of resistance to commonly-used products and the severe restrictions concerning the use of chemicals in agriculture have increased the interest in biological pest control and integrated pest management (IPM) programmes (Vilajeliu, 1989; Avilla *et al.*, 1992; Rieux *et al.*, 1992; Artigues *et al.*, 1996; Vilajeliu *et al.*, 1998; Erler, 2004; Sanchez and Ortín-Angulo, 2012). *Pilophorus gallicus* is a multivoltine mirid whose active stages can be found in in pear orchards between the end of March and the beginning of November (Sanchez *et al.*, 2010, 2011; Sanchez and Ortín-Angulo, 2012). The absence of mobile forms during the winter season suggests either migration to more-suitable areas or hosts, as some species of anthocorids associated with pear orchards do (Scutareanu *et al.*, 1999), or overwintering on pear trees as eggs. Both of these strategies may allow *P. gallicus* to withstand the adverse winter conditions, but they will have different implications if conservation biological pest control methods are adopted. In the first case, the availability of host plants in the vicinity of crops should increase the population of the predator locally and facilitate its migration to pear orchards, while in the second case, non-harmful management strategies should be adopted to preserve overwintering populations on pear trees.

In most environments, organisms face seasonal fluctuations that constitute a major component in their evolution, involving a variety of physiological and behavioural adaptations (Begon *et al.*, 2006). Insects have the ability to modify their life cycle in response to unfavourable environmental factors, such as extremely-low or high temperatures, the lack of convenient sites for reproduction, the scarcity of food or the presence of predators (Tauber *et al.*, 1986). Migration to other, more-suitable places or entering dormancy (quiescence or diapause) are two of the most-typical strategies used by insects to overcome adverse conditions (Dingle, 1978; Tauber *et al.*, 1986; Taylor and Karban, 1986; Saunders, 2010). In both cases, there is a suspension of growth, development or reproduction and, although they may share many physiological similarities (Kennedy, 1961; Dingle, 1978), they normally differ in the life stage involved. Migrant individuals are usually alate adults, whereas individuals may enter into quiescence or diapause in any stage of development, from early stages of embryogenesis to adults. Some organisms may adopt more than one strategy, such as *Danaus plexippus* (Lepidoptera: Nymphalidae) or *Picromerus bidens* (Hemiptera: Pentatomidae) (Musolin, 1996; Musolin and Saulich, 2000; Perez and Taylor, 2004). The population fluctuations in space and time due to migration and dormancy deserve a special consideration as integral parts of the biology of insects, especially in pest and predator species due to the importance of understanding their life-cycles and predicting their population dynamics (Nylin, 2001).

In temperate regions, mirids generally overwinter as eggs, although several species withstand the cold period as adults (Schuh and Slater, 1995; Musolin and Saulich, 1999; Wheeler, 2001; Higuchi and Takahashi, 2005; Shintani and Higuchi, 2008). Embryonic dormancy is commonly manifested through quiescence or diapause, but their repercussions for population dynamics differ significantly. Quiescence is an immediate physiological response to external factors, contrary to diapause, which is a state of genetically-determined suppression of development that is programmed far in advance and often lasts longer than the period of unfavourable conditions (Beck, 1980; Dolling, 1991; Bale and Hayward, 2010). In temperate climates, short photoperiod appears to be a primary cue (*token stimuli*) for winter diapause induction, while low temperatures maintain the dormant status and high temperatures with long photoperiod contribute to the termination of the diapause and the subsequent development (Ruberson *et al.*, 1998; Denlinger, 2002; Saunders *et al.*, 2004; Berkvens *et al.*, 2008; Feng *et al.*, 2012; Bonte *et al.*, 2016).

Besides the adverse winter conditions, insects in arid climates face high temperatures and low humidity that may compromise their survival during summer. To cope with such circumstances, several species of hemipterans enter a summer diapause called aestivation (Masaki, 1980; Musolin and Saulich, 1999, 2000; Pires *et al.*, 2000). In continental southern Spain temperatures in winter reach several degrees below zero and summer is harsh, maximum temperatures exceeding 40°C (http://siam.imida.es). Therefore, it is quite likely that insects living in this environment, such as *P. gallicus*, will either adopt behavioural strategies or will have developed mechanisms to overcome adverse periods, or both. Knowledge of those strategies and biological mechanisms in *P. gallicus* is utterly important because of their influence on its population dynamics and, thus, on its efficacy as a biological pest control agent.

The objective of this work was to investigate the biological strategy used by *P. gallicus* to overcome periods of unfavourable environmental conditions, such as winter and summer, in pear orchards in southern Europe. In the first place, we were interested to know whether *P. gallicus* disappears from pear orchards in winter, at the end of the vegetative period of pear trees, and colonises orchards again in spring, or if, in contrast, it remains in pear orchards and resumes its activity in spring. To achieve this aim, we followed the population dynamics of *P. gallicus* in pear orchards during several years. The absence of mobile forms in late autumn and winter suggests two main possibilities: (1) emigration of populations from pear orchards to overwintering sites at the end of the season; in that case, the founder population in the following cycle will be composed of adults moving from overwintering sites to pear orchards, and the time series of nymphs would be delayed in relation to those of adults; (2) overwintering as eggs on pear trees; in that case, the first generation would start with the emergence of nymphs and the first adults would show up after the nymphs had completed their development. In this latter case, we wanted to see if the eggs suffered some kind of quiescence or diapause. We were also interested to know if some kind of reproduction arrestment or diapause took place in summer. We knew from biological studies that high temperatures, above 30°C, affect negatively the nymphal development and reproduction of *P. gallicus* (Ramírez-Soria *et al.*, 2017). To achieve these objectives, we sampled females of *P. gallicus* along three different seasons of the year and studied their reproductive state and the condition of the eggs they laid. We expected that most of the females from the first generation, present in spring when environmental conditions are favourable, would lay eggs and that these eggs would not be of the quiescent or diapausing type. In contrast, in autumn, when adverse winter conditions are approaching, we expected that the females would lay mainly quiescent or diapausing eggs. As stated above, diapausing eggs need to be exposed to specific conditions to resume their development, while quiescent eggs will immediately resume their development or speed it up when transferred to optimum conditions.

2. MATERIALS AND METHODS

2.1. Population dynamics of P. gallicus

2.1.1. Location and crop management

The study was carried out in three commercial pear orchards (A, B and C) located in the "Rambla del Judío", in the municipality of Jumilla, Murcia (Spain), from 2008 to 2010. The orchards were planted with the cultivar "Ercolini" between 2001 and 2004, using "Castell" for cross-pollination. The area of each orchard was approximately 1 ha, with ten rows of approx. 200 pear trees each; the trees were trained in trellises, with 4 m between rows and 0.8 m between trees within rows. Integrated pest management (IPM) was applied for the control of *C. pyri* and other pests. More details of the spraying calendar may be found in Sanchez and Ortín-Angulo (2012), where Sabinar, Control-1 and Ventorillo correspond to orchards A, B and C, respectively.

2.1.2. *Sampling of insects*

Sampling was carried out in the three orchards from February 2008 until November 2010. The frequency of sampling was weekly from the beginning of March to the end of August, and every two weeks during the rest of the year. The sampling technique involved beating branches (2-4 cm in diameter) over a 28-cm-diameter funnel with a 100-ml plastic bottle at the bottom to collect the sample (Photo 4 Annex of photos). The branches were hit three times at the base with a wooden stick; in 2008, three independent samples of 20 beats each were collected from each plot on every sampling date; in 2009 and 2010 the sample size was increased to 30 beats per sample. The samples were taken in refrigerated containers to the laboratory and observed under a stereomicroscope to count *P. gallicus* nymphs and adults.

2.2. ***Study of the reproductive state of P. gallicus females and the condition of eggs***

Adults of *P. gallicus* were collected in pear orchards located in the municipality of Jumilla (Murcia, Spain) in three different seasons (mid-May, mid-July and mid-September), corresponding to the three annual peaks of abundance, in 2012 (Sanchez and Ortín-Angulo, 2012; observations from the previous trial). Throughout the manuscript we will refer to these three demographic peaks as “generations”, for simplicity, although we are aware of the overlapping of generations after the first one. The average temperature and daylength for the study area were recorded for the week before each collecting event (http://siam.imida.es). Twenty-five pairs of females and males were set up for each of the three generations; each couple was placed in a 2000-ml translucent-plastic cylindrical container with a 9-cm-diameter mesh on the top and a *P. communis* seedling, approximately 20 cm tall, as the laying substrate (Photo 3 Annex of photos). *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs were offered *ad libitum* as factitious prey on 1-cm^2 sticky papers every 2-3 days. All the containers were kept in plant growth chambers (Binder KBWF-750, Tuttlingen, Germany) at

25°C ± 1°C, 16L:8D photoperiod and 70 ± 10% relative humidity (RH). The oviposition of *P. gallicus* females was registered during one week. The couples were removed on the seventh day and the eggs were counted under a stereomicroscope; then, the saplings with eggs were kept in plant growth chambers at 25°C, 16L:8D and 70 ± 10% RH. The emergence of the nymphs was observed three times a week in the following 30 days; each time, the nymphs were counted and removed. On the 30th day since the end of oviposition, the seedlings were observed with binoculars to check the presence of non-emerged eggs. Non-hatched eggs could be either "non-viable" or "diapausing" because the average hatching time at 25°C is 11.9 ± 0.1 days, with a maximum of 14 days (Ramírez-Soria *et al.*, 2017). In the latter case the breaking of the dormancy would happen only if the eggs were placed in the right conditions for their re-activation (*sensu* Hodek, 1996). Saplings with non-emerged eggs were kept in plant growth chambers under diapausing re-activation conditions: 90 days at 6°C, 16L:8D photoperiod and 60 ± 10% RH. The conditions for the breaking of the likely diapause were chosen based on several sources of information: (1) some works on embryonic diapause mention low temperature as the usual way to bring the diapause stage to an end (Zimmermann, 1984; Musolin and Saulich, 1996; Saulich and Musolin, 1996; Shintani and Higuchi, 2008; Lundgren, 2011); (2) preliminary trials showed that non-emerged *P. gallicus* eggs after the incubation time hatched after going through a chilling period of 90 days at 6°C with a 16L:8D photoperiod (Ramírez-Soria *et al.*, unpublished).

After the 90 days the seedlings with the eggs were returned to the previous conditions: 25°C ± 1°C, 16L:8D and 70 ± 10% RH. The eclosion of eggs was observed three times a week during the following 40 days, and the nymphs were counted and removed. We called "diapausing" eggs those hatching after the diapause-breaking treatment and "non-viable" the non-hatching ones. The seedlings were observed under a stereomicroscope to check the status of the eggs before they were discarded.

Finally, females were typified according to their reproductive response, as: (1) "non-diapause inducing", when they laid exclusively non-diapausing eggs; (2)

"diapause inducing", when they laid only diapausing eggs; and (3) "mixed", if both types of eggs were laid by the same female.

2.3. *Statistical analyses*

The population dynamics data collected during the three consecutive years in the three pear orchards were analysed by cross correlation to determine the correlation indexes and lags between the *P. gallicus* nymph and adult time series. The fecundity and fertility of the females collected in the field were compared among the three generations using ANOVA. Fecundity was the total number of eggs laid per female and fertility the number of viable (non-diapausing + diapausing) eggs laid per female during the week of observation. The Tukey test ($\alpha = 0.05$) was used for the separation of the mean fecundity and fertility among generations. The proportions of the different types of egg (viable, diapausing and non-diapausing) out of the total number of eggs laid per female were compared among the three generations using ANOVA, and the Tukey test was employed for the separation of the means. The values were transformed by the square root of the arcsine to account for heteroscedasticity. The proportions of females with different egg-laying responses were compared among the three generations using a Binomial test. All the analyses were performed using R statistical software (R Development Core Team, 2016).

3. RESULTS

3.1. *Study of P. gallicus population dynamics*

Nymphs were systematically the first mobile instar of *P. gallicus* to show up in the three pear orchards during the three years of observations (Figure 1). The first nymphs were generally registered between weeks 12 and 16, corresponding to the period from the end of March to the end of April. Adults always appeared with a three- to five-week delay in relation to nymphs (around mid-May), with a population dynamic trend similar to that of the nymphs but, generally, with lower abundance peaks. The phase shift between the time series of *P. gallicus* nymphs and adults ranged from 0 to 4 weeks, with maximum correlation coefficient values ranging between 0.444 and 0.849. The analyses of the cross-correlations between the nymph and adult annual time series are summarised in Table 1. The population dynamics of *P. gallicus* showed a characteristic pattern, with three abundance peaks: (1) in spring, when temperatures are mild and the photoperiod is increasing; (2) in summer, which generally corresponds to the greatest population peak of the year, when days are the longest and warmest; (3) in autumn, with decreasing temperatures and photoperiod. Around mid-October (weeks 40 to 43), both nymphs and adults were no longer found in the samplings.

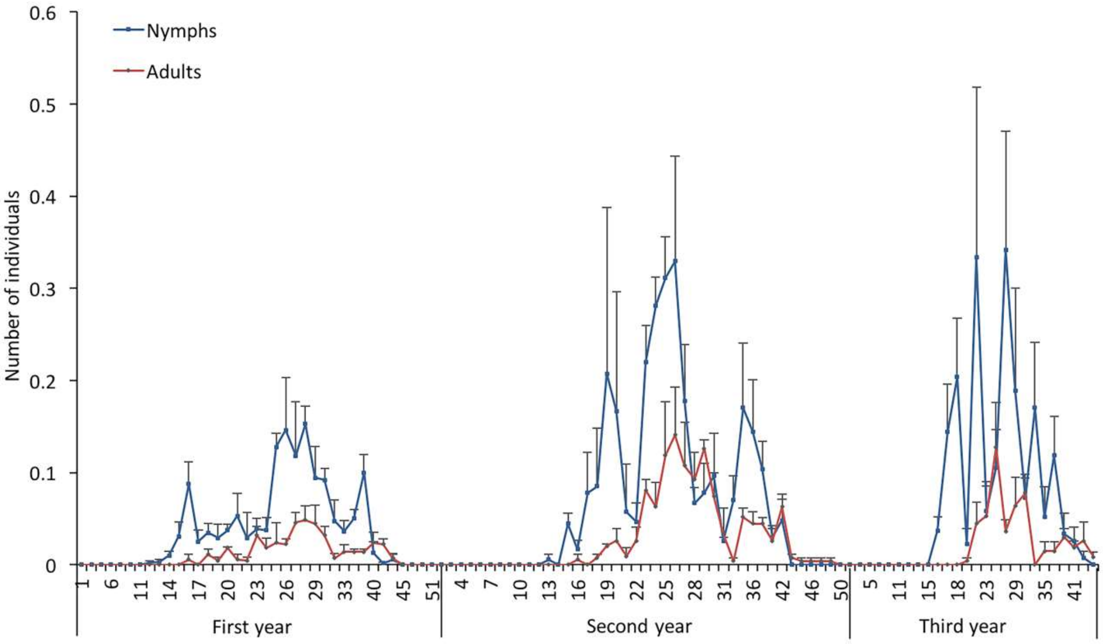

Figure 1. Population dynamics (mean number of individuals per beat ± standard error) of *Pilophorus gallicus* nymphs (A) and adults (B) in pear orchards from southern Europe. The numbers in the X-axis represent weeks of samplings.

Table 1. Maximum correlation coefficients and corresponding phase shifts for cross-correlation analyses of *Pilophorus gallicus* nymph and adult time series in three pear orchards (A, B and C) over three consecutive years.

	Correlation coefficients			Phase shift (weeks)		
	1st year	2nd year	3rd year	1st year	2nd year	3rd year
A	0.773	0.719	0.560	-1	-5	-2
B	0.444	0.748	0.477	-2	-3	-2
C	0.650	0.849	0.654	-4	0	-4

3.2. *Study of the reproductive state of P. gallicus females and the conditions of eggs*

Figure 2 shows the total number of eggs laid per *P. gallicus* female during the week of observation, for the first, second and third generations. The fecundity was significantly different among generations (*F* = 9.04; df = 2, 57, *P* < 0.001). The number of eggs per female decreased from 43.9 ± 3.7 (mean ± SE) in the first generation to 34.5 ± 3.3 and 26.3 ± 2.5 in the second and third generations, respectively. Significant differences were found between generations one and three (Tukey test, *P*< 0.001), but generation two was not significantly different from the first or third (Tukey test, *P* = 0.061 and *P* = 0.073, respectively) (Figure 2). In the same way, the fertility differed significantly among generations (*F* = 7.14; df = 2, 57, *P* < 0.01). The number of viable eggs dropped from 16.1 ± 2.8 (mean ± SE) in the first generation, which represents 35.0 ± 3.52% of the total eggs, to 9.3 ± 1.3 in the second (26.4 ± 2.77%) and 7.0 ± 1.2 in the third (27.5 ± 3.34%). The number of fertile eggs per female was significantly greater in the first generation than in the second and third (Tukey test, *P* < 0.05 and *P* < 0.01, respectively) (Figure 2). However, even if the proportion of viable eggs decreased from the first to the second and third generations, no significant differences were found in the proportion of viable eggs among generations (*F* = 1.83; df = 2, 57, *P* = 0.17).

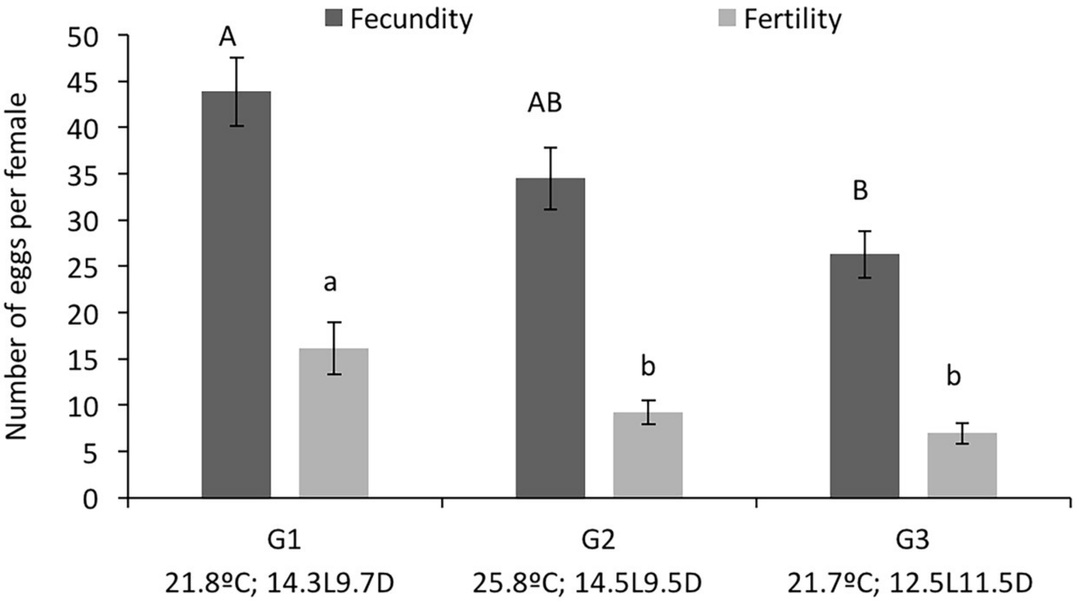

Figure 2. Average fecundity and fertility of *Pilophorus gallicus* females during one week of oviposition for the spring (G1), summer (G2) and autumn (G3) generations. Different letters indicate significant differences between generations: capital letters for fecundity (Tukey test $P < 0.001$) and lower-case for fertility (Tukey test, $P < 0.05$). Temperature and photoperiod refer to the average values of the week before the date of collection of the females.

The females of *P. gallicus* laid three kinds of egg: non-diapausing, diapausing and non-viable. Non-diapausing eggs hatched within 15 days of the end of the laying week, at 25°C and a 16L:8D photoperiod. Nymphs from diapausing eggs, those hatching after being exposed to diapause-breaking conditions (90 days at 6°C and 16L:8D), started to emerge 22.3 ± 0.5 days after being brought back to 25°C and 16L:8D. Both non-diapausing and diapausing eggs were laid in the three generations, but the proportion of each varied significantly among the generations (non-diapausing eggs: $F = 20.92$; df = 2, 57, $P < 0.001$; diapausing eggs: $F = 28.49$, df = 2, 57, $P < 0.001$) (Figure 3). Non-diapausing eggs were more frequent than diapausing ones in the first and second generations, whereas the opposite occurred in the third generation. In the first generation, the proportion of non-diapausing eggs was 30.8 ± 4.5%, whereas 4.2 ± 2.1% of the eggs were diapausing. In the week before the collection of *P. gallicus* females, in the field the average temperature was 21.8°C and the photoperiod was 14.3L:9.7D. In the second generation the proportion of non-diapausing eggs (20.4 ± 3.6%) was a little lower than in the first generation, but not significantly so (Tukey test, $P = 0.152$). The percentage of diapausing eggs in the second generation was increased in relation to the first (6.0 ± 1.8%), but not significantly so (Tukey test, $P = 0.643$). The average day length in the week before the collection date (14.5 h) was similar to that of the first generation, although the temperature was higher (25.8°C). In the third generation, the non-diapausing eggs constituted 2.5 ± 1.5% and the diapausing eggs 25.0 ± 2.9%. The percentages of both kinds of egg differed significantly between the first and third generations (Tukey test, non-diapausing eggs: $P < 0.001$; diapausing eggs: $P < 0.001$) and between the second and third generations (Tukey test, non-diapausing eggs: $P < 0.001$; diapausing eggs: $P < 0.001$). The average temperature registered in the week before the collection of the females in the third generation was 21.7°C, and the day length (12.5 h) was shorter than for the first and second generations.

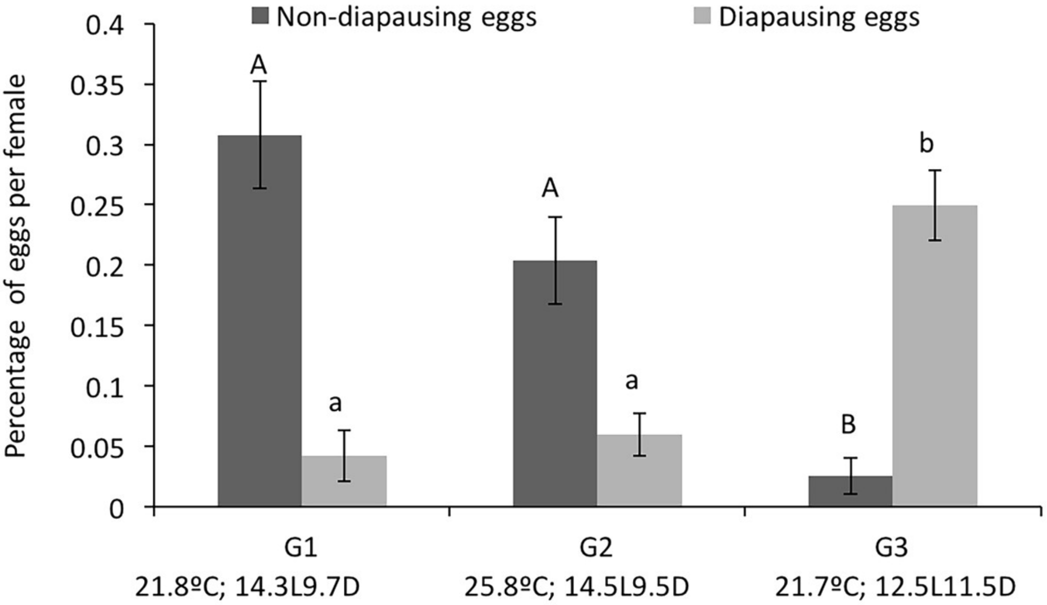

Figure 3. Proportions of non-diapausing and diapausing eggs, out of the total number of eggs laid per female, for the spring (G1), summer (G2) and autumn (G3) generations. Different capital letters indicate significant differences in the proportion of non-diapausing eggs between generations. Different lower-case letters indicate significant differences in the proportion of diapausing eggs between generations (Tukey test, $P < 0.001$). Temperature and photoperiod refer to the average values of the week before the date of collection of the females.

Three kinds of female, according to their reproductive response, were found among the sampled individuals: "non-diapause inducing", "diapause inducing" and "mixed" (those with a dual response). However, the proportions of the different types of females varied across the generations (Figure 4). Females that laid exclusively non-diapausing eggs ("non-diapause inducing") were the most abundant in the first (66.7%) and second (50.0%) generations, but were absent in the third. No significant differences in the proportion of "non-diapause inducing" females were found between the first and second generations ($\chi^2 = 0.48$, df = 1, $P = 0.491$). In contrast, females that laid diapausing eggs exclusively ("diapause inducing") increased from 13.3% in the first generation to 85.7% in the third. Significant differences in the proportion of "diapause inducing" females were found between the first and third generations ($\chi^2 = 15.75$, df = 1, $P < 0.001$) and between the second and third ($\chi^2 = 14.24$, df = 1, $P < 0.001$), but not between the first and second ($\chi^2 = 0.22$, df = 1, $P = 0.64$). Besides, some other females laid both kinds of egg ("mixed"). The proportion of "mixed" females was similar in the three generations and did not show any apparent trend: 20%, 25% and 14.3% for the first, second and third generations, respectively (Figure 4). No significant differences in the proportion of "mixed" females were found among the generations (first-second generations: $\chi^2 \approx 0$, df = 1, $P = 1$; first-third generations: $\chi^2 \approx 0$, df = 1, $P = 1$; second-third generations: $\chi^2 = 0.27$, df = 1, $P = 0.60$).

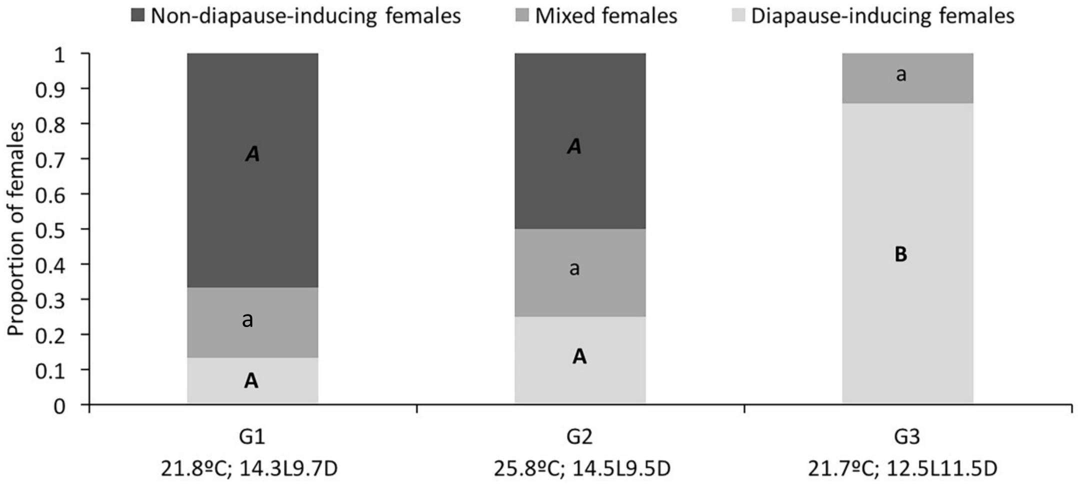

Figure 4. Proportions of the different kinds of female, according to their reproduction response, for the spring (G1), summer (G2) and autumn (G3) generations. Different letters for each type of female indicate significant differences in the proportions between the generations (Binomial test, $P < 0.05$). Temperature and photoperiod refer to the average values of the week before the date of collection.

4. DISCUSSION

Pilophorus gallicus is a multivoltine predator of small arthropod pests that establishes resident populations in pear orchards in southern Europe (Sanchez and Ortín-Angulo, 2012). This work assesses that after the winter absence of mobile stages, the first forms to show up are always nymphs followed by adults with a few weeks delay, which indicates the overwintering of the species as eggs on pear trees. However, this does not exclude the possibility that part of the population emigrates from pear trees to oviposite in other host plants and recolonises pear orchards in late spring-summer after nymphs have completed their development. The population decline in pear orchards after the summer peak could be due, at least in part, to the migration of adults. These migratory movements have been reported for other predatory hemipterans, such as *Anthocoris nemoralis* (Hemiptera: Anthocoridae), which builds up its populations in hedgerows in spring and moves to pear orchards at the beginning of summer (Scutareanu *et al.*, 1999). The population dynamics in *P. gallicus* are greatly regulated by the variation in the type of eggs laid by the females over the different seasons of the year. The non-diapausing eggs prevail in spring and summer, while most of the eggs laid in autumn are of the diapausing kind. As reported for other insect species with embryonic diapauses (Zimmermann, 1984; Musolin and Saulich, 1996; Saulich and Musolin, 1996; Shintani and Higuchi, 2008; Lundgren, 2011), these diapausing eggs will only hatch after going through a chilling period, which should increase the chances of survival of the nymphs as they will emerge after the unfavourable winter period. The occurrence of an aestivation mechanism, either in eggs or in adults, was rejected as all the females collected in summer were fertile and their reproductive response was similar to that of the first generation. We registered much lower hatching rates than those reported for other predatory hemipterans. For instance, the egg hatching of the congeneric *Pilophorus typicus* Distant (Hemiptera: Miridae) was 80.5% at 25°C (Nishikawa *et al.*, 2010); hatching rates for *Macrolophus pygmaeus* Rambur and *Macrolophus caliginosus* Wagner [Syn. *Macrolophus melanotoma* (Costa)] (Hemiptera: Miridae) at 25°C were about 70% (Perdikis and Lykouressis, 2004;

Perdikis *et al.*, 2007); Sanchez and Lacasa (2002) reported that about 95% of the *Orius laevigatus* and *Orius albidipennis* (Hemiptera: Anthocoridae) eggs hatched at 25°C. However, it has to be taken into account that all these cases referred to non-diapausing eggs. Although most Miridae from the temperate region are univoltine species that overwinter in the eggs stage (Schuh and Slater, 1995), we have not been able to find any reference in the specialised literature about hatching rates for any species diapausing as eggs in the largest family of Heteroptera. Highly variable hatching values ranging from 21% to 92% were reported for other hemipterans diapausing as eggs (i.e. *Cryptotympana facialis* Ståland, *Graptopsaltria nigrofuscata* Motschulsky (Hemiptera: Cicadidae)) (Moriyama and Numata, 2008). Diapause may be a costly process in terms of survival because of the exposition to extreme temperatures and extended longevity of diapausing individuals (Musolin and Numata, 2003). Part of the egg mortality registered in the present work was due to the loss of eggs laid on leaf petioles when the saplings were transferred to low temperature and dropped their leaves. On the other hand, the low hatching rates could be as well an artefact due to the small size of the saplings used as laying substrate; eggs embedded on thin branches could have suffered from high dehydration and injuries in general (Wheeler, 2001).

Diapause is a programmed suppression of development that is genetically determined; it is induced by environmental cues that precede unfavourable conditions, it often lasts longer than the adverse period and requires specific conditions for its termination (Beck, 1980; Dolling, 1991; Hahn and Denlinger, 2007; Hand *et al.*, 2016). This definition certainly fits the *P. gallicus* eggs typified as "diapausing" because they did not hatch in standard conditions (25°C, 16L:8D), but did so after being exposed to a chilling period and then brought back to standard conditions. We also believe that diapause in *P. gallicus* is maternally induced because all the eggs were laid under the same standard conditions in the laboratory but the diapause incidence varied depending on the season females were collected. Maternally induced diapause is a well known phenomenon in insects with facultative diapause. In species with this kind of embryonic dormancy, such as *Bombix mori* L. (Lepidoptera: Bombycidae) and *Orgyia antique* L. (Lepidoptera:

Lymantriidae), the process is regulated by a hormone produced in the suboesophageal ganglion of females exposed to the triggering environmental conditions (Fukuda, 1951; Hasegawa, 1951; Kind, 1965). The photoperiod is quite likely one of the environmental factors triggering the oviposition of diapausing eggs in *P. gallicus*. Females collected in spring and summer experienced long photoperiods (more than 14 hours of light) and laid mainly non-diapausing eggs, while most of the eggs laid by the females collected in autumn (photoperiod 12.5L:11.5D) were of the diapausing type. Low temperatures are also known to induce diapause (Fukumoto *et al.*, 2006), and may contribute alone or in synergy with the photoperiod to the regulation of the diapause-inducing response in *P. gallicus* females. Diapause is a mechanism that synchronises the active forms of the species with seasonal periods that ensure rapid development and high survival (Ruberson *et al.*, 1998; Saunders, 2012; Hand *et al.*, 2016). The overwintering of *P. gallicus* as diapausing eggs certainly may be regarded as a mechanism of the species to overcome the adverse winter period.

The diapause mechanism in *P. gallicus* is not a straightforward one; it has several notable features that make it particularly interesting and peculiar. In the first place, the diapausing response is not univocal and, although the females laid mainly non-diapausing eggs in spring and diapausing ones in autumn, both kinds of egg were registered in all seasons. This miscellany of eggs has been reported also by other authors (Kogure, 1933; Zimmermann, 1984; Shiga and Numata, 1997; Feng *et al.*, 2012), and from it arise questions about the significance of the presence of diapausing eggs in favourable conditions and their role in the long-term survival of the species. The laying of diapausing eggs under favourable conditions substrate some biotic potential to the species, but saving some individuals for the following annual cycle at each generation will increase the probability of persistence of populations in the eventuality of extremely-high mortalities due to abnormal environmental fluctuations (Remmert, 1988; Fangsen and Kallenborn, 1993). Therefore, the presence of *P. gallicus* females inducing diapause in eggs at times when the conditions are favourable for the development of the species could be explained by the advantage of those genotypes in the eventuality of negative environmental factors compromising the survival of the population. Among the main risks that *P. gallicus* may face are: (1) the unpredictability of weather conditions of the Mediterranean climate, with frequent heavily destructive storms in summer and autumn (González-Hidalgo *et al.*, 2011) that may impact negatively insect populations (Wheeler, 2001); (2) the likely increase of predation risk in the latest generations; for instance, the abundance of spiders in pear orchards in southeast Spain increases at the beginning of summer and remains high until the end of the season (Sanchez and Ortín-Angulo, 2012); and (3) the increase of disease incidence at the end of the season; we have observed in pear orchards the occurrence of individuals infested with some pathogens that cause them to swell and die (Supp. material S1). Overall, the reproductive strategy of *P. gallicus* may be considered as an example of bet-hedging (*sensu* Stearns, 1976), where "not laying all the eggs in one basket" may increase the probability of persistence of populations.

Another noticeable feature of the diapausing mechanism in *P. gallicus* is the existence of a high proportion of "mixed" females able to lay both diapausing and non-diapausing eggs. Only a few previous works have reported the existence of females with dual oviposition response, or that switch from laying one type of egg to the other (Kudô and Kurihara, 1988; Shiga and Numata, 1997; Musolin and Saulich, 2000). "Mixed" females will contribute to bet-hedging in the first generations but they will lay as well a proportion of non-diapausing eggs in the last one, whose survival will be greatly conditioned by the severity of the winter conditions. The existence of "mixed" females, or even the likely existence of non-diapause inducing females non sensitive to changes in photoperiod, could be explained by the advantage that not entering into diapause could confer to those individuals in areas with mild winters. The eclosion of non-diapausing eggs in winter will be limited by the minimum thermal threshold of the species (12.4°C) (Ramírez-Soria *et al.*, 2017), but once the thermal requirements are completed, nymphs will emerge and, if non exposed to deleterious temperatures, they will develop and contribute with their offspring to the population earlier and at higher numbers than the descendants from diapausing eggs. After the diapause completion period, diapausing eggs need longer development times and suffer from higher mortality rates than non-diapausing ones (Ramírez-Soria *et al.*, 2017). Females producing diapausing eggs may also be in disadvantage in warm climates, or under the current climate change scenario, because they may not reach the necessary chilling conditions for the eggs to break the diapause. In the end, all these factors are expected to give a numerical advantage to "mixed" and non-diapause inducing genotypes in population from areas with mild winters. We predict a variation in the proportion of *P. gallicus* females with different kind of reproduction strategies in function of climatic conditions. Sanchez *et al.* (2013) found variations in the proportion of *Myzus persicae* Sulzer (Hemiptera: Aphididae) genotypes with different reproduction modes linked to winter temperatures; holocyclic clones with sexual reproduction and overwintering as eggs were more abundant in northern areas with cold winter, while anholocyclic clones with obliged parthenogenesis predominated in coastal areas with mild winters.

Diapause has a strong effect on the population dynamics of natural enemies and, thus, on the regulation of the abundance of target pests in crops within integrated and biological pest control programmes (Nylin, 2001). Diapause in natural enemies has been regarded usually as negative - especially in biological pest control in greenhouses, which depends on inoculative releases - because it limits their establishment and population growth, and thus their efficacy as pest control agents (Gillespie and Quiring, 1993, 2005; Musolin and Ito, 2008; Bahşi and Tunç, 2012; Bonte *et al.*, 2016). However, diapause may represent an advantage in permanent crops, such as fruit orchards, when long-term conservation biological pest control strategies are adopted. The early emergence of *P. gallicus* nymphs from overwintering eggs in pear trees, concomitantly with the population increase of *C. pyri*, may result in better control of the pest than if predator were to immigrate from external hosts (Sanchez and Ortín-Angulo, 2012). For instance, immigration of *A. nemoralis* from hedgerows to pear orchards takes place too late for effective control of the pear psyllids (Scutareanu *et al.*, 1999; Erler, 2004). The different overwintering strategies of these two predators make necessary the adoption of different management practices to enhance conservation biological control. Strategies based on the immigration of predators from hedgerows are expected to benefit from increased abundance and proximity of host plants. On the other hand, in the case of predators overwintering in orchards, special care should be taken to protect resident populations.

Very little is known about the biology of the predatory mirid *P. gallicus* and this work gives, for the first time, some insights into the reproductive strategy of the species. The existence of an embryonic diapause allows *P. gallicus* to overcome the adverse winter period and may serve as bet-hedging for the persistence of populations, in case environmental factors produce unusual and extremely-high mortalities. This mechanism is intrinsically linked to the short- and long-term survival of the species, and has great implications for biological pest control. More work has to be done to understand both the factors that induce in females the laying of diapausing eggs and those that regulate the capacity of some females to lay both diapausing and non-diapausing eggs. A graphical abstract of this chapter has been included as supplementary material (Supp. Material S2).

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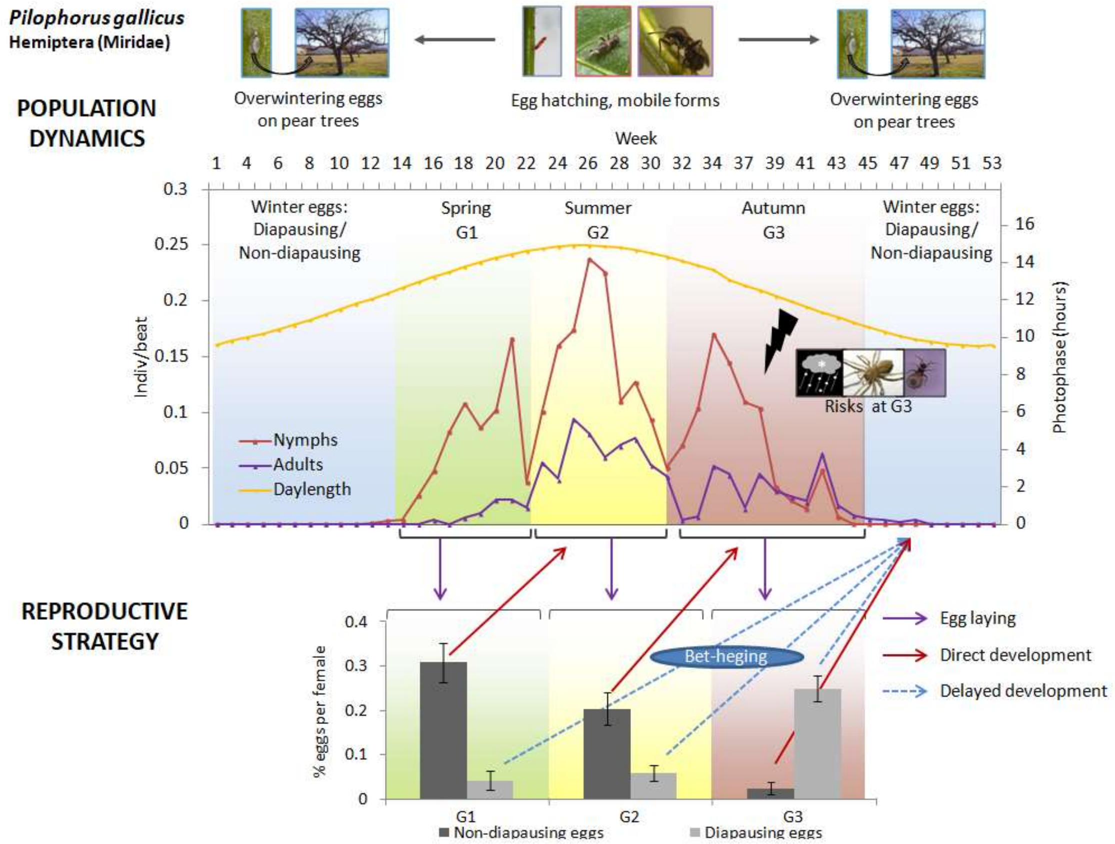
SUPPLEMENTARY MATERIAL

Supplementary material S1. Nymph N5 of *Pilophorus gallicus* collected in autumn in pear orchards in Jumilla. It is infested with some unidentified pathogens that cause the individuals to swell and die.



Supplementary material S2. Graphical abstract. Population dynamics and seasonal variation in the embryonic dormancy of *Pilophorus gallicus*. This mirid has a multivoltine cycle and overcomes winter low temperatures entering an embryonic dormancy on pear trees. Diapausing eggs are mainly laid in the early autumn, along with the decrease of the daylength; they will not hatch before the following spring, after going through the winter low temperature period. A few diapausing eggs are laid as well in spring and summer, revealing a bet-hedging strategy to avoid the risks associated with the last annual generation (heavy rains, high predation rates, pathogens). Some of the eggs laid by the last generation are non-diapausing, their development depends on the severity of the winter.

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CHAPTER 3

Induction and termination of embryonic diapauses in the predatory mirid *Pilophorus gallicus* (Hemiptera: Miridae)

Photo: Elena López Gallego

CHAPTER 3: Induction and termination of embryonic diapause in the predatory mirid *Pilophorus gallicus* (Hemiptera: Miridae)

ABSTRACT

Pilophorus gallicus Remane (Hemiptera: Miridae) is a predator of small arthropods in pear orchards (*Pyrus communis* L. (Rosaceae)). In the Mediterranean area, it has a multivoltine life-cycle, and enters into a facultative embryonic diapause to overcome the winter season. Facultative diapause is regulated by environmental factors, whose role in population dynamics is essential because they determine the presence of active individuals in the field. The objectives were to investigate the effect of (1) photoperiod and temperature on the induction of embryonic diapause in *P. gallicus*, and (2) temperature, photoperiod and exposure time on the completion of the diapause phase in eggs. The induction of diapause was triggered by short photoperiod (9L:15D) whereas temperature did not have a significant effect. The completion of diapause in eggs required the exposure to low temperature while photoperiod did not have a significant effect. At 6°C, a low number of eggs hatched at 50 days of exposure; a significant increase was registered between 50 and 70 days, without significant differences between 70 and 150 days of exposure. The longer cold exposure the more synchronized nymphs emerged. Population of *P. gallicus* was composed by females with three reproductive strategies (diapause inducing, non-diapause inducing, mixed); the laying of diapausing eggs at long photoperiod is regarded as a bet-hedging strategy.

Key words: facultative embryonic diapause, induction, termination, photoperiod, temperature, bet-hedging, mass rearing.

1. INTRODUCTION

Many species of insect have complex life cycles, especially in variable climates where they have to cope with broad daily and/or seasonal fluctuations of abiotic and biotic factors (e.g temperature, humidity, photoperiod, predation, competition) (Danks, 2002; Tauber *et al*., 1986). Insects have adapted to predictable environmental variation by modifying their metabolic-physiological processes or through behavioural responses; these adaptations can be direct responses to external factors or triggered by endogenous rythms (Saunders, 2002). In regions with temperate and continental climate, winter is an unfavourable season for the life of many organisms. Migration and dormancy are two common strategies employed by insects to deal with stresses of winter (Dingle, 1978; Saunders, 2010; Tauber *et al*., 1986; Taylor and Karban, 1986). Diapause is a widespread kind of dormancy; it allows insects to synchronize their activity with the availability of resources and suitable conditions for development and reproduction (Bale and Hayward, 2010; Danks, 1987; Denlinger and Lee, 2010; Tauber *et al*., 1986; Tauber and Tauber, 1976). The diapausing state involves reduced morphogenesis, decreased metabolic rate, and/or development of stress tolerance mechanisms (e.g. cold-hardening) (Koštál, 2006; Tauber and Kyriacou, 2001). Diapause can be either obligatory or facultative. Obligatory diapause is characterized by an annual onset irrespective of the external influences; it is expressed in univoltine species and is genetically programmed rather than being cued by the environment (Andrewartha, 1952; Masaki, 2002; Musolin and Ito, 2008; Musolin and Saulich, 1996). In contrast, facultative diapause is only initiated after the detection of a specific signal or the combination of several of them (token stimuli). Facultative diapause is usually associated with multivoltine species and depends on the prevailing climate conditions. Diapause plays an essential role in insect population dynamics, because it regulates the presence of active individuals in the field. The study of specific environmental signals triggering diapause is essential to understand life-cycle regulation in insects.

Diapause is a dynamic process that follows a pattern of well-defined events. Despite the inter- and intra-specific variations (Andrewartha, 1952; Masaki, 2002; Musolin and Ito, 2008; Musolin and Saulich, 1996), the expression of diapause is generally divided into three main phases: (1) pre-diapause, (2) diapause and (3) post-diapause quiescence (Koštál, 2006) (Supp. Material S1). Progression through the phases of diapause is regulated by the interaction between environmental factors and the physiological responses of the insect (Denlinger, 2002). In the pre-diapause phase, an individual with facultative diapause becomes diapause-destined by the effect of the token stimuli (e.g. photoperiod). Upon completion of the pre-diapausing phase, appropriately programmed insects will enter into the diapause phase. The diapause phase lasts until some specific requirements are fulfilled, such as exposure to a prolonged period of low temperatures (Higaki and Ando, 2005; Hodek, 1996, 2002; Moribe *et al.*, 2010; Saulich and Musolin, 2012; Tauber *et al.*, 1986; Yamaguchi and Nakamura, 2015; Zhou *et al.*, 2016). The need for well-defined stimuli to overcome diapause prevents premature termination and allows a synchronized re-activation of the diapausing population (Koštál, 2006). However, some times the diapause phase may be completed before the environmental conditions are suitable for the development of the species. In such cases, insects remain in a state of post-diapause quiescence as long as the limiting factor (e.g. low temperature) prevails under the development threshold, thus avoiding exposure to environments that may cause high mortality to individuals terminating diapause earlier than the restoration of favorable conditions (Beck, 1980; Broufas and Koveos, 2000; Saulich and Musolin, 2012; Son *et al.*, 2007).

Pilophorus gallicus Remane (Hemiptera: Miridae) is a predator of small arthropod pests in pear orchards (*Pyrus communis* L. (Rosaceae)) (Sanchez and Ortín-Angulo, 2012). This mirid bug has a multivoltine life cycle with about three generations per year in southern Europe and demographic peaks taking place in spring, summer, and autumn (Ramírez-Soria *et al.*, 2018; Sanchez and Ortín-Angulo, 2012). Unfavourable winter conditions are overcome by entrance into a facultative embryonic diapause: *Pilophorus gallicus* females lay mainly non-diapausing eggs in spring and summer, whereas in the autumn they lay predominantly diapausing eggs

(Ramírez-Soria *et al*., 2018). Little is known about regulation of diapause in *P. gallicus,* although, variation in the developmental status of the eggs (diapausing or non-diapausing) laid by females through the seasons suggests that diapause may be triggered by shortening daylength and/or low temperatures in the fall. The emergence of nymphs in spring, after the winter chilling period, suggests the need of a period of low temperature for diapause completion, a common occurrence for species from the temperate zone diapausing in the egg stage (Higaki and Ando, 2005; Javahery, 1994; Moribe *et al*., 2010; Yamaguchi and Nakamura, 2015; Zhou *et al*., 2016; Zimmermann, 1984). *Pilophorus gallicus* eggs are embedded in pear tree tissues; this intimate contact with plant tissues may allow an association between egg physiology and pear tree phenology (Wheeler, 2001), thus the conditions needed to overcome the diapause phase may be related to the vernalization requirements for pear trees (Pliego Condiciones DOP Pera de Jumilla p.12, www.carm.es). Knowledge about diapause expression in a predatory species becomes very useful for the understanding of its population dynamics and the prediction of its impact on the control of prey populations.

There were two main objectives in this work. (1) To investigate the influence of photoperiod and temperature on the induction of embryonic diapause in *P. gallicus*. Our working hypothesis was that *P. gallicus* will lay a higher proportion of diapausing eggs at short rather than at long photoperiods, and at lower rather than at higher temperatures, with a likely interaction between temperature and photoperiod. (2) To determine the influence of temperature, photoperiod and exposure time on the completion of the diapause phase. We hypothesized that diapausing eggs will need to be exposed to a period of time at low temperatures to complete the diapause phase. We predicted interactions between temperature and photoperiod, and between temperature and exposure time.

2. MATERIALS AND METHODS

2.1. Completion of the diapause phase in P. gallicus

The fact that *P. gallicus* inserts their eggs in pear tree tissues makes difficult the assessment of diapause incidence by externally visible characteristics (Ramírez-Soria, personal observation). Therefore, the characterization of the egg status was established on the basis of their hatching when exposed to standard conditions. Eggs that did not hatched at 2-3 times the development time at standard conditions (11.9 ± 0.1 days at 25°C ± 1 °C, 16L:8D and 60 ± 10% RH) (Ramírez-Soria *et al*., 2017), were considered either as non-viable or diapausing. To identify the diapausing eggs and distinguish them from inviable ones, it was necessary to know the requirements needed for the completion of the diapause. That is why, despite the chronological sequence of diapause process (induction --> termination), the assays were carried out in the reverse order. Completion of the diapause phase in *P. gallicus* eggs was assayed at two temperatures (6 and 25°C), two photoperiods (10L:14D and 16L:8D) and four durations of exposure (50, 70, 90, and 150 days) for each combination of temperature and photoperiod. The number of nymphs emerged and eggs hatched per female were recorded after completion of diapause for each of the different combinations of temperature, photoperiod, and exposure time. The eclosion time was registered for every nymph emerged.

Diapausing eggs were obtained from females of *P. gallicus* collected in mid-September in pear orchards located in the municipality of Jumilla (Murcia, Spain). *Pilophorus gallicus* females were known from previous studies to lay mainly diapausing eggs in autumn (Ramírez-Soria *et al*., 2018). Fifty females and fifty males were placed in a mesh-walled, wooden-framed cage (80 x 40 x 40 cm) with 40 *P. communis* seedlings as egg-laying substrate. *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs provided by Biobest N.V. were supplied on sticky papers (Post-it® note; 3M, St Paul, MN, USA) as factitious prey. The cage was placed in a plant growth chamber (Binder KBWF-750, Tuttlingen, Germany) at 25°C ± 1 °C with a 16L:8D photoperiod and 60 ± 10% relative humidity. The seedlings were replaced weekly by new ones during three consecutive weeks. The pear saplings with the eggs were placed into another plant growth chamber under the same conditions (25°C ± 1 °C, 16L:8D and 60 ± 10% RH) and observed three times a week during the following 30 days to remove the nymphs emerged from non-diapausing eggs. Thereafter, pear seedlings were observed under stereomicroscope to count the unhatched eggs, which could be either in diapause or inviable.

Saplings with the putative diapausing eggs were divided into four groups with a similar number of eggs (30 saplings and 600 eggs approximately per group), and each group of trees was placed under one of the four combinations of temperature and photoperiod: (1) 6 ± 1°C and 10L:14D; (2) 6 ± 1°C and 16L:8D; (3) 25 ± 1°C and 10L:14D; (4) 25 ± 1°C and 16L:8D. Each one of these four groups was subsequently divided into four groups with a similar number of eggs and saplings (7–8 trees and 150 eggs approximately, about 20 eggs per tree on average); these saplings were exposed for different lengths of time (i.e. 50, 70, 90 or 150 days) to the four combinations of temperature and photoperiod. During this time, saplings were checked every 3-4 days for the emergence of nymphs.

Once the experimental exposure times had elapsed, pear seedlings were brought back to standard conditions (25°C 16L8D and 60 ± 10% RH) to promote egg hatching. Each sapling was kept in translucent plastic cylinder (diameter, 9 cm; capacity, 2000 ml) covered with a mesh on the top (Photo 3 Annex of photos). The emergence of nymphs was observed everyday over a period of 50 days. Nymphs were counted and removed at each census and their date of emergence recorded; additionally, the number of hatched eggs was counted at the end of the observation period and unhatched eggs were considered inviable.

2.2. *Diapause induction in P. gallicus*

2.2.1. *Insect rearing*

A laboratory stock colony was established from *P. gallicus* collected in pear orchards in the northeast of Murcia (Spain) several months before the start of the experiment. The colony was maintained on *P. communis* seedlings as oviposition substrate placed in mesh-walled wooden-framed cages (40 x 40 x 40 cm) at 25°C ± 1°C and 60 ± 10% relative humidity under long day conditions (16L:8D) (Photo 1 Annex of photos). Insects were fed on *E. kuehniella* eggs provided on sticky papers (Post-it® note; 3M, St Paul, MN, USA).

2.2.2. *Experimental design, conditions and procedures*

The induction of embryonic diapause in *P. gallicus* was assayed at two temperatures (18 and 23°C) and two photoperiods (9L:15D and 16L:8D) in a complete factorial design. The choice of the temperatures assayed was based on the approximate average values observed in the field at the appearance of the first adults in spring (18°C) and the beginning of autumn (23°C) (http://siam.imida.es/; Ramírez-Soria *et al*., 2018). Lower temperatures than 18°C (i.e. 15°C) were known to substantially reduce development and oviposition rates (Ramírez-Soria *et al*., 2017). Photoperiods were chosen based on the extreme values registered annually at the latitude where the insects were collected (http://siam.imida.es/). The number of eggs hatched and nymphs emerged per female were registered.

To generate females and males used in this experiment, approximately four hundred young adults (3 to 10 days-old) from the laboratory colony were split evenly into four cages (40 x 40 x 40 cm) with 20 *P. communis* seedlings as egg laying substrate on each one. These cages were placed in plant growth chambers (Binder KBWF-750, Tuttlingen, Germany) at 25 ± 1°C, 60 ± 10% RH and 16L:8D, and *E. kuehniella* eggs were provided on sticky papers. After 24 hours the adults were removed and the pear saplings were haphazardly assigned to four groups. Each group was transferred to a plant growth chambers at one of the four combinations of temperature and photoperiod tested: (1) 18 ± 1°C and 9L:15D; (2) 18 ± 1°C and 16L:8D; (3) 23 ± 1°C and 9L:15D; (4) 23 ± 1°C and 16L:8D; the relative humidity in the chambers was set up at 60 ± 10% RH. Plants were inspected every day to collect emerged nymphs, which were reared individually until the adult stage at the same conditions that they had been exposed to as eggs. Every nymph was kept in a 100-

ml translucent cup with a pear leaf with the petiole inserted into a 5 mm agar (1%) layer, water-soaked cotton, and *E. kuehniella* eggs on a 1 cm^2 sticky paper. The agar surface was covered with a plastic disc to prevent nymphs from getting stuck (Photo 2 Annex of photos). The pear leaf, the cotton, and the sticky paper with *E. kuehniella* eggs were replaced three times a week. Once the adult stage was reached, females and males that emerged on the same day were put together for 24h for mating in plastic containers (4700 ml capacity) with cotton soaked in water and *E. kuehniella* eggs *ad libitum* at the corresponding experimental conditions.

Pairs of one day-old females and males were taken from the mating container and placed individually in translucent plastic cylinders (diameter, 9 cm; capacity, 2000 ml; covered with a mesh on the top), with *P. communis* seedlings approximately 20 cm high as laying substrate (Photo 3 Annex of photos), at the corresponding combination of temperature and photoperiod. *Ephestia kuehniella* eggs on sticky papers were provided as factitious prey. The oviposition of the couples at 23°C was registered over a period of two weeks, whereas at 18°C oviposition was observed over a period of three weeks because the pre-oviposition period was longer and the number of eggs laid during the first week was low. Seedlings were changed weekly and observed under a stereomicroscope to count eggs. Thereafter, the saplings were brought to standard conditions (25°C, 16L:8D and 60 ± 10% RH) for egg hatching. The emergence of nymphs was observed three times a week in the following 30 days; nymphs were counted and removed during each observation period. According to the criterium used in the termination trial, non-hatched eggs after 30 days of observation were considered either as non-viable or diapausing. Thereafter, saplings were placed under the conditions for the completion of the diapause; these conditions were established based on the results of the completion of the diapause phase experiment.

After going through the conditions for the completion of diapause, seedlings were returned to standard conditions: 25°C ± 1 °C, 60 ± 10% RH and 16L:8D. Emergence of nymphs from diapausing eggs was observed three times a week over 50 days, and nymphs were counted and removed at each census. Once nymphs had stopped emerging, saplings were observed under the stereomicroscope to count the number of hatched eggs. The remaining unhatched eggs were considered inviable.

2.3. *Statistical analyses*

In the completion of the diapause phase assay (experiment 1), the effects of temperature, photoperiod, exposure time and their interactions on the number of nymphs emerged and diapausing eggs hatched was tested by a complete factorial ANOVA. Tukey's HSD test (α = 0.05) was used for the separation of means. The number of nymphs emerged and diapausing eggs hatched were ln (x+1) transformed to account for heteroscedasticity. The patterns of the emergence of nymphs over the time (i.e. the time the diapausing eggs took to hatch after being transferred to standard conditions) for each exposure time (50, 70, 90, 150 days) at cold temperature (6°C) were fitted to sigmoid curves following the approach of Kostal and Havelka (2001):

$$y = \frac{100}{1 + 10^{[(Et_{50} - x) * Slope]}}$$

Where x stands for the days after transfer to 25°C 16L:8D, y is the cumulative nymph emergence, Et_{50} is the time necessary for emergence of 50% of nymphs, and *Slope* describes the slope of the central part of the sigmoid curve; the slope of the curve is considered an indicator of the level of synchrony of nymph emergence (Dong *et al.*, 2013). The parameters of the equation (*Slope* and Et_{50}) were estimated by nonlinear regression using the function 'nls' ('MASS' package) (R Development Core Team 2017 version 3.4.2). Comparisons of the mean hatching times among exposure times and photoperiod was done using one-way analysis of variance (ANOVA) followed by Tukey HSD test (α = 0.05) for the separation of means.

In the diapause induction trial (experiment 2), the effects of temperature and photoperiod on the percentages of diapausing and non-diapausing eggs during the whole period of observation were tested using a two-way ANOVA. Differences among means in the ANOVA were established with Tukey's HSD test (α = 0.05).

Additionally, females were classified according to their egg-laying response as: (1) "non-diapause inducing" when they laid exclusively non-diapausing eggs; (2) "diapause inducing" when they laid only diapausing eggs; (3) "mixed" if the two types of eggs were laid by the same female; and (4) "infertile" when none of the eggs they laid hatched. Multinomial logistic regression was used to predict the probability of each female reproductive response based on photoperiod and temperature. The Likelihood Ratio Test on Multinomial Models (function 'multinom', 'nnet' package) (R Development Core Team, 2017; version 3.4.2) was used to estimate p-values representing the detectability of an effect for each factor: we compared a full model (with photoperiod and temperature) against reduced models without the effects in question (function 'anova', package 'stats'). In each case, a factor was considered to have a significant effect if the difference between the likelihood of these two models was significant ($P < 0.05$). A Chi-squared goodness of fit test (function 'chisq.test', package 'stats') was used to check the suitability of the multinomial logistic regression model. All the analyses were performed using R statistical software, version 3.4.2 (R Development Core Team, 2017).

3. RESULTS

3.1. Factors influencing the completion of diapausing eggs in P. gallicus

A low temperature (6°C) period was required for diapause completion, whereas photoperiod had no effect. No nymphs emerged from the eggs kept at 25°C at any of the four durations of exposure ranging from 50 to 150 days, neither at short (10L:14D) nor at long (16L:8D) photoperiods; the observation under stereomicroscope confirmed the absence of hatched eggs (Figure 1). At 6°C, both at 10L:14D and 16L:8D, there was a significant increase in the number of eggs hatched and emerged nymphs from 50 to 70 days of exposure time (Tukey test, $P < 0.05$) but the values were similar for 70, 90 and 150 days (Tukey test, $P > 0.05$) (Figure 1). In summary, both the number of nymphs emerged and the number of diapausing eggs hatched were significantly influenced by temperature (Nymphs: $F = 39.3$; df = 1, 172; $P < 0.001$. Eggs: $F = 60.1$; df = 1, 172; $P < 0.001$) and time of exposure (Nymphs: $F = 11.4$; df = 3, 172; $P < 0.001$. Eggs: $F = 14.0$; df = 3, 172; $P < 0.001$), but not by photoperiod (Nymphs: $F = 0.143$; df = 1, 172; $P = 0.706$. Eggs hatched: $F = 0.490$; df = 1, 172; $P = 0.485$). The interaction between temperature and photoperiod was not significant (Nymphs: $F = 0.127$; df = 1, 172; $P = 0.722$. Eggs hatched: $F = 0.461$; df = 1, 172; $P = 0.498$), whereas the one between temperature and exposure time had a significant effect both on the number of nymphs emerged and eggs hatched (Nymphs: $F = 11.0$; df = 3, 172; $P < 0.001$. Eggs hatched: $F = 13.8$; df = 3, 172; $P < 0.001$).

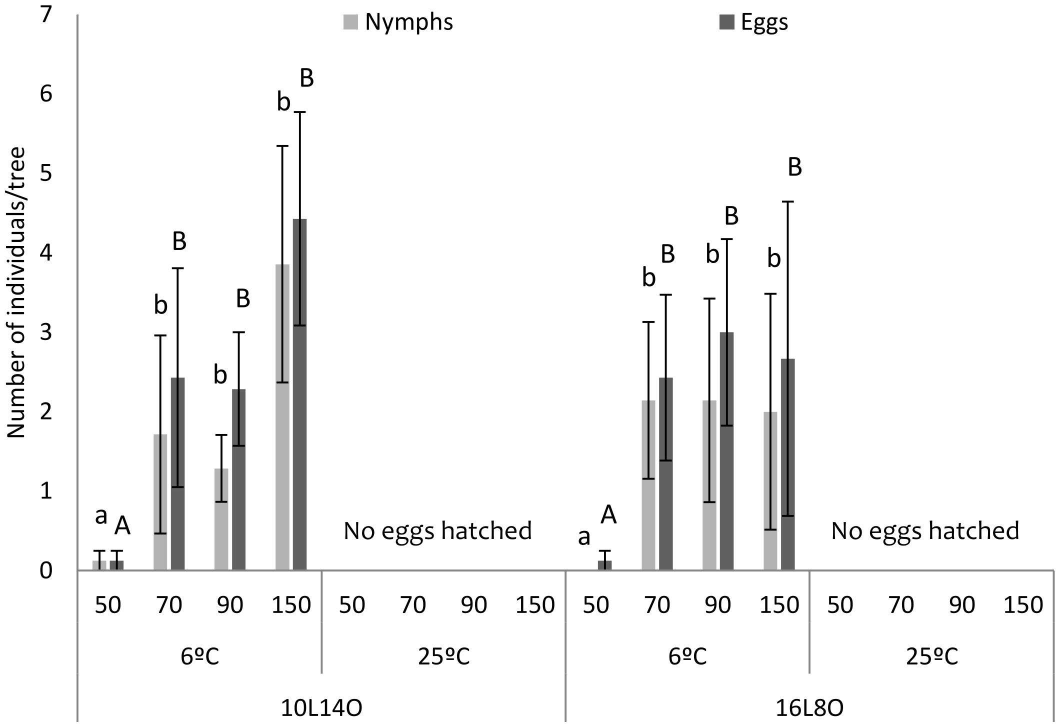

Figure 1. Number of eggs hatched (diapausing eggs) and emerged nymphs per tree depending on the photoperiod (10L:14D, 16L:8D) and temperature (6°C, 25°C) conditions. Different letters denote significant differences between the conditions assayed (Tukey Test $P < 0{,}05$).

Time of exposure had a significant influence on the eclosion time of diapausing eggs ($F = 15.45$; df = 3, 85; $P < 0.001$), but photoperiod did not ($F = 2.078$;

df = 1, 87; *P* = 0.153); the longer the duration of the chilling period was, the shorter average eclosion time (Table 1). However, the shortened incubation time do not really compensate for the extra chilling time, as an increase of 60 days (from 90 to 150) only reduced in 5 days the Et_{50} (from 19.48 to 14.28 days respectively) (Table 1). The range of eclosion times became more narrow as well with longer exposure times, decreasing from 21-49 days at t=50 to 11-28 days at t=150 (Figure 2). The nymph emergence patterns shown in Figure 2 and the function slopes reported in Table 1 indicate that longer cold exposure leads to higher synchronization of egg hatching.

Table 1. Parameters of the model for *Pilophorus gallicus* nymph emergence after the transfer of diapausing eggs from chilling conditions (6°C) to 25°C 16L:8D at different exposure times.

Treatment			Parameters of sigmoid curve	
Temperature (°C)	Exposure time (days)	Eclosion time (mean±SE)	ET50 (mean±SE)	Slope (mean±SE)
6	50	30.50±4.33 a	26.38±1.82	0.07±0.02
	70	26.64±1.93 a	24.10±0.43	0.07±0.01
	90	22.57±1.65 a	19.48±0.32	0.15±0.02
	150	15.70±0.60 b	14.28±0.20	0.29±0.04

Diapausing eggs were kept under chilling conditions (6°C) for different exposure times and photoperiods. As photoperiod was not a significant factor it was not taken in account for these analyses. Following the methodology of Kostal and Havelka (2001), the Et50 (the duration for 50 % nymph emergence) and slope (of the linear component of the sigmoid curve) were calculated. Values within a column followed by the same letter are not significantly different (One-way ANOVA was followed by Tukey HSD test).

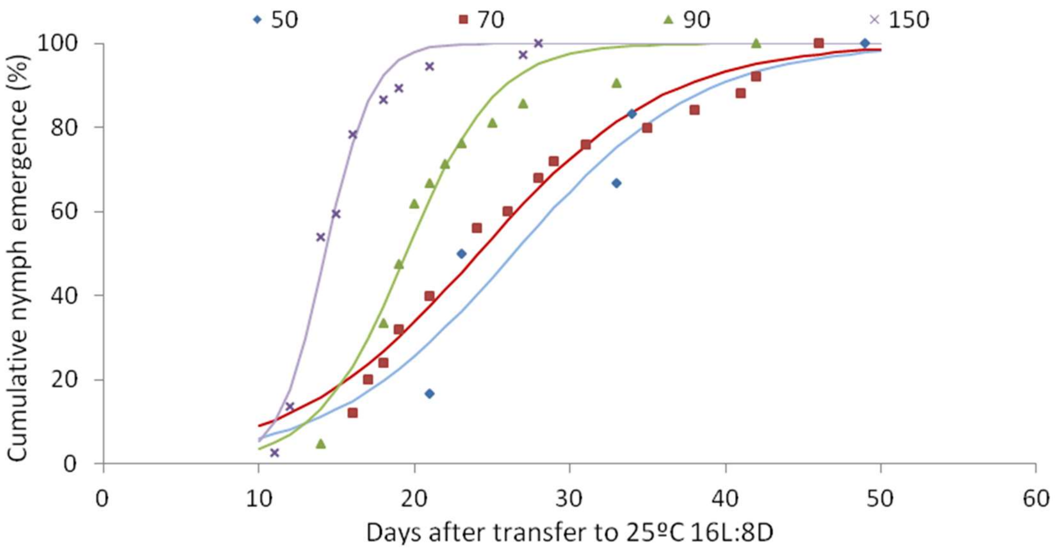

Figure 2. Cumulative emergence of *Pilophorus gallicus* nymphs following the transfer of diapausing eggs from low temperature conditions (6°C) to standard conditions (25°C, 16L:8D) at different exposure times (50, 70, 90 and 150 days).

The diapause completion conditions chosen for the following trial were 90 days at 6°C and 16L:8D. Despite the lack of significant differences among 70, 90, and 150 days for the amount of diapausing eggs hatched, we considered 90 days of exposure time as a good compromise for the experimental conditions. Although no differences were found on egg diapause completion between the long and short photoperiod, the long photoperiod was preferred as a caution measure because short photoperiods are known to have diapause-inducing effects (Jin *et al.*, 2016; Musolin *et al.*, 1999; Musolin and Saulich, 2000).

3.2. Factors influencing the induction of diapause in P. gallicus eggs

Photoperiod had a significant effect on the percentage of diapausing eggs laid by *P. gallicus* females (F = 17.3; df = 1,113; P < 0.001). Females at short photoperiod (9L:15D) laid exclusively diapausing eggs, but under long photoperiod (16L:8D) a small proportion of the eggs laid was diapausing (Figure 3). Temperature had no effect on the induction of the diapause in eggs (F = 0.210; df = 1,113; P = 0.648), without a significant interaction between photoperiod and temperature (F = 0.006; df = 1, 113; P = 0.937). On the other hand, both photoperiod (F = 22.9; df = 1, 113; *P* < 0.001) and temperature (*F* = 14.7; df = 1, 113; *P* < 0.001) had a significant influence on the percentage of non-diapausing eggs laid by females. Under long photoperiod, the proportion of non-diapausing eggs at 23°C (37.92 ± 0.05%) was higher than at 18°C (20.38 ± 0.04%) (Figure 3). The interaction photoperiod:temperature was significant for the amount of non-diapausing eggs (*F* = 7.28; df = 1, 113; *P* < 0.01). The percentage of viable eggs per female (non-diapausing + diapausing) did not differ significantly among photoperiods (*F* = 2.90; df = 1,113; *P* = 0.091), but temperature had a significant effect (*F* = 6.55; df = 1,113; *P* < 0.05), being the proportion of viable eggs higher at 23°C than at 18°C (Figure 3). Overall, a higher percentage of viable eggs per female was registered at 23°C 16L:8D (44.30 ± 0.05% per female) than at 18°C 9L:15D (26.78 ± 0.03% per female); a significant effect of the interaction photoperiod:temperature was detected (*F* = 3.96; df = 1,113; *P* < 0.05).

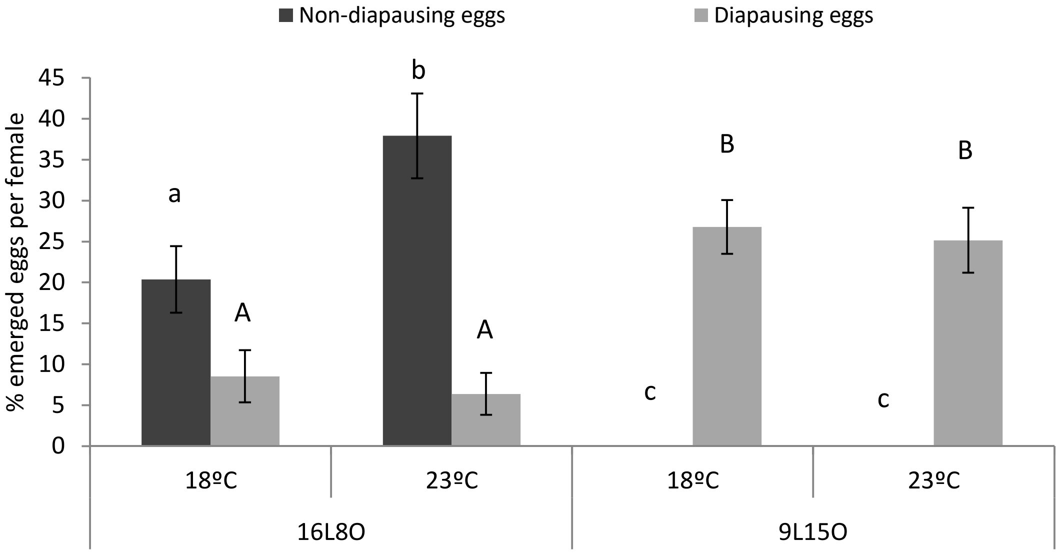

Figure 3. Percentage of emerged eggs (number of non-diapausing and diapausing eggs out of the total number of eggs laid) per female during the whole laying period, at different photoperiods (16L:8O, 9L:15O) and temperatures (18°C, 23°C). Different low case letters denote significant differences in percentage of non-diapausing eggs. Different capital letters denote significant differences in percentage of diapausing eggs. (Factorial ANOVA, *P* < 0,05).

The reproductive response of the females was significantly influenced by photoperiod (Likelihood Ratio Test, *LR* = 88.0; *P* < 0.001) but not by temperature (*LR*

= 5.01; *P* = 0.171), without a significant interaction photoperiod:temperature (*LR* = 0.002; *P* = 1.00). At the short photoperiod, only diapause inducing (81.7%) and infertile females (18.3%) were registered, whereas under long photoperiod females with all four kinds of reproductive response (non-diapause and diapause inducing, mixed and infertile) were present, being the non-diapause inducing and the mixed ones the most frequent (40.4% and 28.1% respectively) (Figure 4). The predictions of the model were close to the experimental proportions of females with different reproductive responses; under short photoperiod, diapause inducing females were clearly the predominant (0.817), whereas under long photoperiod the non-diapause inducing and the mixed females were expected to be the most abundant (0.403 and 0.281, respectively). The predictions of the model explain with a high degree of confidence the experimental variation in the percentage of the different kinds of females in function of the photoperiod (χ^2 = 1.78e-08; df = 3; *P* = 1).

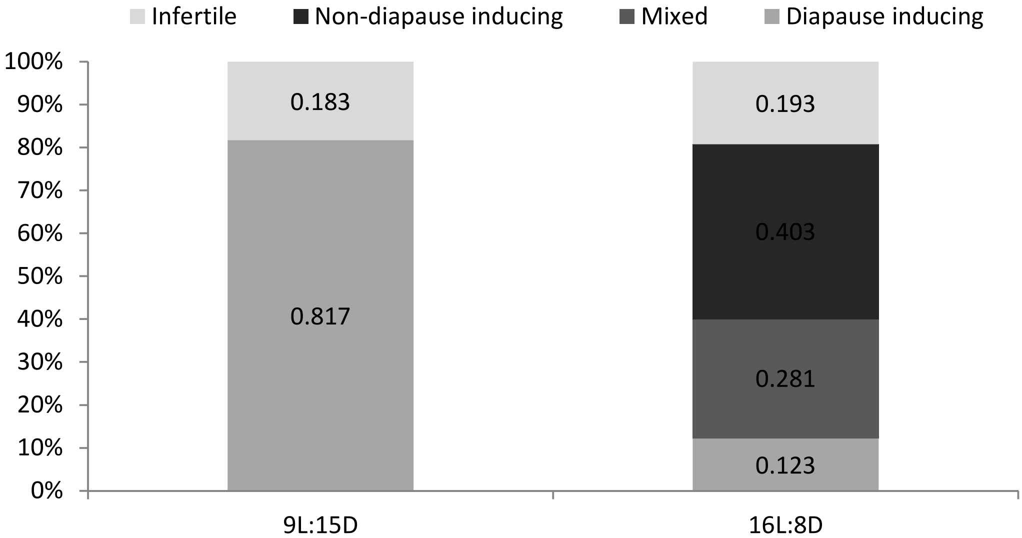

Figure 4. Proportion of females with different types of reproductive response in function of photoperiod. Bars represent the experimental values and the figures the predictions according to the multinomial logistic regression model.

4. DISCUSSION

Both photoperiod and temperature influence the regulation of the embryonic diapause in *P. gallicus*. The induction of diapause was triggered by the short photoperiod (9L:15D) whereas none of the temperatures tested (18 and 23°C) had a significant effect. This confirms the role of short daylength as token stimuli for diapause induction in *P. gallicus*, as suggested by Ramírez-Soria *et al.* (2018). Overwintering diapause is one of the most typical mechanisms of photoperiodic life-cycle control in multivoltine insects from the temperate zone (Denlinger, 2002; Koštál, 2011; Saunders, 2002; Saunders and Bertossa, 2011). The long-day photoperiodic response (*sensu* Beck, 1980) has been widely reported in hemipterans with reproductive diapause, which is the most common type of diapause among the species belonging to this order (Hodkova, 2015; Ikeda Kikue and Numata, 2001; Lundgren, 2011; Musolin *et al.*, 2011; Numata, 2004; Saulich and Musolin, 2009; Spurgeon and Brent, 2015). The role of short photoperiod in Hemipteran diapause induction has been demonstrated as well both for nymphal (Hou *et al.*, 2016; Musolin and Saulich, 1996; Tanaka *et al.*, 2002) and embryonic diapause (Jin *et al.*, 2016; Musolin *et al.*, 1999; Musolin and Saulich, 2000). The parental generation can be sensitive to diapause inducing daylength in the immature or adult stages and, thus, can produce diapausing or non-diapausing progeny depending on the signals received (Saunders, 2002). The results of this work corroborate the predictions made in early works about the maternal induction of diapause in *P. gallicus* (Ramírez-Soria *et al.*, 2018). The photosensitivity of the parental generation has also been reported in other insect species with embryonic diapause, such as *Bombix mori* L. (Lepidoptera: Bombycidae), *Dianemobius nigrofasciatus* Matsumura (Orthoptera: Gryllidae), *Chortoicetes terminifera* Walker (Orthoptera: Acrididae), *Locusta migratoria* L. (Orthoptera: Acrididae), *Aedes albopictus* Skuse (Diptera: Culicidae) (Deveson and Woodman, 2014; Schiesari *et al.*, 2011; Shiga and Numata, 2017; Suman *et al.*, 2015; Tanaka, 1994). The use of daylength as token stimuli allows a timely annual onset of diapause, as its regular seasonal cycle represents a reliable ‘clock’ which announces the approach of the unfavourable season (Danks, 1987; Tauber *et al*., 1986); this avoids late-season ineffective reproduction and increase the probability of survival of the species in regions with rigorous winters (Dos Santos *et al*., 2015).

Temperature did not have a significant influence on the induction of embryonic diapause, at least in the range assayed (18-23°C). Temperature is usually the dominant cue in latitudes where the annual variation in daylength is not so evident (i.e. tropical and subtropical areas) (Denlinger, 1991; Groeters, 1994). This has been demonstrated in several species, such as the pentatomid *Andrallus spinidens* (F.) (Hemiptera: Pentatomidae) (Shintani *et al*., 2010), or some tropical flies (Denlinger, 1986). In species from the temperate zone, temperature generally acts as a mere modifier of the photoperiodic effect (Saulich and Musolin, 2012). Contrary to our expectations, temperature did not interact with the effect of photoperiod on the induction of diapause in *P. gallicus.* In some other Hemipteran species, such as the mirid bugs *Apolygus lucorum* Meyer-Dür and *Adelphocoris suturalis* Jakovlev (Hemiptera: Miridae) (Feng *et al*., 2012), and the bean bug *Riptortus clavatus* Thunberg (Hemiptera: Alydidae) (Kobayashi and Numata, 1995) temperature was not found to have a significant effect on the photoperiodic response. In contrast, in the cabbage beetle *Colaphellus bowringi* Baly (Coleoptera: Chrysomelidae) (Xue *et al*., 2002) and the corn stalk borer *Sesamia nonagrioides* Levebvre (Lepidoptera: Noctuidae) (Eizaguirre *et al*., 1994; Fantinou and Kagkou, 2000), high temperatures (≥ 25°C) reduced or averted the diapause-inducing effect of the photoperiodic signal. The modulation of the photoperiodic response by temperature has also been reported in many hemipterans such as *Trigonotylus caelestialium* Kirkaldy (Hemiptera: Miridae) (Higuchi and Takahashi, 2005), *Stenotus rubrovitatus* Matsumura (Hemiptera: Miridae) (Shigehisa, 2008), *Laodelphax striatellus* Fallen (Hemiptera: Delphacidae) (Wang *et al*., 2014), and *Orius insidiosus* Say (Hemiptera: Anthocoridae) (van den Meiracker, 1994). Contrary to photoperiod, temperature is a more variable factor and do not represent an accurate predictor of the approach of the unfavourable season (Danks, 1987; Tauber *et al*., 1986). The lack of influence of temperature on photoperiodic diapause induction in *P. gallicus* allows the species to enter diapause at the right time to survive through winter, irrespectively of the fluctuations of temperatures in autumn. Finally, temperature had a clear effect on the amount of non-diapausing eggs laid by *P. gallicus,* being significantly lower at 18°C than at 23°C; this could be related to higher mortality eggs experience at low temperatures (i.e. 15°C) (Ramírez-Soria *et al*., 2017).

Population of *P. gallicus* is composed by females with different reproductive strategies. Under short photoperiod, diapause inducing females were predominant (81.7%), whereas the reproductive responses under long photoperiod were more diverse: non-diapause inducing females were the most abundant (40.4%) followed by mixed (28.1%) and diapause inducing (12.3%). The percentage of infertile females was similar for both photoperiod conditions (Figure 4). In many Hemiptera diapause incidence is practically 100% and 0% under inducing or averting conditions, respectively (van den Meiracker, 1994). However, this study shows that *P. gallicus* response is not always univocal. The existence of diapause inducing and mixed females under long photoperiod explain the unexpected presence of diapausing eggs obtained under averting conditions (8.54% and 6.38% eggs per female at 16L8D, 18°C and 23°C respectively). The presence of diapause-inducing females under non-diapause inducing conditions suggests the existence of univoltine individuals in the population that undergo an obligatory diapause (Numata and Nakamura, 2002; van den Meiracker, 1994; Xue *et al*., 2002). Mixed females laying simultaneously diapausing and non-diapausing eggs has also been reported among other species, such as *Choristoneura rosaceana* Harris, (Lepidoptera: Tortricidae), *Trigonotylus coelestialium* Kirkaldy (Hemiptera: Miridae), *Dianemobius nigrofasciatus* Matsumura (Orthoptera: Trigonidiidae) and *Picromerus bidens* L. (Heteroptera: Pentatomidae) (Hunter, 1997; Hunter and Mcneil, 1997; Kudô and Kurihara, 1988; Musolin and Saulich, 2000; Shiga and Numata, 1997). The variation in the proportion of the different reproductive strategies will have an impact on population dynamics. Univoltine and mixed females laying diapausing eggs too early in the season may result in the loss of reproductive opportunity in the late season (Shimizu and Kawasaki, 2001) or entail costs on post-diapause fitness (Ellers and Van Alphen, 2002; Ito, 2007; Kroon and Veenendaal, 1998; Sadakiyo and Ishihara, 2012; Shou Sadakiyo and Ishihara, 2012). These observations seem to be related to a "bet-hedging" reproductive strategy, allowing a distribution of diapausing eggs in time as a form of risk spreading for the population whenever it faces difficulties in the timing of diapause induction (Fangsen and Kallenborn, 1993; Hodek and Michaud, 2008; Ramírez-Soria *et al.*, 2018). These spreading of diapausing eggs along the growing season will increase the probability of persistence of the population; however, it also decreases the biotic potential of the species as a biological control agent of pests (e.g. aphids, psylla).

The completion of diapause in *P. gallicus* relies on the exposure to low temperature (e.g. 6°C) during a period of a certain length (e.g. 70 days); contrary to our hypothesis, photoperiod did not have a significant influence on the termination of diapause in *P. gallicus* eggs. The requirement of a low-temperature exposure for diapause termination in *P. gallicus* prevents eggs laid early in the fall from initiating development before winter. The results are in agreement with the population dynamics of *P. gallicus*: the diapausing eggs become more prevalent in autumn, when photoperiod shortens (from 13L:11D onwards), and these eggs do not hatch before next spring, after undergoing through the cold winter period (Ramírez-Soria *et al.*, 2018). The need of a chilling period during embryonic dormancy to synchronize diapause termination with favorable conditions, has also been reported in several insect orders (e.g. Coleoptera, Diptera, Hymenoptera, Orthoptera, Phasmida) (Bosch and Kemp, 2003; Fisher, 1997; Higaki and Ando, 2005; Moribe *et al.*, 2010; Smith and Jones, 1991; Yamaguchi and Nakamura, 2015; Zhou *et al.*, 2016). A significant effect was registered for the time of exposure to low temperatures, with the proportion of egg hatching greatly increasing between 50 and 70 days but remaining fairly constant at higher exposure times. In contrast, for some species, the effect of time exposure is stronger than the one of low temperature, showing an increase in eclosion rates with the time spent under chilling conditions (e.g. *Pieris napi* L. (Lepidoptera: Pieridae)); *Bactrocera minax* Enderlein (Diptera: Tephritidae)) (Dong *et al.*, 2013; Lehmann *et al.*, 2017). Our results suggest that diapausing eggs in *P. gallicus* probably complete diapause before the end of the winter, but the climatic conditions do not allow the resumption of the development until later (aprox. mid-March), when the thermal requirements are satisfied (Ramírez-Soria *et al.*, 2017, 2018). We predict that *P. gallicus* eggs spend the late winter in a state of post-diapause quiescence to avoid the risk of high mortality that could occasion an early emergence (Moriyama and Numata, 2008; Nielsen *et al.*, 2016; Numata, 2004; Saulich and Musolin, 2007; Zhou *et al.*, 2016). On the other hand, a longer exposure to cold conditions shortened and synchronized the eclosion time of diapausing eggs in *P. gallicus* (i.e. Et_{50} ranges from 26.38 ± 1.82 to 14.28 ± 0.20 days and *Slope* ranges from 0.07± 0.02 to 0.29 ± 0.04 at 50 and 150 days, respectively). This is in agreement with studies that demonstrate that a longer low temperature period decreases the hatching time and synchronizes post-diapause emergence (Bosch and Kemp, 2003; Dong *et al.*, 2013). Even if nymphs emerged from diapausing eggs were not followed in this trial, complementary works showed shorter post-diapause eclosion times for males than for females (Ramírez-Soria personal observation), which has also been reported for the green-veined white *Pieris napi* L. (Lepidoptera: Pieridae) (Forsberg and Wiklund, 1988; Lehmann *et al.*, 2017). Finally, the necessity of a determined low-temperature period to complete diapause makes the overwintering populations vulnerable to the climate change. Various reports have predicted that global air temperature will increase by 1.8–4.0°C, on average, over the next 100 years (Pachauri *et al.*, 2014). Consequently, warmer and shorter winter season could negatively affect the survivorship of diapausing eggs and thus ultimately reduce the initial spring populations (Bale and Hayward, 2010; Xiao *et al.*, 2016).

This work supply, for the first time, the basis for the understanding of diapause expression in *P. gallicus*. The singular existence of different reproductive responses, which contributes to a bet-hedging strategy, suggests in this species a high potential to adapt to different environmental conditions. Additionally, from an applied point of view, this kind of studies may represent an interesting asset for the biocontrol industry. Insight into the factors regulating embryonic diapause in *P. gallicus* may be used for the handling of this insects; for instance, to reduce the costs of continuous mass rearing (Ruberson *et al.*, 1998) or the negative effects of logistic issues (e.g. storage, shipping or releases) on natural enemy's performance (Kostal *et al.*, 2001). In this sense, experiments dealing with post-diapause fitness in *P. gallicus* would be desirable. Ultimately, further knowledge about how *P. gallicus* integrates photoperiod in its diapausing response (e.g. critical daylength) will improve the understanding of the population dynamics and the management of the species as a biological control agent.

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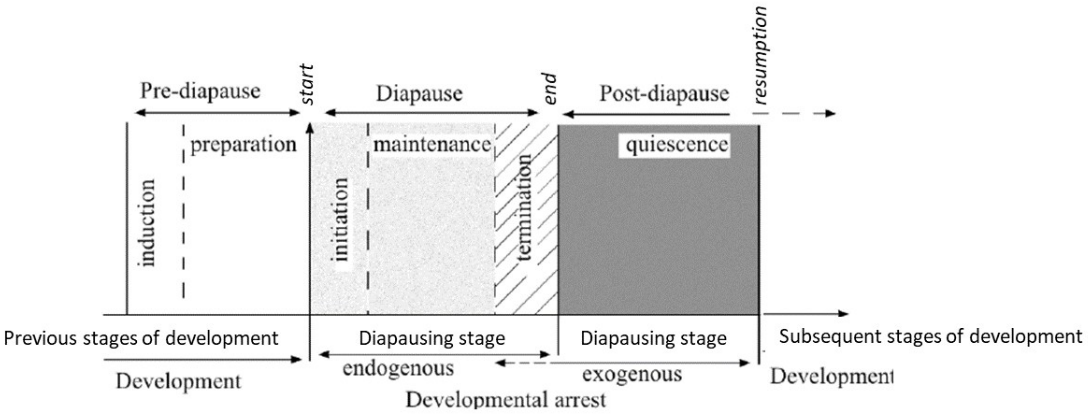
SUPPLEMENTARY MATERIAL



Supplementary material S1. Ecophysiological phases of insect diapause. Modified after Kostal (2006).

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CHAPTER 4

Study of photoperiodism in diapause induction of the predatory mirid *Pilophorus gallicus* (Hemiptera: Miridae)

Photo: Elena López Gallego

CHAPTER 4: Study of photoperiodism in diapause induction of the predatory mirid *Pilophorus gallicus* (Hemiptera: Miridae)

ABSTRACT

Pilophorus gallicus (Hemiptera: Miridae) is a multivoltine predator living on pear orchards ecosystem (*Pyrus communis* L. (Rosaceae)) in southern Europe. To overcome winter, *P. gallicus* enters into a facultative embryonic diapause, triggered by short photoperiod. Knowledge about its photoperiodic response will contribute to predict its population dynamics in the field. The objectives were: (1) to draw its photoperiodic response curve and estimate its critical daylength; (2) to investigate the effect of the direction of change in daylength (increasing or decreasing) on its diapause response; (3) to study the reversibility of egg-diapausing laying in females. Photoperiod had an effect on egg and nymphal development times, but no particular trend was detected. The critical daylength was estimated at 13h53min. The direction of change in photoperiod (i.e. increasing or decreasing) did not have a significant effect on the incidence of the embryonic diapause. Females laying diapausing eggs start laying non-diapausing ones when transferred to long photoperiods. Three reproductive responses were observed in the egg-laying of females: diapauses inducing, predominant at short photoperiods (below 13h53min); non-diapause inducing, mainly at long photoperiods (above 13h53min); and mixed. This study shows the plasticity of *P. gallicus* diapause expression enabling the species to adapt to a variable climate conditions. The findings of this work could be applied to biological control programs and mass rearing.

Key words: facultative embryonic diapause, critical daylength, direction of change in daylength, reversibility, ecological plasticity, population dynamics.

1. INTRODUCTION

Insects can use Earth's natural cycles of daylight and darkness as a reliable source of environmental information to make behavioral decisions and to regulate life-history processes (Bale and Hayward, 2010b; Saunders, 2002; Tauber and Kyriacou, 2001; Tauber *et al.*, 1986). The ability to detect seasonal changes in daylength and to respond to them has enabled arthropods to colonize regions that would otherwise have been uninhabitable (Saunders, 2011). Diapause is a common photoperiodic response employed by insects to survive adverse periods of the year (Tauber and Kyriacou, 2001); it is a widespread type of dormancy genetically determined, induced in advance to the arrival of the unfavourable period, and that may last longer than the adversity (Danks, 2002). The diapausing state is characterized by reduced morphogenesis, decreased metabolic rates, and/or increased stress tolerance mechanisms (e.g. cold-hardening) (Koštál, 2006; Tauber and Kyriacou, 2001). Diapause can be either obligatory or facultative. Obligatory diapause typically occurs in insects with univoltine life-cycles, where every individual undergoes diapause regardless of external conditions. On the contrary, facultative diapause is associated with multivoltine species that alternate generations where all or nearly all the individuals enter diapause, triggered by specific environmental cue (*token stimuli*), with generations where most individuals develop and reproduce directly (Danks, 1987; Denlinger, 2002; Hahn and Denlinger, 2007; Tauber *et al.*, 1986). The facultative winter diapause of most predatory Hemiptera from temperate latitudes is regulated by photoperiod, being averted under long daylengths and induced under short photoperiods below a critical length (Hand *et al.*, 2016; Musolin and Saulich, 1996; Ruberson *et al.*, 1998; Saunders *et al.*, 2004).

Knowledge about the influence of annual daylength in diapause incidence is of special interest to predict insect population dynamics in the field. The variation in the percentage of diapausing individuals in a population in relation to photophase is species-specific and it is known as the *diapause induction response curve*. The switch from a non-diapausing population to a diapausing one occurs in a narrow range of daylengths above and below a critical value. The *critical daylength,* estimated from the diapause induction response curve, represents the photoperiod at which half of the individuals of the population enters into diapause and thus the timing of the switch from a reproducing population to a diapausing one (Musolin, 2007; Shigehisa, 2008; Tauber *et al*., 1986). Critical photoperiod has important ecological significance in insects living in the temperate zone, because it has a direct influence on the synchronization of their life-cycle with seasonal climatic variation, and it allows or hinders the successful establishment of the species (Musolin and Numata, 2003). Short critical daylengths would entail a late timing of diapause induction, when temperatures are too low, placing at risk survivorship of the late generation (Musolin, 2007; Musolin and Numata, 2004); in contrast, long critical photoperiod would induce diapause too early, preventing the species to take fully advantage of the growing season, and may have negative effects in post-diapause fitness (Bean *et al*., 2012; Ellers and Van Alphen, 2002; Hultine *et al*., 2015; Kroon and Veenendaal, 1998).

The concept of critical daylength assumes the photoperiodic response in terms of absolute daylength, that is, the insect does not respond to a rate of change in daylength over time (Beck, 1980). In latitudes with a long growing season (e.g. temperate climate), insects may experience the critical photoperiod twice, once when daylength is increasing in spring and once when it is decreasing in autumn; however the population is triggered to enter into a diapausing state in autumn rather than in spring. Three distinct processes are known to originate these seasonal differences in diapause induction. On the one hand, in some cases temperature can modify the effect of photoperiod in the induction of diapause, thus different ambient temperatures at these two moments of the year may have an influence on photoperiodic response (Beck, 1980; Eizaguirre *et al*., 1994; Saulich and Musolin, 2014). On the other hand, some published works have suggested an influence of the rate of change in daylength on diapause induction (Adkisson and Roach, 1971; Saunders, 2002; Gillespie and Quiring, 2005; Deveson and Woodman, 2014). Tauber and Tauber (1970) demonstrated how *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) entered diapause after experiencing decreasing photoperiods above the critical daylength, and averted diapause when exposed to increasing photoperiods under the critical daylength. But the response to the rate of change of daylengths is not very common among insects (Beck, 1980; Danks, 1987). Some species have a prolonged photosensitivity and are able to respond to different levels of photoperiods along their life-cycle. In these species, entering into diapause may not be irrevocable, and progression into the diapause-developmental trajectory may be altered along the developmental stages (Higuchi and Takahashi, 2005; Hunter and Mcneil, 1997). Several authors showed that the transition to the diapause phase may not be achieved if the photoperiodic conditions change from short- to long-day during the development of the insect (Bell and Adkisson, 1964; Eizaguirre *et al*., 1994; Numata and Hidaka, 1982; Saunders, 1965; Shintani, 2009). Species with facultative diapause and long development time are interesting organisms to assess plasticity in diapause induction and their ability to fit their response to current environmental conditions.

Pilophorus gallicus (Hemiptera: Miridae) is a multivoltine species living on pear orchards ecosystem (*Pyrus communis* L. (Rosaceae)) in southern Europe (Ramírez-Soria *et al*., 2018; Sanchez and Ortín-Angulo, 2012). It is a generalist predator of small arthropods, with approximately three annual population peaks at spring, summer and autumn (Ramírez-Soria *et al*., 2018; Sanchez and Ortín-Angulo, 2012). To overcome unfavourable winter conditions, *P. gallicus* enters into a facultative embryonic diapause triggered by short photoperiod (Ramírez-Soria *et al*., 2018; Chapter 3 of this thesis dissertation). However, this mirid experiences short photoperiods at two different times during its annual cycle, once in the fall when diapause is induced and once in the spring when populations resume development. The difference is that females that develop from nymphs to adults in spring start developing at short daylength (from 13h approximately) and reach the adult stage at long photoperiods (above 14h30min), while in autumn they start developing at longer photoperiods and complete the nymphal stage at shorter ones (Supp. material, S1). Previous works showed that temperature does not have an influence on short photoperiod induction (Chapter 3 of this thesis dissertation). This seems to indicate either that the direction of the photoperiod (i.e. increasing or decreasing) has an effect on the induction of the egg-laying (diapausing or non-diapausing) of *P. gallicus* females, or that the laying of *P. gallicus* females is determined by the photoperiodic conditions at which females are exposed rather than by the photoperiodic conditions at early nymphal instars.

Photoperiodic response of *P. gallicus* can determine the structure of the population in the crop. Therefore, the understanding of how *P. gallicus* integrates the photoperiodic signals (i.e., the critical daylength; if it responds to an increase or a decrease in daylength; the reversibility of the egg-diapausing laying in females) will contribute to the understanding of the biological cycles and population dynamics of the species. The three main objectives in this work were: (1) To study the diapause response of *P. gallicus* under different daylenghts and to draw the photoperiodic response curve, to ultimately determine the critical daylength for this species. Our working hypothesis was that there will be a high diapause incidence at short photoperiods, followed by a sharp decrease at daylength close to the critical day-length, and low diapause incidence at long photoperiods. (2) To investigate the extent to which the direction of change in daylength (increasing or decreasing) may have an effect on the diapause response of *P. gallicus.* We predict that increasing photoperiod will stimulate in females the production of non-diapausing eggs, whereas a decreasing photoperiod will lead to diapausing egg-laying. (3) To study if the egg-diapausing laying is a reversible condition in *P. gallicus* females. Diapause induction is a developmental trajectory that is triggered at a sensitive stage; in this work we test if females developed under short photoperiod conditions and laying diapausing eggs will lay non-diapausing eggs after being transferred to long day conditions. We hypothesized that *P. gallicus* females are sensitive to *token stimuli*, thus, even if they have developed under diapause inducing conditions, they can avert diapause in their egg progeny if exposed to non-diapause inducing conditions at the adult stage.

2. MATERIALS AND METHODS

2.1. Insect rearing

Pilophorus gallicus were collected in pear orchards located in Jumilla (Latitude: 38°23'N, Longitude: 1° 14'W) (Murcia, Spain) several months before the start of the assay. A laboratory stock colony was established to provide the individuals to be used in the experiments. The insects were placed in mesh-walled wooden-framed cages (40 x 40 x 40 cm), using *P. communis* saplings as laying substrate, kept at 25°C ± 1 °C and 60 ± 10% relative humidity under long day conditions (16L:8D) (Photo 1 Annex of photos). *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs provided by Biobest N.V. were supplied on sticky paper (Post-it® note; 3M, St Paul, MN, USA) as factitious prey.

2.2. Critical daylength assay

The laying of *P. gallicus* females (diapausing or non-diapausing) was studied at six photoperiod levels (11L:13D; 12L:12D; 13L:11D; 14L:10D; 15L:9D; 16L:8D). This photoperiods are within the daylength range of variation at the area of study (Latitude: 38°23'N, Longitude: 1°14'W) from mid-March to the beginning of November. The hatching and development times were registered for every nymph emerged; fecundity, the number of non-diapausing and diapausing eggs per female were recorded for each photoperiod tested.

To obtain the females and males used in this experiment, approximately six hundred 3- to 10-day old adults from the laboratory colony were divided into six mesh-walled wooden-framed cages (40 x 80 x 40 cm, wide x long x high) with thirty-six *P. communis* seedlings as laying substrate and *E. kuehniella* eggs on sticky papers *ad libitum.* The cages were placed in plant growth chambers (Binder KBWF-750, Tuttlingen, Germany) at 25±1°Cand 60±10% of relative humidity for one day to obtain a uniform cohort of eggs. After 24h, the adults were removed and saplings were haphazardly assigned to six plant growth chambers (Binder KBWF-750, Tuttlingen, Germany) set up at the corresponding six photoperiods tested, 25 ± 1°C and 60 ± 10% of relative humidity. The seedlings were inspected daily to take the emerged nymphs and to register hatching times. Nymphs were reared individually under the same experimental conditions they were collected, in a 100-ml translucent cup with a pear leaf on a 5-mm agar (1%) layer, soaked cotton and *E. kuehniella* eggs as food. The agar surface was covered with a plastic disc to avoid nymphs getting stuck (Photo 2 Annex of photos). Water and food were provided three times a week. Once nymphs reached the fifth instar, they were checked daily until they reached the adult stage. The development time and sex were registered for each individual, and adults that emerged the same day were kept together for mating in plastic containers (4700 ml capacity), with cotton soaked in water and *E. kuehniella* eggs *ad libitum* during three days in the same plant growth chamber where they had developed.

Once the mating period was completed, pairs of three-day old males and females were put together and placed individually in translucent plastic cylinders (9 cm in diameter, 2000 ml in volume, and a ventilation mesh on the top), with *P. communis* seedlings approximately 20 cm high as laying substrate, and fed on *E. kuehniella* eggs on sticky papers (Photo 3 Annex of photos). A minimum of 28 couples per photoperiod were set up and kept under the same conditions they developed as nymphs for two weeks. The pear seedlings were changed weekly and observed under stereomicroscope to count the eggs. Thereafter they were brought to standard conditions (25 ± 1°C, 16L8D and 60± 10% of relative humidity), and they were examined three times a week during 30 days to register the emergence of nymphs from non-diapausing eggs. Then, hatched and non-hatched eggs were counted under a stereomicroscope and the seedlings with non-hatched eggs (diapausing or unviable) were placed under diapause completion conditions (90 days at 6°C, 16L8D and 60 ± 10% relative humidity) (Chapter 3 of this thesis dissertation; Ramírez-Soria *et al*., 2018).

After the cold period, the saplings were returned to 25°C, 16L:8D and 60 ± 10% of relative humidity. Saplings were observed three times a week over a period of at least eighty days to monitor the emergence of nymphs from diapausing eggs.

Finally, seedlings were inspected under a stereomicroscope to count hatched and non-hatched eggs, the last ones were considered as non-viable.

2.3. *Effect of the direction of change in daylength on P. gallicus* embryonic *diapause*

The laying of *P. gallicus* females (diapausing or non-diapausing) was assayed at photoperiods with increasing and decreasing trends. Two photophase regimes were chosen within the range of diapause-inducing photoperiods, according to the critical daylength assay, to test just for the effect of the daylength trend. At increasing daylength the photoperiod went from 12L:12D to 13L:11D in 50 days at a pace of 1.2 minutes per day. At decreasing daylength the photoperiod went the opposite way, from 13L:11D to 12L:12D, at the same pace. The 50 day period of decreasing/increasing photoperiod covered the development of eggs and nymphs (11.9 ± 0.1 and 14.8 ± 0.1 days at 25°C, 60±10% RH respectively) (Ramírez-Soria *et al.*, 2017) and adult reproduction. The variables recorded were: hatching and development time for every nymph emerged; fecundity, number of diapausing and non-diapausing eggs per female.

Individuals used in this experiment were obtained from the laboratory colony. Approximately two hundred adults (3 to 10-days-old) were introduced into a mesh-walled wooden-framed cage (40 x 80 x 40 cm) with forty *P. communis* seedlings and *E. kuehniella* eggs *ad libitum* as factitious prey. The cage was kept in a plant growth chamber (Binder KBWF-750, Tuttlingen, Germany) at 25 ± 1°C, 16L:8D and 60 ± 10% RH for 24 hours in order to get a uniform cohort of eggs. Then, adults were removed and the saplings were evenly divided in two plant growth chambers at 25°C ± 1°C, 60± 10% of RH, and the above mentioned increasing or decreasing photoperiods. The seedlings with one-day old eggs placed under increasing or decreasing conditions were inspected everyday to collect the nymphs emerged, which were reared individually following the same methodology described for the critical daylength trial. Hatching time, development time and sex were registered for each individual. The adults that emerged the same day were placed for three days in a plastic container (4700 ml) with cotton soaked in water and *E. kuehniella* eggs, and kept under the same increasing/decreasing photoperiod conditions.

Once the mating period had elapsed, couples of males and females were placed individually in translucent plastic cylinders (diameter, 9 cm; volume, 2000 ml; ventilation mesh on the top) with *P. communis* seedlings and *E. kuehniella* eggs on sticky papers (Photo 3 Annex of photos). Forty couples were set up for each of the experimental conditions assayed; the pairs that had developed at decreasing or increasing daylenght were maintained at their respective conditions. The oviposition of *P. gallicus* was registered during twelve days; pear seedlings were changed every six days and *E. kuehniella* eggs added every 3-4 days. The replaced seedlings were observed under a stereomicroscope to count the eggs and then transferred to standard conditions (25°C ± 1°C, 16L:8D and 60± 10% of RH). The procedure followed to determine the number of diapausing, non-diapausing and unviable eggs was the same as for critical daylength assay.

2.4. *Reversibility of diapause induction in P. gallicus*

The reversibility of embryonic diapause induction in *P. gallicus* was studied through the observation of female's sensitivity to photoperiod: females collected from the field in autumn (i.e. under short photoperiod conditions) were brought to the laboratory and placed under short or long photoperiod (9L:15D and 16:L8D) and constant temperature (23°C). The number of non-diapausing and diapausing eggs per female was recorded.

Adults of *P. gallicus* were collected in mid-October in pear orchards located in the municipality of Jumilla (Murcia, Spain). Females collected at this time of the year produce predominantly diapausing eggs (Ramírez-Soria *et al*., 2018). Fifty couples were set up individually in cylindrical containers (diameter, 9 cm; volume, 2000 ml; covered with a ventilation mesh on the top) with *P. communis* seedlings as oviposition substrate and *E. kuehniella* eggs as factitious prey (Photo 3 Annex of photos). Half of the couples were placed at short photoperiod (9L:15D), to simulate the field photoperiod they came from, and the other half were placed at long photoperiod (16L:8D) to observe the effect of the switching in photoperiod on the egg laying response. Couples were kept in two different plant growth chambers, both at 23°C ± 1°C and 60 ± 10% RH. Oviposition was recorded over a three-week period. Seedlings were replaced every week and observed under the stereomicroscope to count the eggs. The number of diapausing, non-diapausing and inviable eggs was estimated following the same methodology described for the critical daylength trial.

2.5. *Statistical analyses*

In the critical daylength assay (experiment 1), ANOVA was used to test the effect of photoperiod on egg hatching and nymphal development time of *P. gallicus* (‘aov’ function, package ‘stats’ in R) (R Development Core Team, 2017). Tukey’s HSD post hoc test (α=0.05) was used for the separation of the means (‘TukeyHSD’ function, package ‘stats’ in R). The nymphal survivorship and sex ratio were compared among the photoperiods by the binomial proportion test using the function ‘prop.test’ (‘stats’ package) in R (R Development Core Team, 2017). The relationship between the percentage of diapausing eggs (Y) and daylength in hours (x) was estimated by the logistic equation $Y = a + \frac{(b-a)}{1+e^{\frac{c+x}{d}}}$. The parameters of the equations were estimated by nonlinear regression using the function ‘nls’ (‘MASS’ package) in R (R Development Core Team, 2017). Females were typified according to their egg-laying response as: (1) “diapause inducing” when they laid exclusively diapausing eggs; (2) “non-diapause inducing” when they laid only non-diapausing eggs; (3) “mixed” when they laid both diapausing and non-diapausing eggs; and (4) “infertile” when none of the eggs they laid hatched. The variation in the percentage of diapause-inducing females in relation to daylength was performed using the same logistic equation and analytical procedure as for the diapausing eggs.

The effect of increasing or decreasing daylength (experiment 2) on the hatching time, development time, fecundity and fertility was tested by ANOVA using the ‘aov’ function (‘stats’ package in R). The percentages of diapausing and non-diapausing eggs were transformed by the natural logarithm to account for heteroscedasticity; the mean values were compared using ANOVA test (‘aov’ function, ‘stats’ package in R). Females were typified according to their egg-laying response as in the analysis of critical daylength data (non-diapause inducing, diapause inducing, mixed and infertile); the proportion of the different reproductive responses was compared by the binomial proportion test using the function ‘prop.test’ (‘stats’ package) in R.

Finally, in the assay to determine the reversibility of embryonic diapause induction in *P. gallicus* females (experiment 3), the effect of photoperiod switching on the number of diapausing and non-diapausing eggs laid per female was tested using repeated measures ANOVA (‘aov’ function from ‘stats’ package), including week of observation and female as random factors to account for temporal pseudoreplication and inter-subject variation, respectively. As in the critical daylength assay, females were classified as (1) “non-diapause inducing”, (2) “diapause inducing”, (3) “mixed” and (4) “infertile”. The change from diapausing to non-diapausing laying in mixed and non-diapause inducing females was examined along the weeks. In all cases, females that did not lay eggs were removed from the analysis. All the analyses were performed using R statistical software, version 3.4.2 (R Development Core Team, 2017).

3. RESULTS

3.1. *Critical daylength assay*

Embryonic and nymphal development times were significantly influenced by the length of the photophase (Egg: $F = 41.7$; df = 5, 973; $P < 0.001$; Nymph: $F = 29.7$; df = 5, 973; $P < 0.001$). The shortest nymphal development times were registered at extreme photoperiods (11L:13D and 16L:8D), with no significant differences between them (Tukey test, $P > 0.05$). The highest development times were registered at 14L:10D and 15L:9D (Table 1). No differences were found between males and females neither for egg ($F = 1.06$; df = 1, 371; $P = 0.304$) nor for nymphal development times ($F = 3.89$; df = 1, 371; $P = 0.065$). Significant differences were observed in sex ratio and survivorship among some of the photoperiods, but no apparent trend was observed in relation to the variation of the photophase length (Table 1).

Table 1. Average egg hatching and nymphal development times (days ± standard error), survivorship, and sex ratio for *Pilophorus gallicus* under different photoperiods. n is sample size; mean values followed by the same letter in the same column are not significantly different ($P > 0.05$).

Photoperiod	n	Hatching time	Development time (NI to Adult)	Sex ratio	Survivorship
11L:13D	159	12.64 ± 0.07 a	26.60 ± 0.09 ab	0.625 a	0.805 ab
12L:12D	251	12.26 ± 0.06 b	26.97 ± 0.09 ac	0.487 bc	0.777 ab
13L:11D	155	12.41 ± 0.09 ab	27.08 ± 0.13 c	0.447 b	0.852 b
14L:10D	239	13.37 ± 0.07 c	27.80 ± 0.10 d	0.559 abc	0.787 ab
15L:9D	232	13.19 ± 0.08 c	27.49 ± 0.10 d	0.571 ac	0.763 a
16L:8D	220	12.66 ± 0.07 a	26.42 ± 0.08 b	0.522 ab	0.723 a

Pilophorus gallicus females laid predominantly diapausing eggs at short-day conditions, whereas those at long-day conditions laid mainly non-diapausing eggs (Figure 1). Variation in the percentage of diapausing eggs was satisfactorily explained with a logistic model in funtion of day-length ($R^2 = 0.997$, Table 2). The model predicts a sharp decrease in the percentage of diapausing eggs at about 14L:10D. The critical daylength, that is the photoperiod at which 50% of the eggs are in diapause, was estimated at 13 h and 53 min (Figure 1).

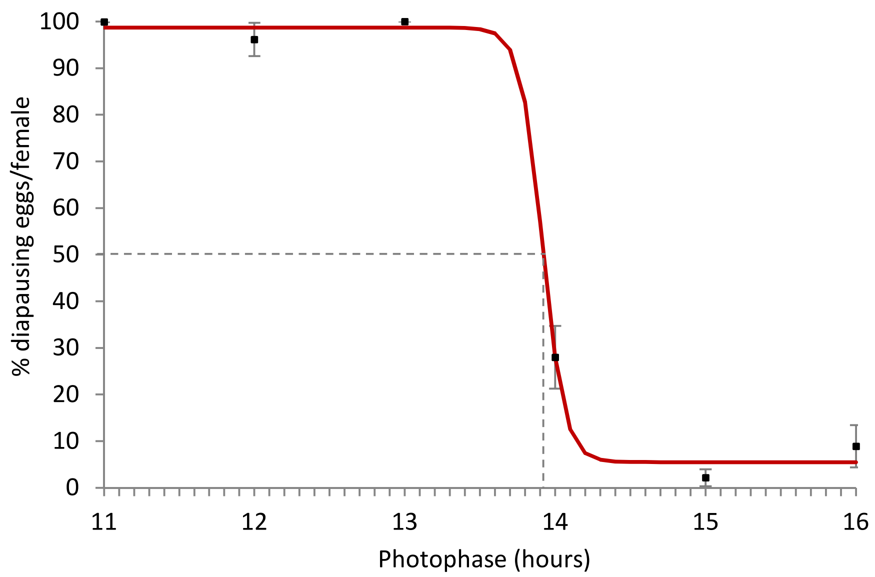

Figure 1. Photoperiodic response for the embryonic diapause induction in *Pilophorus gallicus*. The dots represent the experimental values (percentage of diapausing eggs per females ± standard error) and the curve the prediction according to the logistic equation. Dashed line shows the critical day-length.

Table 2. Parameters (estimate ± standard error) of the logistic function ($\boldsymbol{Y = a + \frac{(b-a)}{1+e^{\frac{c+x}{d}}}}$), where (Y) stands for the percentage of diapausing eggs and (x) for photoperiod.

Parameter	Estimate	Standard Error	t-value	P
a	5.51	2.75	2.01	0.18
b	97.87	2.74	35.79	7.80e-04
c	-13.88	3.19	-4.35	0.04
d	0.10	2.81	0.04	0.97

Fecundity (*F* = 27.0; df = 5, 177; *P* < 0.001) and fertility (*F* = 29.6; df = 5, 177; *P* < 0.001) were significantly influenced by photoperiod. The minimum mean values for fecundity (54.37 ± 3.28 eggs per female) and fertility (6.40 ± 1.17 eggs per female) were observed at 12L:12D. The maximum fecundity (124.20 ± 6.16 eggs per female) and fertility (46.03 ± 4.34 eggs per female) values were registered at 15L:9D (Table 3). However, there was no apparent trend in fecundity and fertility in relation to the variation of the photophase length. The number of ovipositing females was high and ranged from 77% at 15L:9D to 97% at 13L:11D (Table 3).

Table 3. Number and percentage of ovipositing females, fecundity (eggs per female ± standard error) and fertility (nymphs per female ± standard error) of *Pilophorus gallicus* for the whole period of observation (two weeks) under different photoperiods. n is number of females assayed; mean values followed by the same letter within each column are not significantly different (Tukey test, *P* > 0.05).

Photoperiod	n	Ovipositing females	Fecundity	Fertility
11L:13D	28	27 (96.4%)	77.44±4.83 ae	23.26±3.03 a
12L:12D	38	30 (78.9%)	54.37±3.28 b	6.40±1.17 b
13L:11D	37	36 (97.3%)	61.50±3.35 ab	6.86±1.44 b
14L:10D	38	34 (89.5%)	102.56±6.08 cf	31.18±2.79 d
15L:9D	39	30 (76.9%)	124.20±6.16 d	46.03±4.34 e
16L:8D	31	26 (83.9%)	92.31±6.41 ef	36.38±4.43 de

Reproductive response of *P. gallicus* females varied at the different photoperiods assayed: at short photoperiods (i.e. 11L:13D to 13L:11D) most of the females showed a clear egg-diapause induction response, while at long photoperiods (i.e. 14L:10D to 16L:8D) most of the females were of the non-diapause-inducing type. The percentage of diapause inducing females decreases steeply around 14L:10D (Figure 2), in a similar way to the trend observed for the percentage of diapausing eggs (Figure 1). The logistic model predicts satisfactorily the variation in the percentage of the diapause-inducing females in function of the daylength ($R^2 = 0.994$, Table 4). At 11L:15D, 14L:10D, 15L:9D and 16L:8D there was a nearly constant percentage of females with a mixed response, without any apparent trend in relation to the variation in the length of the photophase (Figure 2). The percentage of diapausing eggs laid by those females decreased from 97.5% at 11L:13D to 13.8% at 16L:8D (Figure 3). No significant differences were found neither between the proportion of diapausing eggs nor non-diapausing eggs laid by mixed females at photoperiods longer than the critical daylength (14L:10D, 15L:9D and 16L:8D) (Binomial test, *P* > 0.05).

Table 4. Parameters (estimate ± standard error) of the logistic function ($\boldsymbol{Y} = \boldsymbol{a} + \frac{(\boldsymbol{b}-\boldsymbol{a})}{\boldsymbol{1}+\boldsymbol{e}^{\frac{c+x}{d}}}$), where (Y) stands for the percentage of diapause-inducing females and (x) for photoperiod.

Parameter	Estimate	Standard Error	t-value	*P*
a	0.91	3.70	0.25	0.83
b	85.49	3.70	23.12	0.002
c	-13.51	0.12	-115.25	<0.001
d	0.31	0.07	4.59	0.04

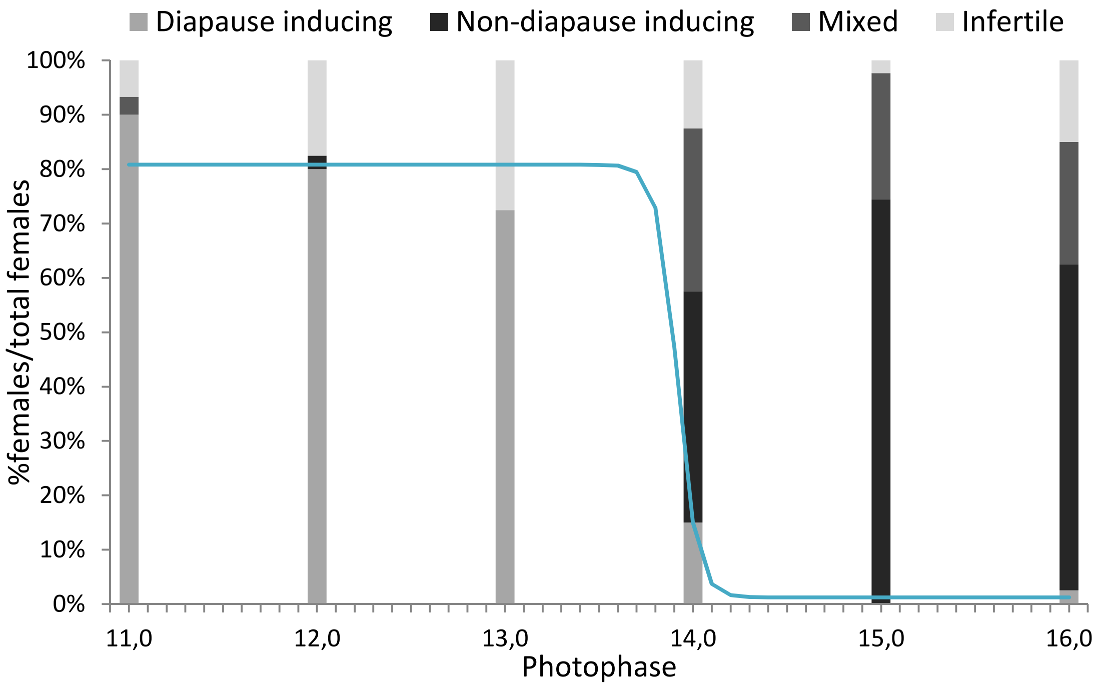

Figure 2. Proportion of females with different types of reproductive response in function of photophase length in hours. Bars represent the experimental values and the curve the predictions according to the logistic equation for diapause-inducing females.

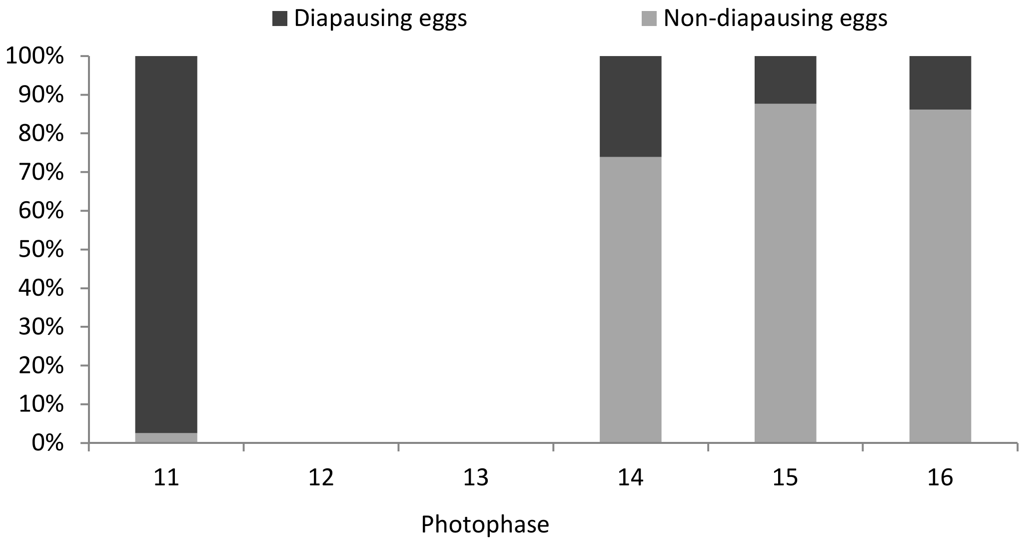

Figure 3. Variation in the egg-laying condition of mixed females depending on the photoperiod.

3.2. Effect of the direction of change in daylength on P. gallicus diapause

Neither the direction of change of daylength (increasing/decreasing) (*F* = 0.002; df = 1, 371; *P* = 0.965) nor sex (*F* = 1.06; df = 1, 371; *P* = 0.304) had a significant effect on the egg-hatching time. In contrast, the nymphal development time (NI to Adult) was slightly influenced by the photoperiod trend (*F* = 19.47; df = 1, 371; *P* < 0.001) and sex (*F* = 13.81; df = 1, 371; *P* < 0.001). The interaction sex:photoperiod was not significant (*F* = 3.743; df = 1, 371; *P* = 0.054), but females at decreasing photoperiods took significantly longer to develop than at increasing ones (Tukey test, P < 0.001) (Table 5). The sex ratio (i.e. the proportion of females) (Increasing: 0.512; Decreasing: 0.595) and survivorship rates (Increasing: 0.714; Decreasing: 0.770) were not significantly affected by the direction of change in the photoperiod (Binomial test, χ^2 = 2.24, df = 1, *P* = 0.134 and χ^2 = 1.76, df = 1, *P* = 0.184, respectively).

Table 5. Average egg hatching and nymph development times (days ± standard error) of *Pilophorus gallicus* under increasing and decreasing photoperiods. n is the number of individuals assayed; mean values followed by the same letter within each column are not not significantly different (Tukey test, *P* > 0.05).

Photoperiod direction	Sex	n	Hatching time	Development time (NI to Adult)
Increasing photoperiod	Female	111	12.95 ± 0.084 a	14.42 ± 0.11 a
	Male	106	13.21 ± 0.102 a	14.22 ± 0.08 a
Decreasing photoperiod	Female	94	13.13 ± 0.077 a	15.00 ± 0.10 b
	Male	64	12.98 ± 0.093 a	14.41 ± 0.10 a

Fecundity and fertility were significantly higher under the increasing photoperiod (Fecundity: *F* = 12.5; df = 1, 78; *P* < 0.001; Fertility: *F* = 15.5; df = 1, 78; *P* < 0.001). Fecundity decreased from 71.35 ± 2.82 to 58.53 ± 2.29 eggs per female and fertility from 28.15 ± 2.32 to 15.83 ± 2.10 hatched eggs per female at increasing and decreasing photoperiods, respectively (Figure 4). The percentage of diapausing eggs was significantly affected by the direction of daylength change (*F* = 4.56; df = 1, 78; *P* < 0.05), but not the percentage of non-diapausing ones (*F* = 2.313; df = 1, 78; *P* =

0.132). Diapausing eggs were clearly predominant over the non-diapausing ones, with a higher percentage at increasing (35.71 ± 3.23% eggs per female) than at decreasing (26.08 ± 3.35% eggs per female) photoperiods. In contrast, the amount of non-diapausing eggs per female was very low at the two photoperiodic conditions (Increasing: 4.18 ± 1.78% eggs per female; Decreasing: 1.20 ± 0.93% eggs per female) (Figure 4).

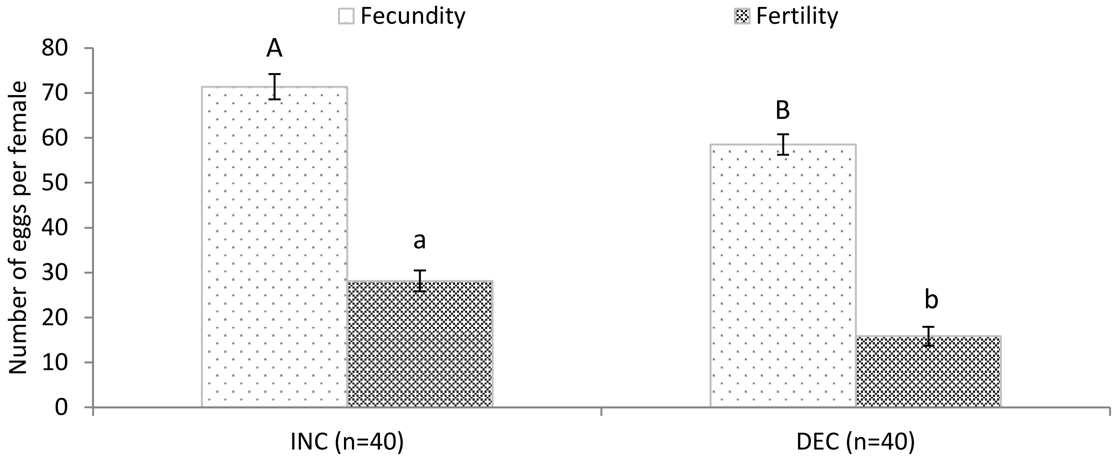

Figure 4. Average fecundity and fertility of *Pilophorus gallicus* for the whole period of observation (twelve days) under increasing (INC) and decreasing (DEC) photoperiod. n is the number of females assayed; capital different letters denote significant differences in fecundity; different low case letters denote significant differences in fertility (ANOVA *P* < 0,001).

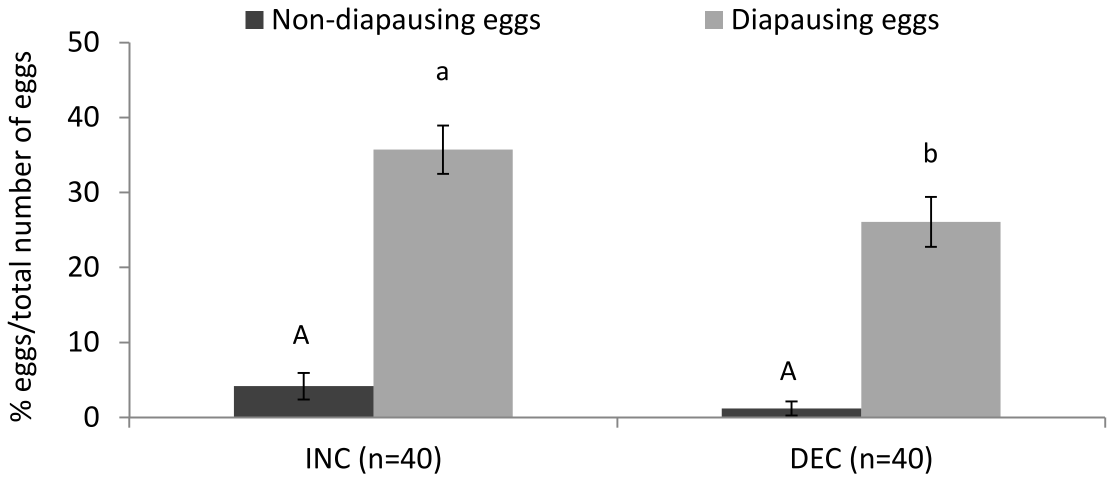

Figure 5. Percentage of diapausing and non-diapausing eggs laid per *Pilophorus gallicus* female during the whole period of observation (twelve days) under increasing (INC) or decreasing (DEC) photoperiod. n is the number of females assayed; capital different letters denote significant differences in non-diapausing laying; different low case letters denote significant differences in diapausing laying (ANOVA *P* < 0,01).

Photoperiodic trend did not have a significant effect on the proportion of the females with different reproductive response (Binomial test, $P > 0.05$). The egg-laying response was similar at increasing and decreasing photoperiods, as most of the females were typified as "diapause inducing". Among the forty females under decreasing conditions, only one of them was "mixed" and another one "non-diapause inducing"; under increasing conditions, only three females out of forty were "mixed" and one was "non-diapause inducing".

3.3. Reversibility of diapause induction in P. gallicus

This experiment demonstrated the sensitivity of *P. gallicus* females to the change of photoperiod conditions. Females taken from the field at short photoperiod and kept under similar photoperiod conditions in the laboratory (9L:15D) laid exclusively diapausing eggs (Figure 6). In contrast, when females collected from the field were placed under long photoperiod (16L:8D), both diapausing and non-diapausing eggs were laid (Figure 6). The laying was exclusively diapausing during the first week, but from the second week of long-day exposure onwards, there was a slight increase in the number of non-diapausing eggs per female and a decrease in the number of diapausing ones (Figure 6). The switching of photoperiod had a significant effect both on the number of diapausing ($F = 6.47$; $df = 1{,}53$; $P < 0.05$) and non-diapausing eggs ($F = 13.5$; $df = 1{,}53$; $P < 0.001$); females transferred from short to long photoperiods laid more non-diapausing eggs and less diapausing eggs than females kept at short photoperiods.

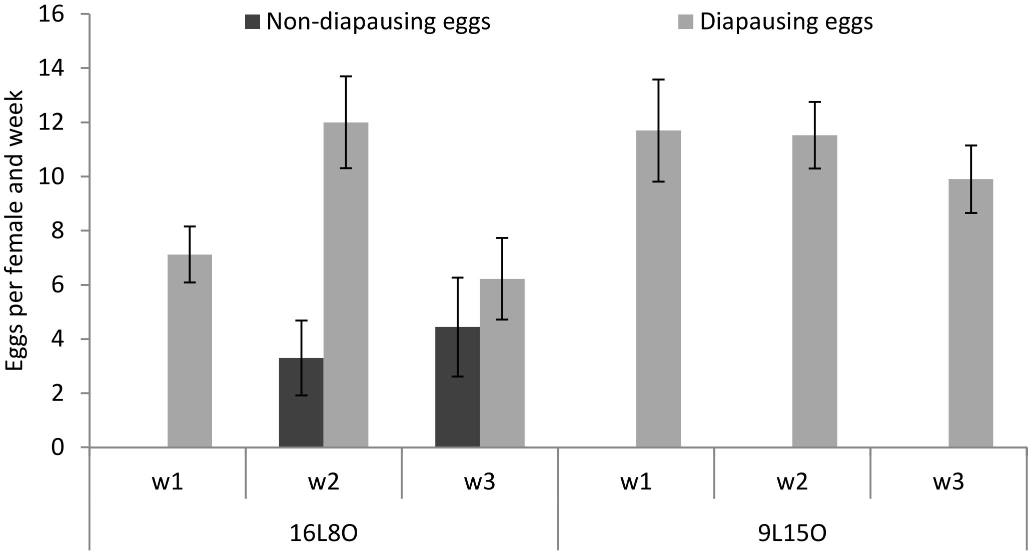

Figure 6. Number of the different kinds of eggs laid in successive weeks by females collected from the field under short daylength and kept under different photoperiods (short: 9L15D or long: 16L8D).

In the second week of long-day exposure, the 30% of females that laid non-diapausing eggs had a mixed strategy and also laid diapausing eggs, while in the third week 30% of the females had a mixed response and 10% of them laid only non-diapausing eggs (Figure 7). The proportion of non-diapausing eggs laid by females with a mixed or non-diapause inducing response increased from 34.9 ± 0.1% to 73.9 ± 0.01% between the second and third week, respectively (Figure 8).

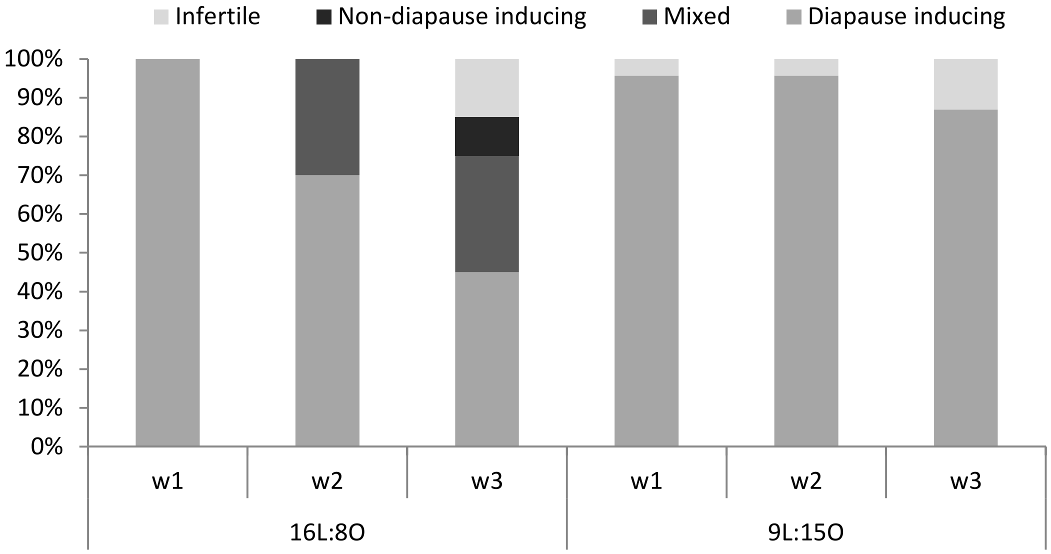

Figure 7. Variation in the proportion of the different kinds of female, according to their reproductive response, along the 3 week observation when switched from a short to a long photoperiod (16L:8D) or kept at short photoperiods (9L:15D).

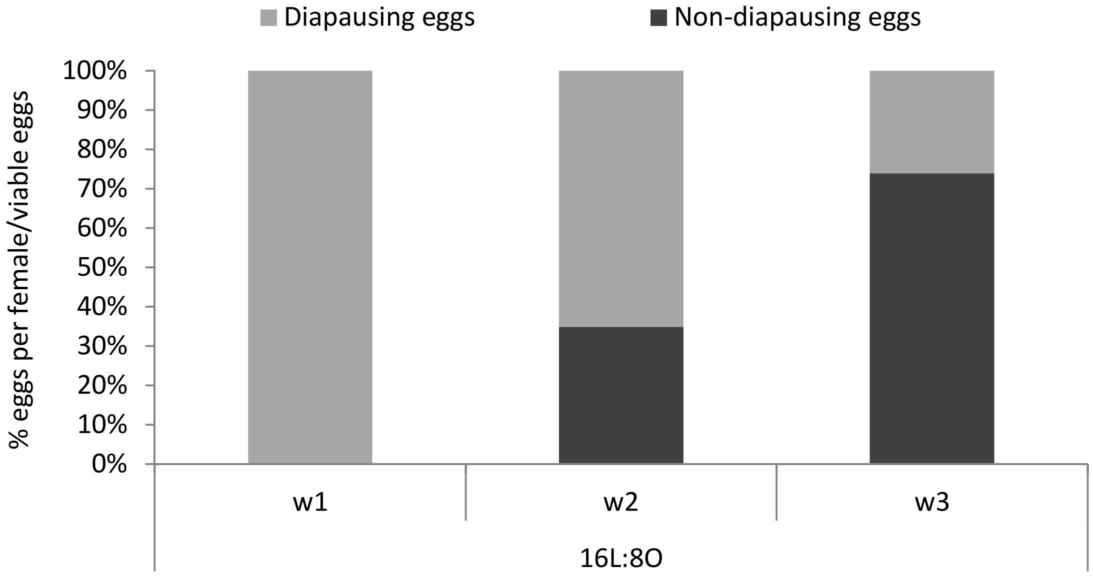

Figure 8. Variation in the proportion of eggs (diapausing or non-diapausing) laid by females that changed their reproductive response (from diapause-inducing to mixed and non-diapause inducing) along the weeks after the switch from short field photoperiod to long laboratory photoperiod (16L:8D).

4. DISCUSSION

In the present work, we showed that photoperiodic induction of diapause in *P. gallicus* takes place at daylengths below a critical value. The photoperiodic response curve and the critical daylength of *P. gallicus* - estimated at 13h53min - are consistent with the timing of diapause observed under field conditions (Ramírez-Soria *et al.*, 2018). For instance, from mid-April onwards (photoperiods above 14L:11D) there is a non-diapausing egg-laying response in females which originates the presence of active forms (nymphs and adults) in the second generation, whereas from mid-September onwards (photoperiods below 14L:11D) females produce a prevalent diapausing progeny which leads to the absence of active forms in winter (Supp. material, S1). The critical daylength of *P. gallicus* allows a suitable synchronization with the environmental conditions of its area of distribution.

Natural photoperiod reaches the critical daylength at the beginning of the autumn (week 33-34), avoiding the emergence of nymphs that, under the temperature regime around these dates, will not likely reach the adult stage and reproduce (Ramírez-Soria *et al*., 2017; Supp. material S2). Other multivoltine mirids from similar latitudes (i.e. similar photoperiodic regime) with embryonic diapause, such as *Trigonotylus caelestialium* Kirkaldy (Hemiptera: Miridae) and *Stenotus rubrovittatus* Matsumura (Hemiptera: Miridae), have similar critical daylengths, between 13L:11D and 14L:10D at 25°C (Higuchi and Takahashi, 2005; Shigehisa, 2008). In general, multivoltine hemipterans from middle latitudes have critical daylengths that range from 12h to 14h, depending on their area of distribution (Bahşi and Tunç, 2012; Gillespie and Quiring, 2005; Musolin *et al*., 2011; Saulich and Musolin, 2009). Species or populations from higher latitudes, where the growing season is shorter and average temperature lower, have longer critical daylength that enable them to enter diapause earlier, before the arrival of the cold period (Bahşi and Tunç, 2012; Gillespie and Quiring, 2005; Musolin *et al*., 2011; Saulich and Musolin, 2009). Some works reported the effect of temperature on the photoperiodic response, modifying the diapause response curve (Eizaguirre *et al*., 1994) and making the critical photoperiod longer under low temperatures (Beck, 1980; Shintani and Higuchi, 2008; Taylor and Karban, 1986). In previous studies, we proved that a decrease of 5°C (from 23 to 18°C) did not modify diapause incidence in *P. gallicus* at 9L:15D. However, we cannot generalize this statement and further trials should be done for other photoperiods in the range of critical daylength and at higher temperatures. Finally, the low fertility obtained for *P. gallicus* at 12L:12D and 13L:11D – diapause inducing photoperiods – makes think about the cost of diapause, resulting in a low survivorship of diapausing eggs, which has already been discussed by Ramírez-Soria *et al.* (2018).

Contrary to our working hypothesis, increasing the length of the photophase did not reduce the proportion of diapausing eggs; surprisingly, females that developed at increasing photophases laid a higher percentage than females at decreasing photoperiods; however, the fertility was higher than the one observed under constant photoperiods (12L:12D and 13L:10D in critical daylength trial). As suggested by Blake (1963), the lengthening days may have a positive influence on the rate of diapause completion, given the higher fertility obtained compared to constant and decreasing daylengths. Regarding the mechanisms of diapause induction, our results show that this mirid relies on the absolute length rather than on the photoperiod trend. This response is in agreement with the results reported in the critical daylength trial, which indicates that the diapause induction is dependent on daylengths below the critical value 13h53min. Thus, the direction of change of photoperiod is not considered as responsible for the different variation in the embryonic dormancy of *P. gallicus* observed in autumn and spring in the vicinity of the critical daylength. Similarly, Corbet (1956) studied larval diapause in the emperor dragonfly *Anax imperator* Leach (Odonata: Aeshnidae), which takes place in late summer but not in spring when nymphs are subjected to analogous daylengths but with opposite direction of change; laboratory experimentation did not allow to conclude that shortening daylengths induce diapause. The direction of change of photoperiod is rarely used as the primary cue for diapause incidence and in most cases absolute levels of daylength rather than changing ones are effective (Adkisson *et al*., 1963; Danks, 1987; Dingle, 1978; Eizaguirre *et al*., 1994; Kikukawa *et al*., 2008). However, some authors have reported an influence of increasing or decreasing photoperiods on diapause induction (Adkisson and Roach, 1971; Deveson and Woodman, 2014; Saunders, 2002; Tauber and Tauber, 1970). Beck (1980) and Danks (1987) mentioned several examples of species where the induction of diapause by decreasing daylength has been claimed, however they do not always considered this studies absolutely convincing. According to the photoperiodic counter theory, the incidence of diapause in *P. gallicus* might be proportional to the number of the short days experienced (Kikukawa *et al*., 2008; Saunders, 2002). Moreover, the response may not be to changing daylengths *per se*, but related to the sensitivity to the photoperiod across the development instars of the insect. In this sense, shortening daylengths may result in a high incidence of diapause if the latter stages of development are photosensitive, so that the daylengths below the critical level are the ones to which the insects respond (Beck, 1980).

Pilophorus gallicus females laying diapausing eggs started to lay non-diapausing eggs when they were transferred to long photoperiods. This photosensitivity of the adult stage may explain the different reproductive responses of *P. gallicus* females in spring and autumn. The critical daylength takes place in spring and autumn 1-2 weeks before the first and the latest adult population peak respectively (Supp. material, S1); thus, the first *P. gallicus* nymphs emerging in the field in spring may develop under photoperiods below the critical daylength (diapause inducing), but they are exposed to long photoperiods at the adult stage. In contrast, in fall, females may have developed at photoperiods above the critical daylength as juveniles but are exposed to short photoperiods when they start laying eggs (Supp. material, S1). Likewise, the pink bollworm *Pectinophora Gossypiella* Saunders (Lepidoptera: Gelechiidae) did not respond to changing daylengths but to absolute levels, and the effects of short-day exposure in the early instars could be cancelled by exposure of the subsequent instars to long days (Adkisson *et al*., 1963). Some other authors registered the reversibility in diapause induction when individuals ceased to be exposed to the triggering environmental factors (Bell and Adkisson, 1964; Eizaguirre *et al*., 1994; Higuchi and Takahashi, 2005; Hou *et al*., 2016; Numata and Hidaka, 1982; Saunders, 1965; Shintani, 2009; Tanaka, 1994; Uchidoi and Takeda, 1997). But in other species, diapause can be strongly determined on the first days of development (e.g. Indian meal moth *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae)) (Kikukawa *et al*., 2008). The photosensitivity of *P. gallicus* females allows them to revert the short daylength diapause inducing effect experienced during their nymphal development when exposed to long photoperiods. These results demonstrate that diapause induction pathway in *P. gallicus* females is not fixed at early developmental stages. In contrast, diapause is an irreversible condition in the *P. gallicus* egg, which can only be overcome after a minimum period of low temperatures (e.g. 90 days at 6°C) (Chapter 3 of this thesis dissertation).

As Bradford and Roff (1993) assesed, the study of life history traits variation at an individual level can provide valuable biological information of the species that could be missed at a population level approach. In the vicinity of the estimated critical daylength (13h53min), the composition of the female population changed substantially, from an almost homogeneous group of diapause-inducing females at 13L:11D to a heterogeneous group of diapause-inducing, non-diapause inducing and mixed females at 14L:10D (Figure 2). These results show that most of the population is photosensitive, however there is a proportion of females who does not respond to photoperiod. This variability in the expression of diapause might be due to genetic variation maintained by interannual variability selection in the optimal diapause strategy (Hairston Jr and Dillon, 1990). The population dynamics of the species will be subjected to the proportion of the different reproductive strategies in the population. On the one hand, females with diapause inducing and mixed responses under photoperiods above 14L:10D contribute to a diapausing laying which can be considered as an example of bet-hedging, understood as a genetically determined loss of individual fitness in the current generation which allows a long-term increased fitness (*sensu* Stearns, 1976). On the other hand, females with non-diapause inducing and mixed strategies under short photoperiods will lay non-diapausing eggs which have little chances to survive winter, but in the context of climate change, where autumn season is getting warmer and winter period shorter (Pachauri *et al.*, 2014), they can represent the chance to develop an extra generation (Groeters, 1994; Jaworski and Hilszczański, 2013). Life cycle flexibility of *P. gallicus* seems to have evolved to exploit a variable climate where it can face unpredictable environmental events.

The results of this work extend the knowledge about diapause expression in *P. gallicus*, and offer ultimately practical applications on the mass rearing of the species and its management in biological control programs. It has been demonstrated that *P. gallicus* has a complex reproductive strategy with embryonic diapause induced by short photoperiods and high variability in the response of females within populations. Thus, the mass rearing of this insect could be optimized by photoperiod management, and the possibility of the selection of different strains of females with different response to photoperiod. The critical daylength and the sensitivity of the adult stage are useful features to be considered for successful releases in spring or autumn (van den Meiracker, 1994). The logistic models obtained relating photoperiod to diapause incidence provide baselines to facilitate future studies of environmental and geographical influences on diapause in this species, and to investigate the evolutionary history of *P. gallicus*.

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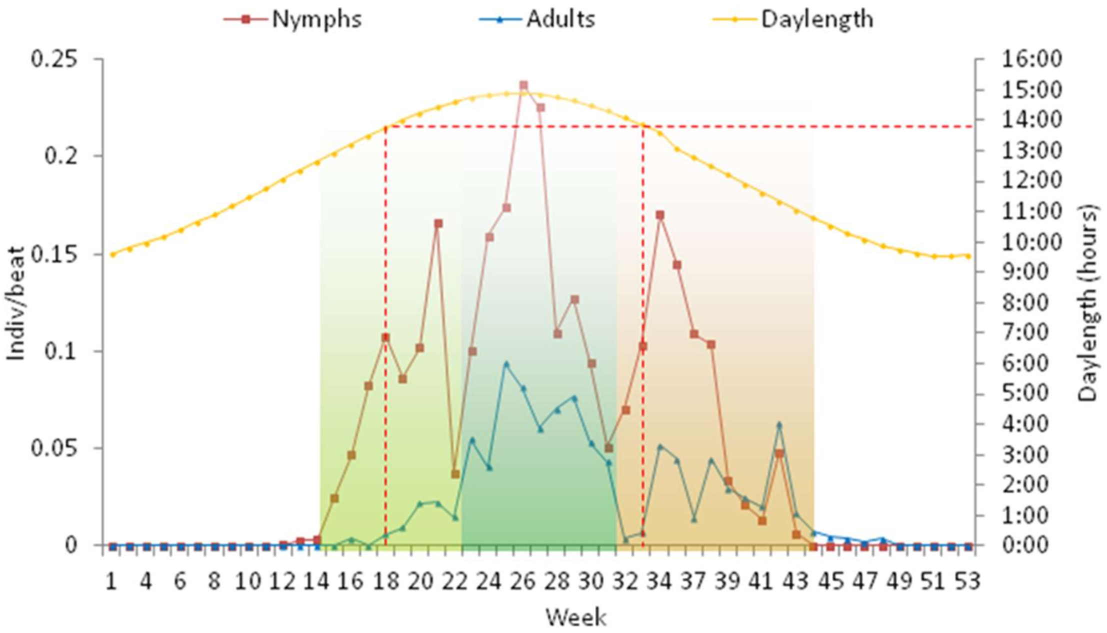
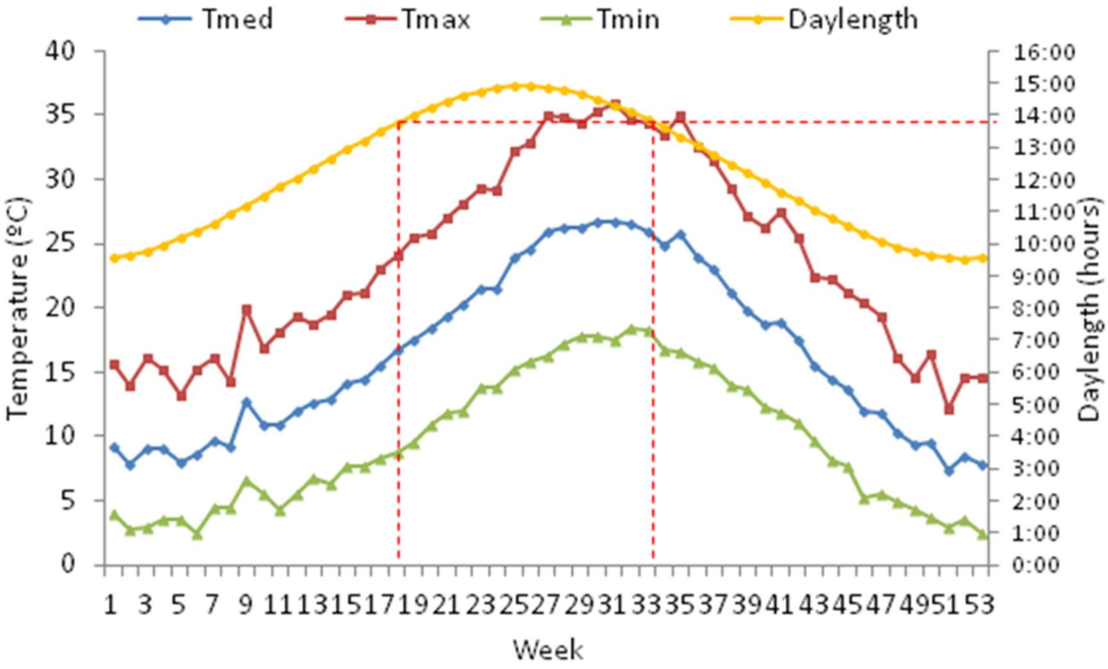
SUPPLEMENTARY MATERIAL



Supplementary material S1. Annual photoperiod and population dynamics (average number of individuals per beat from February 2008 until November 2010) of *Pilophorus gallicus* nymphs and adults in pear orchards from southern Europe (adapted from Ramírez-Soria *et al*., 2018). Dashed line shows dates where the critical day-length takes place.



Supplementary material S2. Annual photoperiod and weekly averages of the temperature registered at the Jumilla (Murcia, Spain) climatic station (JU71, Las Encebras; 38°23' 40.01"N, 1°14'21.58"W) from 2007 to 2011 (http://siam.imida.es/) (adapted from Ramírez-Soria *et al*., 2017). Dashed line shows the dates where critical day-length takes place.

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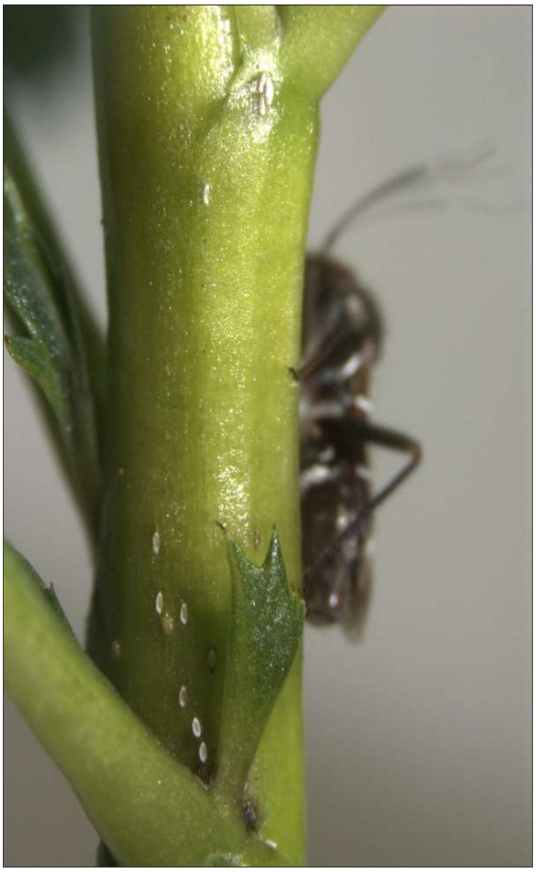
DISCUSIÓN GENERAL



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DISCUSIÓN GENERAL

El presente trabajo contribuye al conocimiento del ciclo biológico, la dinámica poblacional y la biología reproductiva de *Pilophorus gallicus* Remane (Hemiptera: Miridae). El mírido depredador *P. gallicus* tiene un ciclo anual multivoltino, estando presentes las formas activas en el cultivo del peral entre mediados de marzo y finales de octubre. El umbral inferior de desarrollo de *P. gallicus* se ha estimado en 12.01°C (N1 a Adulto) y la temperatura óptima en 25°C. La última generación anual, cuyos adultos muestran un pico poblacional a la entrada del otoño, hace una puesta eminentemente diapausante, permitiendo a la especie sobrevivir a las bajas temperaturas invernales. La diapausa en *P. gallicus* es de tipo facultativo, siendo inducida por fotoperiodos cortos y necesitando un determinado periodo de bajas temperaturas (e.g. 90 días a 6°C) para terminarla; una vez completada, los huevos permanecen en un estado de quiescencia a la espera de temperaturas superiores al umbral mínimo y el cumplimiento de los requisitos térmicos para emerger. El fotoperiodo crítico, que induce la diapausa en un 50% de la población, ha sido estimado en 13 horas y 53 minutos. Se ha demostrado la insensibilidad de esta especie frente a la dirección del fotoperiodo (creciente o decreciente). Asimismo, se ha demostrado la reversibilidad de la capacidad de las hembras de *P. gallicus* de inducir la diapausa en huevos: hembras inductoras de la diapausa ovipositaron huevos no diapausantes cuanto fueron transferidas de fotoperiodos cortos a largos. Este trabajo ha puesto de manifiesto la existencia de diferentes respuestas reproductivas en hembras de *P. gallicus* (inductoras y no-inductoras de la diapausa, y mixtas). La capacidad de las hembras mixtas e inductoras de la diapausa de poner huevos diapausantes en condiciones no inductoras (fotoperidos largos) puede ser considerada como una estrategia “bet-hedging” en la que *P. gallicus* “no pone todos los huevos en la misma canasta” para aumentar la probabilidad de persistencia de la población en caso de advenimiento de condiciones desfavorables atípicas. La incidencia de la diapausa en las poblaciones de *P. gallicus* viene determinada por la proporción de hembras con diferente respuesta al fotoperido.

El hecho de que *P. gallicus* emplee la diapausa como mecanismo de supervivencia frente a las condiciones adversas invernales implica una cierta predictibilidad de su emergencia o aparición en el cultivo. El interés de este trabajo reside en el conocimiento de la influencia de la temperatura y el fotoperiodo sobre la biología de *P. gallicus*, que puede ser empleado para predecir la emergencia de las ninfas en campo y su dinámica poblacional. En este trabajo se ha demostrado que el fotoperiodo es el principal factor inductor de la diapausa, no habiéndose encontrado una influencia de la temperatura en el rango estudiado (18-23°C, 9L:15O). El fotoperiodo es un "reloj" inequívoco, por ello la dependencia de este tipo de señal para la inducción de la diapausa incrementa la probabilidad de supervivencia. El fotoperiodo corto desencadena la puesta diapausante en hembras de *P. gallicus* antes de la llegada de las condiciones adversas, aun cuando las temperaturas se mantengan cálidas (23°C), y evita una reproducción tardía que podría en riesgo la supervivencia de la población en invierno. Asimismo, la necesidad de los huevos diapausantes de un determinado periodo de frío para completar el proceso impide su emergencia antes de la estación invernal. Sin embargo, la dependencia de las bajas temperaturas para terminar la diapausa podría aumentar la vulnerabilidad de la especie frente al cambio climático. En el contexto actual, el calentamiento global es inequívoco, siendo las tres últimas décadas el periodo más cálido de los últimos 1400 años en el hemisferio norte (Pachauri *et al*., 2014). Las fluctuaciones de las temperaturas invernales (e.g. incremento de los valores medios, advenimiento de eventos más extremos) podrían tener consecuencias negativas sobre una población diapausante de *P. gallicus*. Un invierno cálido puede suponer el incumplimiento de los requerimientos de frío, lo que daría lugar a una primera generación de *P. gallicus* mermada. En dichas circunstancias, teniendo en cuenta que se trata de un depredador generalista que puede desempeñar un papel importante en el control de fitófagos en el peral (e.g. pulgón, psila), sería conveniente el monitoreo de sus poblaciones primaverales para reforzarlas con sueltas en caso necesario. En contraposición, el incremento de las temperaturas invernales permitiría el desarrollo de huevos no diapausantes y la selección de hembras no inductoras de la diapausa o con una oviposición mixta. Así, la variabilidad en la respuesta fotoperiódica encontrada en las poblaciones de *P.*

gallicus aumenta la probabilidad de supervicencia de la especie frente a la variación en las condiciones ambientales.

Numerosos estudios han demostrado la importancia de la consideración de la diapausa en el control integrado de plagas, y cada vez se otorga más interés a la biología estacional de los insectos. Por definición, la diapausa es un mecanismo de sincronización entre los estadíos activos de la especie y las condiciones favorables para su desarrollo y reproducción. Para un control eficiente de plagas, es fundamental la sincronización de los estadíos activos de la plaga y del depredador. Fisher *et al.* (1999) reportaron la ineficacia de los ácaros fitoseidos *Amblyseius cucumeris* Oudemans y *Amblyseius barkeri* Hughes (Acarina: Phytoseiidae) frente al control de *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) debido a la diapausa invernal de los depredadores y la ausencia de esta en las poblaciones plaga, que se incrementaban libremente durante el invierno. Por otro lado, Huffaker (1971) puso de manifiesto el fracaso del control de *Pieris rapae* L. (Lepidoptera: Pieridae) por parte del parasitoide *Cotesia* (*Apanteles) glomeratus* L. (Hymenoptera: Braconidae) a causa de la terminación de la diapausa más temprana de este último, y por tanto la falta de disponibilidad de huéspedes para continuar reproduciéndose. En el presente estudio, la dinámica poblacional de *P. gallicus* muestra como las primeras ninfas emergen de los huevos diapausantes a mediados de marzo, y los adultos de la primera generación aparecen a finales de abril-mayo (Ramírez-Soria *et al.*, 2018). Una de sus principales presas, *Cacopsylla pyri* L. (Hemiptera: Psyllidae), termina su diapausa reproductiva a finales de enero, pero permanece en un estado de quiescencia térmica a la espera de temperaturas favorables para la puesta; a mediados de marzo empiezan a emerger las primeras ninfas y en abril-mayo la primera generación de adultos no-hibernantes (Lyoussoufi *et al.*, 1994; Nin *et al.*, 2012; Rieux y Faivre d'Arcier, 1990). Asimismo, Sanchez y Ortín-Angulo (2012) pusieron en evidencia el paralelismo de las dinámicas poblacionales de *P. gallicus* y *C. pyri* en los campos de peral en Jumilla. El hecho de que *P. gallicus* entre en diapausa en el cultivo del peral implica la emergencia de los huevos diapausantes *in situ*, fundamental para la sincronización con las poblaciones incipientes de psila y el control temprano de éstas, limitando su crecimiento y por tanto el daño al cultivo.

Esto resulta una ventaja comparativa con respecto a los Antocóridos (e.g. *Anthocoris Nemoralis* F.), cuya migración implica un retraso en la llegada al cultivo y consecuentemente un escaso control de las poblaciones primaverales de psila (Artigues *et al*., 1996; Erler, 2004; Scutareanu *et al*., 1999). La hibernación en el cultivo es un aspecto a considerar en las estrategias de control biológico por conservación. En este sentido, habría que plantear el manejo del cultivo evitando prácticas que pudiesen mermar las poblaciones hibernantes de *P. gallicus*, como por ejemplo tratamientos fitosanitarios agresivos y podas. Aunque los estadíos diapausantes pueden ser más resistentes frente a insecticidas (Buès *et al*., 1999; Denlinger, 2008; Van de Baan and Croft, 1991), es conveniente el uso de productos respetuosos con la fauna auxiliar; el aceite parafínico ha sido empleado con éxito contra *C. pyri* sin afectar significativamente las poblaciones de *P. gallicus* (observación personal; Sanchez and Ortín-Angulo, 2012b). Por otro lado, la localización de los huevos diapausantes en ramas y troncos del peral cuestiona la idoneidad de las podas intensas en invierno, sugiriendo su sustitución por podas ligeras a lo largo del año. Denlinger (2008) reportó como la poda invernal de los manzanos en República Checa dificultaba el establecimiento de los ácaros fitoseidos en el cultivo, ya que sus poblaciones diapausantes se localizaban precisamente en las partes eliminadas.

Por último, el conocimiento de los factores que controlan la diapausa puede representar una herramienta muy útil para optimizar la producción de insectos, lo cual sería interesante para las biofábricas productoras de enemigos naturales. Así como la diapausa obligatoria suele suponer un obstáculo para la cría masiva (e.g. *Ragholetis pomonella* Walsh, *Ragholetis* cerasi L., *Bactrocera minax* Enderlein, *Thaumetopoea pityocampa* Schif), la diapausa facultativa puede convertirse en una oportunidad de mejora, ya que se puede manipular – inducir, terminar - en función de la necesidad (FAO/IAEA, 2016; Ruberson *et al*., 1998). Dada la variabilidad anual en la demanda de insectos por parte de los agricultores, no es rentable una producción continua a lo largo del año. El control de los factores de inducción y terminación de la diapausa permiten someter la cría al estado de latencia en periodos de baja demanda, y reactivarla y mantenerla en continua reproducción en momentos de alta demanda. Esto supondría un ahorro considerable de esfuerzo y costes en el mantenimiento de las colonias, e incluso podría ayudar a conservar el potencial reproductivo de la especie. Por ejemplo, Chang *et al*. (1996) observó en *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) que la intervención de la diapausa evitaba la degeneración asociada a la cría continuada (e.g. disminución de la tasa de reproducción, aumento del tiempo de desarrollo). Por otro lado, la existencia de variabilidad de estrategias reproductivas en las poblaciones de *P. gallicus* permitiría plantear la selección y cría de determinadas razas. Finalmente, más allá de la producción de insectos, la logística para su comercialización puede conllevar ciertas situaciones de estrés para los organismos (e.g. hipoxia, temperaturas desfavorables) tanto durante su almacenamiento como su transporte y su liberación en campo. El hecho de que el estado diapausante presente mayor resistencia frente al estrés ambiental (Kostal *et al*., 2001) puede ser potencialmente interesante para incorporarlo en estas actividades, con el objetivo final de optimizar la performance del enemigo natural una vez liberado en campo.

En resumen, los resultados conjuntos de esta tesis doctoral aportan unos conocimientos de base sobre la biología estacional de *P. gallicus* y herramientas para el manejo de la especie en programas de control biólogico de plagas. La diapausa es una estrategia de adaptación que permite a *P. gallicus* la supervivencia a periodos desfavorables y la sincronización con los recursos necesarios para su desarrollo y reproducción, pero también puede ser útil a nivel industrial. Asimismo surgen nuevas preguntas especialmente en cuanto a la selección genética de razas, la evolución de la diapausa en el contexto de cambio climático, o la viabilidad de la incorporación de este mecanismo en la producción industrial. Este trabajo pone de manifiesto la importancia de los estudios biológicos de las especies que forman parte de los agroecosistemas de cara a desarrollar modelos de producción sostenible.

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CONCLUSIONES – CONCLUSIONS

Foto: Elena López Gallego

CONCLUSIONES

Atendiendo a los estudios presentados en esta memoria de tesis doctoral, cuya finalidad ha sido aportar información sobre la biología estacional y la estrategia reproductiva del mírido depredador *Pilophorus gallicus* Remane (Hemiptera: Miridae), se extraen las conclusiones de conjunto que se exponen a continuación:

1. *Pilophorus gallicus* es una especie multivoltina que presenta aproximadamente tres generaciones anuales activas entre mediados de marzo y finales de octubre en el sureste de la Península Ibérica.
2. El estudio del efecto térmico sobre los parámetros biológicos de *P. gallicus* pone de manifiesto que esta especie reduce altamente la tasa de desarrollo, la supervivencia y fertilidad a bajas temperaturas (15°C) y, en menor medida, la supervivenia y fertilidad a altas temperaturas (30°C). El umbral mínimo y la constante térmica obtenidos para el periodo ninfal (N1 a Adulto) son respectivamente 12.01°C y 198.94 grados día; la temperatura óptima se estima en 25°C.
3. Para hacer frente a las condiciones adversas del invierno, *P. gallicus* ha desarrollado una diapausa embrionaria facultativa, que le permite entrar en reposo a la llegada del otoño. Se rechaza la existencia de una diapausa estival como mecanismo de protección frente a las extremas temperaturas que tienen lugar en el área de estudio durante el verano.
4. El fotoperiodo corto induce la puesta diapausante, habiéndose estimado el fotoperiodo crítico a 25°C en 13 horas y 53 minutos. No se ha encontrado un efecto de las temperaturas (18 y 23°C) sobre la estimulación de la diapausa ni una interacción de la temperatura con el fotoperiodo.
5. Hembras de *P. gallicus* que producen huevos diapausantes pueden revertir dicha condición cuando se transfieren a fotoperiodos largos. En contraste, la diapausa es una condición irreversible en el huevo, que únicamente puede superar tras un periodo mínimo de bajas temperaturas (e.g. 90 días a 6°C).

6. Se ha demostrado que la inducción de la diapausa en *P. gallicus* es insensible al crecimiento o decrecimiento del fotoperiodo.
7. En la población estudiada se han encontrado hembras de *P. gallicus* con diferentes estrategias reproductivas (inductoras y no inductoras de la diapausa de huevos, y mixtas). La existencia de hembras mixtas y hembras inductoras insensibles a los cambios del fotoperiodo sugiere la existencia de un mecanismo de "bet-hedging" o "no poner todos los huevos en la misma canasta", que incrementaría la probabilidad de persistencia de las poblaciones frente al advenimiento de fluctuaciones ambientales átipicas.
8. La expresión de la diapausa en las poblaciones de *P. gallicus* está por tanto condicionada no solo por el fotoperiodo sino también por la proporción de hembras con diferentes estrategias reproductivas.
9. El hecho de que el depredador *P. gallicus* entre en diapausa en el mismo cultivo del peral permite a la especie el establecimiento de poblaciones residentes, siendo un aspecto a considerar en las estrategias de control biológico por conservación. Asimismo, permite una mejor sincronización con las poblaciones primaverales de fitófagos (e.g. *Cacopsylla pyri* L. (Hemiptera: Psyllidae)) y por tanto un control temprano de estas.
10. El estudio de la diapausa representa una herramienta interesante para optimizar los programas de cría masiva y otros procesos asociados en la industria del control biológico.
11. Los resultados obtenidos en esta tesis doctoral constituyen unas bases científicas para la mayor comprensión de la biología y ecología de este insecto y su manejo en sistemas de control biológico de plagas.

CONCLUSIONS

On the basis of the studies presented in this PhD thesis, with the goal of providing information on the seasonal biology and reproductive strategy of the predatory mirid *Pilophorus gallicus* Remane (Hemiptera: Miridae), the following general conclusions can be drawn:

1. *Pilophorus gallicus* is a multivoltine species with approximately three annual generations; it is present in the field between mid-March and the end of October in the southeast of the Iberian Peninsula.
2. The study of thermal effects on the biological parameters of *P. gallicus* shows that this species highly reduces survival, development rates and fertility at low temperatures (15°C) and, to a lower extent, survival rates and fertility at high temperatures (30°C). The lower thermal threshold and the thermal constant for the nymphal period (N1 to Adult) are 12.01°C and 198.94 degree-days, respectively; 25°C was considered as the optimum temperature.
3. To cope with the adverse winter conditions, *P. gallicus* has developed a facultative embryonic diapause, which allows it to enter dormancy at the arrival of autumn. The existence of a summer diapause as a mechanism of protection against the extreme high temperatures that take place in the study area during summer is rejected.
4. Short photoperiod induces the diapausing laying; the critical photoperiod at 25°C has been estimated at 13 hours and 53 minutes. Neither the temperature (18 and 23°C) nor the interaction of the temperature with the photoperiod had a significant effect on the diapause induction.
5. *Pilophorus gallicus* females producing diapausing eggs can reverse this condition when they are transferred to long photoperiods. In contrast, diapause is an irreversible condition in the egg, which can only be overcome after a minimum period of low temperatures (e.g. 90 days at 6°C).

6. It has been demonstrated that the direction of change of daylength (increasing or decreasing) does not have an effect on the induction of diapause in *P. gallicus*.
7. Different reproductive strategies (diapause and non-diapause inducing, and mixed) have been found among the females from the studied population. The existence of mixed females and diapause inducing females insensitive to changes in the photoperiod, suggests the existence of a "bet-hedging" or "not putting all the eggs in the same basket" strategy that would increase the probability of the persistence of populations in case of atypical environmental fluctuations.
8. The expression of diapause in populations of *P. gallicus* is therefore subjected not only to photoperiod but also to the proportion of females with different reproductive strategies.
9. The fact that the predator *P. gallicus* enters diapause in the pear crop allows the species to establish resident populations, being an aspect to consider in the strategies of biological control for conservation. It also allows a better synchronization with the spring populations of phytophagous species (e.g. *Cacopsylla pyri* L. (Hemiptera: Psyllidae)) and therefore an early control of them.
10. The managementof diapause represents an interesting tool to optimize mass rearing programs and other processes related to the biological control industry.
11. The results of this PhD thesis contribute to the understanding of the biology and ecology of *P. gallicus* and its management in biological control programs.

ANEXO DE FOTOS – ANNEX OF PHOTOS

ANNEX OF PHOTOS

Photo 1. *P. gallicus* colony employed for assays.

Photo 2. Translucent cup employed for rearing nymphs individually.

Photo 3. Plastic cylinder emplyed for isolating couples and study their laying.

Photo 4. Funnel and container used for beating sampling.