FACTORES ECOLÓGICOS Y MECANISMOS IMPLICADOS EN LA VARIABILIDAD DE LA INTERACCIÓN ENTRE UN ECTOPARÁSITO GENERALISTA (*Carnus hemapterus*) Y SUS HOSPEDADORES

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Miguel Ángel Calero Torralbo

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Miguel Ángel Calero Torralbo
Estación Experimental de Zonas Áridas
Consejo Superior de Investigaciones Científicas
Carretera de Sacramento, s/n, Cañada de San Urbano
E-04120, Almería, Spain
calero@eeza.csic.es, ma.calero@gmail.com

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http://www.lamazeta.es , http://www.estudiolamazeta.blogspot.com

Estación Experimental
de Zonas Áridas

Departamento de Biología Animal
UNIVERSIDAD DE GRANADA

Factores ecológicos y mecanismos implicados en la variabilidad de la interacción entre un ectoparásito generalista (*Carnus hemapterus*) y sus hospedadores.

Memoria presentada por Miguel Angel Calero Torralbo para optar al Grado de Doctor en Ciencias Biológicas por la Universidad de Granada

Esta tesis ha sido dirigida por el Dr. Francisco Valera Hernández, científico titular y la Dra. Eulalia Moreno Mañas, profesora de investigación de la Estación Experimental de Zonas Áridas (CSIC)

En Almería, a 17 de Mayo de 2011

VºBº del director	VºBº del director
Fdo: Francisco Valera Hernández	Fdo: Eulalia Moreno Mañas

El Doctorando

Fdo. Miguel Ángel Calero Torralbo

A mis padres, a mi hermana.

A todos aquellos que luchan, que aman, que se empeñan en perseguir un sueño.

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"Es tan grande el placer que se experimenta al encontrar un hombre agradecido que vale la pena arriesgarse a no ser un ingrato" – Lucio Anneo Séneca

Con el paso de los años he ido aprendiendo lo importante y fácil que es ser agradecido. No hay una sola persona en este mundo que no necesite alguna vez el reconocimiento de sus seres queridos, de sus compañeros de trabajo, o de cualquier persona que te rodee y cuya trayectoria vital haya sido beneficiada por tu interés y dedicación en el desempeño de la labor diaria. El resultado final de esta tesis ha sido sustentada por el esfuerzo personal del que escribe, pero sin la ayuda profesional, anímica y emocional de decenas de personas que he tenido la suerte de conocer y disfrutar a lo largo de estos años, posiblemente estarías leyendo un trabajo muy distinto, puede que con otro nombre reflejado en la portada, o simplemente te encontrarías enfrente de una hoja en blanco con un contenido por escribir. Es por esto que pido paciencia y comprensión al que lea estas líneas, si considera que su contenido es aburrido, monstruosamente grandilocuente o excesivamente lacrimógeno: están escritas con todo el cariño y orgullo del que se sabe atado irremisiblemente al lazo colectivo que le une con su entorno y del que no quiere o no sabe desprenderse. Sirva este pequeño texto como un sencillo homenaje entresacado de los posos de mi memoria, hacia todas aquellas personas que hicieron posible que este barco llegara a buen puerto.

Siguiendo el libro de ciencia ficción más leído en occidente de todos los tiempos, me gustaría empezar por el Génesis, el germen creador de todo este tinglado: Jamás olvidaré la primera vez que nos encontramos Paco; una clara y fría anticiclónica mañana de invierno cordobesa. Tuviste la amabilidad de desplazarte de tu ruta original para que pudiéramos vernos las caras y empezar a discutir sobre las ideas que te bullían en la cabeza. Me sorprendió tu sencillez y cercanía; han pasado casi ocho años desde que nos conocimos, y cada vez que toco al despacho de tu puerta, todavía me recibes con la misma calidez y energía de aquella vez, el mismo porte, la misma sonrisa, la misma pasión por tu trabajo. Paco Valera, mi director de tesis, gracias por enseñarme tanto, por infectarme con tu pasión por los parásitos y la naturaleza en general, por descubrirme el placer del proceso cotidiano de la investigación (aunque a veces nos deparara desagradables sorpresas), por tus conversaciones en el campo, tus chistes, tus reflexiones en voz alta, por el viento fresco de tu campechanía jiennense. Tu visión de la ciencia y del mundo en general ha sido un gran aporte para mi formación profesional y vital y jamás olvidaré los años pasados a tu lado. Gracias por tu santa paciencia al ver como la tesis se iba alargando irremisiblemente, por creer en mí, por animarme y aguantar mis cabezonerías, enfados, torpezas y demás daños colaterales. Cada vez tengo más claro que en estos tiempos difíciles necesitamos expertos en tecnología, en innovación del conocimiento, personas que con su habilidad ayuden al

desarrollo de esta sociedad, pero aún más importante es disponer de “buena gente” como tú, individuos íntegros, lógicos, con sentido común, que ayuden a detener el auge de la vulgaridad y del culto al atocinamiento intelectual que nos ha tocado vivir.

De Centroeuropa, con semblante tímido y mirada escrutadora, llegó Radovan Václav para transmitirme su amor por las carracas. Esta tesis hubiera sido irrealizable sin su colaboración y ayuda. Durante tres increíbles temporadas de campo compartimos trabajo y asombro en cada paso hollado en el sobrio e imponente paisaje del desierto de Tabernas. Gracias por aguantar estoicamente mis payasadas, canciones y demás repertorio de perogrulladas (aunque conseguí sacarte una sonrisa en más de una ocasión ¿eh?). Tus conocimientos en estadística y tu pulcritud metodológica fueron esenciales para el resultado final de esta tesis; muchas gracias por acogerme en Bratislava durante dos fríos inviernos, por compartir tu pasión, dolor y rabia por las atrocidades sobre el medio ambiente que cada día se cometen en cualquier esquina del mundo, por enseñarme el Danubio y donde se bebe la mejor cerveza de toda Europa Central, Ďakuje Ti veľmi za všetko chlape!

Teresa, nuestra multidisciplinar enfermera-anilladora, también me contagió su amor por la naturaleza y la provincia de Almería durante largas jornadas en el campo. Gracias por tu inestimable ayuda, tu excelencia moral y profesional, tus bromas y socarronerías. Lali, muchas gracias por apoyar y hacer posible esta tesis, por tus ánimos y por las ganas que le echas a la vida, que impregnan casi cualquier cosa que tocas. El “chumbo” te debe mucho y espero que algún día sepan agradecértelo como te mereces.

La veteranía es un grado, y en mi caso me ha servido para conocer y encandilarme de muchísima gente que han pasado o permanecen en nuestro centro y que consiguen que éste funcione como un reloj suizo. Sebas y Ramón consiguieron dejarme alucinado con su eficiencia y dominio de un lenguaje completamente críptico para mi, logrando adicionalmente que mi computadora no cayera en acto de combate en más de una ocasión: desde curaciones a distancia cuasi-milagrosas cuando estaba de estancia en Italia, pasando por el uso de hielo artificial para poder salvar los datos de un disco duro que se recalentaba, o el dominio de técnicas de resurrección de sistemas operativos al borde de la obsolescencia tecnológica. Gracias por estar siempre ahí, cuando he necesitado de vuestra ayuda y consejo. Isabel logró que entendiera de una vez por todas la ingente labor que los bibliotecarios del mundo realizan para el resto de mortales inmersos en la estocasticidad del bombardeo de citas y referencias bibliográficas a la que estamos sometidos diariamente. Gracias por conseguir cualquier artículo que te pedía, aunque estuviera en la biblioteca militar de Vladivostok y fueran del año en que Franco andaba a gatas. Su labor diaria en administración consigue hacernos la vida un poco más fácil a todos los atolondrados que creemos vivir al margen de la burocracia y el papeleo. Gracias a Juan Leiva, Olga, Manolo Arrufat,

Mari Carmen Cazorla, Mercedes, por aguantarnos y comprender nuestro ajado romanticismo. Una mención especial a Andrés Castro, porque el Betis lo merece, por ser una gran persona y por reir con nosotros aunque a veces no le apeteciera mucho…¡ánimo con todo! El día que la mujeres consigan ser partenogenéticas se nos acabo el chollo a los hombres. Parte de la estabilidad y armonía de este centro esta estructurado alrededor del cariño, el amor y el sentido común de mujeres increíbles, talladas a fuego por las heridas irremisibles del tiempo, pero tiernas y sensibles como solo una madre sabe serlo: Velefique debería hacerte hija predilecta Marcela, gracias por todos estos años, por estar siempre a mi lado preguntando y preocupándote por mí, por compartir las noticias de la radio, por demostrarme que la bondad y la sencillez son armas poderosas para combatir la ponzoña de este mundo. Gracias a Luisa, Paquita, Mª Angeles, por hacerme sentir la calidez de su cercanía, por cuidar de todos nosotros. El vasco de Bricomanía es un aficionado al lado de nuestro Javi Toledo, lo mismo te plancha un huevo, que te fríe una camisa…gracias por compartir tus cualidades humanas y profesionales, vaya fichaje que ha hecho el chumbo contigo. Los tumbos y destrozos que he ido provocando por los diversos laboratorios de este centro han sido debidamente amortiguados por gente como Jose María Gaset, Ester Campanario o Alejandro. Gracias a todos por permitirme meter las zarpas en vuestros dominios.

Siguiendo un estricto orden castrense, me gustaría destacar el apoyo y colaboración de algunos investigadores de este centro. Jordi Moya Laraño (¡cada vez que veo tu apellido, me cuesta mas trabajo creer en las casualidades!) ha compartido sus vastos conocimientos en estadística y ecología conmigo. Gracias por tus ánimos y por hacerme ver la diversidad e interés del estudio de los invertebrados. Miguel A. Rodriguez-Gironés, confió en mis incipientes conocimientos en ecología molecular para ofrecerme un puesto de trabajo cuando la beca predoctoral se agotó, gracias por creer en mi y permitirme viajar a lo largo de media Europa. No puedo olvidar a gente como Juan Soler, Jesús Avilés, Jesús Benzal, Desi Parejo, Andrés Barbosa, Javier Cuervo, David Gálvez o Roberto Lázaro que continuamente se han ido interesando por mis avances, animándome y ayudando con sus comentarios e ideas para que mis neuronas se reactivaran de vez en cuando.

Sección becarios “chumberos” y agregados: Aunque este centro es pequeño y somos como una gran familia, debido a mi empeño denodado de no abandonar los pasillos de la EEZA bajo ninguna circunstancia, he tenido la gran fortuna de conocer a cantidad de gente que como yo, cometió la maravillosa locura de iniciar una tesis y lo que es todavía más asombroso: muchos consiguieron terminarla sin el menoscabo de su salud física y mental. Mi nacimiento como becario y mi llegada a Almería allá por el año 2005 fue recibido con el beneplácito y la comprensión de los veteranos que ya andaban por aquí. Leyre y Ernesto, con su simpatía consiguieron contagiarme las ganas de quedarme a vivir en Almería, a pesar de mis irracionales reticencias iniciales. Muchas

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En la parrilla de salida, decenas de personas comenzaron su vía-crucis particular en la investigación en la misma época que yo. Juntos hemos reído, algunas veces llorado, nos hemos emborrachado, hemos soñado en voz alta y nos hemos emocionado al sentir como, lentamente pero con paso firme, lográbamos superar los obstáculos que iban apareciendo a lo largo del camino. Gracias a todos por compartir esperanzas, ánimos, sufrimientos. Iván (nobleza y porte venidos del norte, el más llorón de todos y el que primero terminó), Lupe (mi medio paisana extremeña, te deseo lo mejor), Lauri, Pepa, Rubén, Sebas…Anita, ¡que orgulloso me siento de ti! Se me va a hacer muy difícil mirar por las mañanas al firmamento y no verte sentada ahí enfrente, todo afanosa, con esa luz de genio despistada que irradias. Muchas gracias por todos estos años, por compartir despacho, gozos y sombras. Juamma, un tío hecho y derecho, un modelo a seguir por muchos (y muchas, ya te gustaría ¿eh?), gracias por los ratos pasados a tu lado, consejos y demás conversaciones tanto transcendentales como banales. Con el paso de los años, nuevos becarios, contratados y postdoctorales fueron instalándose en la órbita chumbera. Laura, gracias por todos los momentos que hemos compartido, noches en bares, en la Almedina, musicales; transmítele mis agradacimientos a Mora, tú que entiendes tan bien su lenguaje perruno. Eva de Mas ha sabido hacerme reír más de una vez y ha compartido quebraderos de cabeza y cotilleos en el laboratorio de biología molecular, gracias por todos los momentos de alivio entre gel y gel. Fran, con su alegría y espontaneidad, consiguió que mi cabeza se liberará de vez en cuando, gracias por estos años de convivencia despachil; Ori, tu humor y frikadas varias también fueron un bálsamo para mi en muchos momentos, suerte con tus modelos matemáticos, ¡espero que no se te pegue nada malo! Maite, la continuadora de este trabajo, como Platero, pura resistencia por fuera, pero suave, como de algodón, por dentro, mucho ánimo. Juan, miarma, esas fiestas, conversaciones, esos sueños carraqueros en voz alta no se olvidan facilmente, mucha suerte en tu nueva andadura sevillana. Gracias también a Sonia, Meire, Christian, Sara, Nuria, Olga, Bea, Estefanía…, por compartir tapas y cervezas, charlas en el comedor, por preguntarme y animarme en estos últimos meses de locura, ¡gracias a todos! Mención especial para Luisa y Gustavo, pura simpatía y calidad humana.

Durante dos estancias transcurridas en el norte de Italia, llegué a la conclusión de que en el resto de Europa existe una estación llamada otoño, y disfruté de colores inéditos y paisajes de ensueño en Padova y el resto del Véneto, al mismo tiempo que comenzaba mi formación de bata, que llegaría a aportarme tanto años después. La gente del laboratorio de Lorenzo Zane y Andrea Pilastro, consiguieron que me sintiera uno más durante ese tiempo. Matteo, Clelia, Erica, Chiara, Chiara Bosquetto, Ale, Ilaria…Grazie mille a tutti. Lorenzo y Federica, compartieron paciencia y conocimientos conmigo, ¡esta tesis os debe mucho! El otoño del 2007 no hubiera sido lo mismo sin los workshops gastrónomicos internacionales, los spritzs y las reuniones departamentales de la ONU particular en que se convirtió la residencia en la que pase ese año. Gracias a todos los Galileanos y agregados, Dajana, Ivana, Seran, Daniele, Gilberto, Pepe, Borja, Eider, Martina, Sara, Claude…no os olvidaré jamás. Grazie Kati, per mettere un po di luce nella mia vita quando avevo piu bisogno.

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El tesoro más grande que tengo, mis amigos y familia, a ellos le debo mi estabilidad emocional y el sentirme el hombre más afortunado del mundo por tenerlos a mi lado.

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dos, no sabéis como me reconforta el pensamiento de que siempre estaréis ahí. Paula, no se puede albergar más apego y amor por la vida en todas sus facetas, has estado siempre a mi lado, a pesar de la distancia, y aunque a veces pueda parecer un poco áspero, nunca voy a olvidar cuanto has hecho por mantener encendida nuestra amistad. Pablo, tan pequeño y tan grande a la vez, una de las mejores personas con las que he tenido la suerte de toparme, tu sentido del humor es parejo a la dimensión de tu humanidad. Laura, como los Pokemon, hay que ver cuanto ha evolucionado nuestra amistad, aunque seamos unos dejados, sé que tu guindillismo siempre estará dispuesto a abrirme sus puertas, ¡Obrigadinho!. Kiko, mi hermano científico, parece mentira, pero todo llega. Gracias por acogerme en Granada siempre que lo he necesitado, por hacer de los meses en el Albaicín uno de los periodos de mi vida que recuerdo con más cariño, por tu sapiencia, tu generosidad como persona e investigador, ¡los caballeros negros acaban venciendo siempre! Hermano, nuestro informático afrancesado, las noches golfas del Under, las conversaciones, esos momentos musicales, tu risa contagiosa, la tranquilidad pasmosa con que recibes al mundo, merci…Mis agravios particulares, Beatriz y María, mujeres de bandera de las que uno se tiene que sentir orgulloso, gracias por estos años, por acogerme en Córdoba, Tarifa, Sevilla o donde fuera, por esas conversaciones ajenas a la ciencia y esas copas y cervezas variadas, os debo tanto. Fredy, culillo de mal asiento, gracias por tantos años de amistad, por tu sinceridad y cariño. Elena, gracias por interesarte siempre por mi estado mental y físico, por tu alegría cristalina, por tu estilo fuensantero a prueba de bombas. Alberto, Fredy Moreno, Araceli, Ilde, Inma, Crespo, Isaac, López, Majo, Chaco, Castro, Lucky, Pepelu, María Salinas, Juan Fran, Cata…Todos estos años no hubieran sido lo mismo sin vosotros, gracias, de corazón, a todos.

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el placer del jazz en directo, de las barbacoas en Retamar, de las fiestas compartidas. Tania, mi vecina postiza (¿o el postizo era yo?), llegaste tarde, pero no dudes en que también tienes cabida aquí, tu simpatía y personajismo han conseguido que me hiciera fan tuyo desde casi el día que te conocí. Gracias al resto de almerienses y agregados, por hacerme pasar tantos buenos ratos, por compartir conmigo su arte, su energía vital, su calor y simpatía: Chico, Pepe, Cristina, Sergio, Manu, Iván, Silvia, Paco, Alberto, Alesia,… siempre habrá un hueco para vosotros en mis recuerdos.

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cada vez que veíamos un bicho en la cuneta, por llevarme al campo a coger espárragos, setas o cualquier cosa que pudiera ser recolectable. Mi infancia estuvo marcada por tu cariño por la dehesa y es indudable que fue el pistoletazo de salida que confluye en el trabajo que ahora mismo estáis leyendo. Jamás podré devolveros todo lo que habéis sacrificado por mi, al menos espero haber podido conseguir que os sintáis orgullosos de vuestro hijo. Os quiero.

"*El infierno de los vivos no es algo por venir; hay uno, el que ya existe aquí, el infierno que habitamos todos los días, que formamos estando juntos. Hay dos maneras de no sufrirlo. La primera es fácil para muchos: aceptar el infierno y volverse parte de él hasta el punto de dejar de verlo. La segunda es peligrosa y exige atención y aprendizaje continuos: buscar y saber quién y qué, en medio del infierno, no es infierno, y hacer que dure, y dejarle espacio.*"

Italo Calvino – Las ciudades invisibles.

Índice

RESUMEN

Como todos los seres vivos, los parásitos generalistas están sometidos a diversos factores y mecanismos que controlan el flujo genético y el aislamiento entre grupos o poblaciones de individuos. Al mismo tiempo, diversos procesos, selectivos o no, controlan el grado de diferenciación y divergencia dentro de las poblaciones o conjunto de individuos. El resultado final de la interacción entre estas dos fuerzas (flujo genético entre poblaciones versus divergencia dentro de poblaciones) determina la estructura genética y el grado de diversificación final de los organismos. Dentro del gremio de los parásitos y patógenos en general, los factores y rasgos, tanto de parásitos como de hospedadores, relativos a la interacción entre los dos componentes antagonistas del sistema juegan un papel determinante en la diversificación y tasa de flujo genético de los primeros, debido a la íntima interacción y dependencia de las especies a las que explotan. Los distintos factores y mecanismos que rigen los procesos de diversificación en parásitos generalistas han sido tradicionalmente ignorados en el estudio de la especiación y diferenciación genética en parásitos, en detrimento de sistemas más especializados. Esto es debido en parte a: (1) la idea extendida de que este tipo de sistemas no están expuestos a presiones selectivas diferenciales derivadas de los distintos hospedadores a los que parasita, como sí ocurre en parásitos especialistas, y (2) la asunción de estrategas generalistas totales con que han sido categorizadas multitud de especies parasíticas cuya historia natural y ecología básica se desconoce en gran medida. No obstante, recientes estudios teóricos y trabajos experimentales con diversos sistemas parasíticos aparentemente generalistas comienzan a descartar esta idea preconcebida. La presente tesis pretende contribuir al conocimiento de este tipo de sistemas y ampliar la comprensión sobre la evolución y la ecología de los procesos de diversificación en parásitos. Con tal fin, nuestro trabajo se centra en el estudio del díptero ectoparásito *Carnus hemapterus*, sus hospedadores aviares y diversos aspectos del resultado de la interacción entre ellos; abordando para ello tres objetivos principales: (1) exploración y estudio de diversas características ecológicas y comportamentales de *Carnus hemapterus* y de la interacción de éstas con sus

hospedadores y el ambiente que los circunda, con el fin de identificar factores y mecanismos implicados en la divergencia y diversificación evolutiva de esta especie. (2) Estudio de la variación y plasticidad del ciclo de vida de este ectopárasito y sus consecuencias en la sincronización parásito-hospedador. Por último (3) examinar mediante análisis moleculares de ADN mitocondrial la estructura genética de *Carnus hemapterus*, con el fin de encontrar evidencias de procesos de divergencia y diferenciación a diversas escalas geográficas.

Se estudiaron rasgos claves que pueden afectar la tasa de flujo genético y diferenciación en sistemas parásito-hospedador como la selección de hospedador, la dispersión espacial y temporal (diapausa) del parásito, la sincronía parásito-hospedador, el efecto de la fenología reproductora de los hospedadores y de la temperatura de incubación de éstos sobre el ciclo de vida de *Carnus* y la persistencia y predecibilidad de sus hospedadores en el tiempo.

La capacidad de discernir y seleccionar preferentemente un tipo de hospedador con unas características determinadas, se ha relacionado con la capacidad de transmisión e infección diferencial en párasitos, que a su vez podría determinar la dirección y tasa de flujo genético entre individuos conespecíficos. Para conocer más profundamente las preferencias de hospedador en *Carnus hemapterus* se investigó la selección de hospedador de este parásito en uno de sus hospedadores más frecuentes en nuestra área de estudio, la Carraca Europea *Coracias garrulus*. Los resultados muestran que *Carnus hemapterus* selecciona preferentemente pollos con una mayor respuesta inmune y no emplumados, en detrimento de rasgos como el tamaño o la condición corporal. No obstante, la preferencia por pollos más sanos (mayor respuesta inmune) solo se mantiene cuando la densidad de parásitos dentro del nido es alta (mayor competencia intraespecífica) y la condición física de la nidada es baja (mayor probabilidad de mortalidad dentro del nido). Esto sugiere que esta especie posee una plasticidad en los criterios de selección de hospedador y que para completar su ciclo de vida con garantías prefiere hospedadores persistentes en el tiempo más que la calidad nutricional o el tamaño corporal de éstos.

El seguimiento de la fenología de emergencia y ciclo de vida de *Carnus hemapterus* durante varios años ha permitido registrar la existencia de diapausa prolongada (dormancia de ciclo largo en la cual los individuos pierden una o más oportunidades de reproducirse) en este parásito. Este tipo de diapausa está asociada a especies que se enfrentan a altos grados de impredecibilidad de sus recursos y es considerada una alternativa a la dispersión espacial. Nuestros resultados sugieren que esta estrategia ha surgido debido a la fluctuación y variabilidad espaciotemporal de la distribución de los hospedadores a escala de nido y/o poblacional. En cuanto a la dispersión espacial, los análisis de ADN mitocondrial encontraron una gran correlación entre distancia geográfica y genética a escala continental (Europa). Estos resultados, junto con la expansión demográfica continua detectada y la falta de evidencias moleculares y observacionales de eventos de colonización a larga distancia por medio de transporte y dispersión pasiva, sugieren una capacidad de colonización de nuevos hábitats continua pero restringida a sus propios medios, mediante la fase alada de los imagos recién emergidos.

En el caso de sistemas generalistas como el que nos ocupa, el ciclo de vida de los parásitos y la capacidad de sincronización de éstos con una especie o más de hospedadores podrían limitar las interacciones y el contacto entre individuos dentro y entre distintas especies de hospedadores a través de barreras temporales impuestas por la fenología reproductiva de los hospedadores y por la variación y predecibilidad de ésta en el espacio y el tiempo. En el caso de parásitos con una fase de dormancia estacional, el estudio de la variabilidad y el desarrollo de la diapausa se hace indispensable para conocer cómo varía y se sincroniza el ciclo de vida del parásito con respecto a los de sus hospedadores.

Para conocer el papel y la importancia de la plasticidad fenotípica en la variabilidad y capacidad de sincronización de la fenología de emergencia de *Carnus hemapterus* se realizaron dos experimentos, uno de campo y otro de laboratorio, en el que sometímos a pupas de *Carnus hemapterus* en dormancia provenientes del mismo hospedador a variaciones ambientales mediante dos aproximaciones experimentales distintas: (1) a través de la simulación de un cambio de hospedador más temprano que el original y con un patrón de

incubación distinto, y (2) mediante experimento de translocación de parásitos a dos tipos de ambientes climatológicos distintos. Los resultados de ambos experimentos demostraron que el desarrollo y terminación de la diapausa son rasgos muy plásticos en *Carnus hemapterus* que pueden ser modificado por la temperatura de incubación impuesta por un hospedador más temprano o por los cambios ambientales en su entorno durante la última fase de terminación de la diapausa.

Se estudió la fenología de emergencia en *Carnus hemapterus* y su capacidad de sincronizar su ciclo de vida con varios de sus hospedadores habituales en nuestras latitudes (nivel interespecífico), asi como con la fenología reproductora de uno de sus hospedadores habituales en nuestra latitudes, la carraca *Coracias garrulus* (nivel intraespecífico), con el fin de comparar ambas escalas de sincronización.

A nivel interespecífico, nuestros resultados muestran que existe una emergencia diferencial en el tiempo (emergencia alocrónica) asociada a tres hospedadores distintos habituales de nuestras latitudes (abubilla, *Upupa epops*; mochuelo, *Athene noctua;* carraca, *Coracias garrulus*). Además la distribución de la fenología de emergencia de los individuos asociados a abubilla y carraca están sincronizadas con la fenología reproductora de sus respectivos hospedadores. Esto sugiere la existencia de fenotipos distintos aislados temporalmente que podrían ser el resultado de un proceso de diversificación asociada a la barrera temporal impuesta por las distintas fenologías reproductoras de los hospedadores estudiados.

En cuanto al estudio de la sincronización y fenología parásito-hospedador a escala intraespecífica, la fenología de emergencia de *Carnus hemapterus* varió entre años y tipos de nido. Mantuvo una distribución con asimetría negativa a escala poblacional, con un incremento progresivo en la emergencia de imagos y un alto porcentaje de ellos emergiendo antes de la aparición de los pollos de carraca. A escala de nido, la distribución de la emergencia tuvo una asimetría positiva, con más del 50% de adultos apareciendo en las cuatro primeras semanas. La sincronización parásito-hospedador en general es baja y con valores muy fluctuantes entre años, debido principalmente a la variación de la fenología de

emergencia de *Carnus*, puesto que la fenología reproductora de la carraca no varió entre los años de estudio. Este efecto asimétrico de la variación interanual entre parásito y hospedador podría explicarse por la distinta intensidad con que afecta la variación ambiental a invertebrados heterotermos y vertebrados endotermos. El estudio de la repetibilidad de la fenología reproductora y la tasa de reutilización de los nidos por parte de la carraca reveló valores bajos de predecibilidad de este hospedador entre años. Sin embargo, el tiempo de reproducción de los hospedadores influyó sobre la tasa de sincronización parásito-hospedador, lo que indica que la variación de la fenología de cría del hospedador tiene cierta influencia sobre la fenología de emergencia del parásito. Todos los resultados arriba obtenidos relativos a la sincronía *Carnus*-Carraca sugieren que otros mecanismos no estudiados distintos de la sincronización parásito-hospedador (dispersión espacial, explotación de otras especies) podrían ser importantes para asegurar el éxito infectivo en este ectoparásito dentro del marco temporal estudiado.

La emergencia alocrónica en un parásito generalista puede ser el resultado de un mismo genotipo respondiendo polifénicamente a los distintos hospedadores (generalismo completo o extremo) o bien pueden existir diversos genotipos que han divergido asociados a sus distintos hospedadores y responden de manera diferencial (poliespecialistas). Con el fin de buscar evidencias moleculares que ayuden a despejar la estructura genética y diversificación a gran escala (entre poblaciones) y a escala local (entre hospedadores simpátricos) de este parásito, y ver la contribución real de la emergencia alocrónica como barrera temporal que limita el flujo genético entre grupos asociados a los distintos hospedadores, realizamos un estudio de marcadores mitocondriales en *Carnus hemapterus* en diversas localidades de Europa y Canadá. El análisis mitocondrial revela una acusada estructura filogeográfica de las poblaciones de *Carnus hemapterus* a nivel continental. Diversos eventos geológicos durante el Pleistoceno, como las glaciaciones en Europa o la apertura del estrecho de Bering entre Eurasia y Norteamérica, han podido tener un papel relevante en la conformación de la estructura y diferenciación genética de este parásito a lo largo del área de distribución estudiada. Estos resultados, junto con la correlación positiva entre

distancia geográfica y genética encontrada, indican que esta especie es bastante sensible a las barreras físicas y al aislamiento de sus poblaciones por la distancia, al menos cuando estudiamos escalas geográficas amplias. A escala local, los resultados muestran que la población de *Carnus hemapterus* estudiada en Tabernas presenta una cierta diferenciación genética en simpatría asociada al periodo de emergencia del imago pero independiente del hospedador parasitado, lo que sugiere la existencia de una barrera temporal que impide el libre flujo genético en esta especie.

Los resultados de la presente tesis demuestran la importancia de estudiar procesos de diversificación de parásitos generalistas y la necesidad de tener un amplio conocimiento previo de la historia natural y la ecología de las especies para abordar con fundamento cuestiones más complejas. Serán necesarias futuras investigaciones ecológicas y moleculares que permitan establecer con seguridad el efecto de las barreras temporales y geográficas sobre la diversificación y flujo genético de este parásito, mediante el uso de marcadores moleculares nucleares y/o microsatélites, mayor esfuerzo de muestreo y el uso de potentes técnicas de análisis espacial y epidemiológico.

INTRODUCCIÓN GENERAL

ANTECEDENTES

Los parásitos como objeto de estudio

El parasitismo es la estrategia ecológica más extendida en la naturaleza (De Meeus et al. 1998). En el sentido amplio de la definición de parasitismo (sin ceñirnos al concepto clásico que tradicionalmente se ha centrado en parásitos eucariotas de animales), prácticamente todos los taxones superiores existentes de seres vivos (exceptuando las arqueobacterias, Gill & Brinkman 2011) incluyen múltiples especies parasíticas, tanto animales, plantas y protozoos así como muchas bacterias y hongos y todos los virus (Madigan et al. 2003, Dobson et al. 2008). Todas las especies conocidas de plantas y animales se ven afectadas por algún taxón parasítico asociado (Bush et al. 2001); tan sólo en los seres humanos se ha estimado que las catorce enfermedades parasitarias más importantes hoy en día (sin incluir patógenos como bacterias y virus), afectan anualmente a más de 5300 millones de personas en el mundo (Bush et al. 2001), lo que equivaldría a decir que para un instante dado, aproximadamente el 88% de la población mundial está siendo infectada por algún tipo concreto de parasitosis. Esta ubicuidad y generalización del parasitismo entre todos los seres vivos se debe principalmente a que desde un punto de vista evolutivo, el parasitismo ha aparecido a lo largo de la historia de la vida en la Tierra en múltiples ocasiones y en gran cantidad de taxones distintos, produciendo una ingente diversidad de interacciones entre parásitos y hospedadores, estrategias ecológicas, grados de diversificación y/o rangos de hospedadores distintos (Bush et al. 2001, Poulin 2007).

Los parásitos ostentan dos características esenciales que los hacen organismos especialmente interesantes para llevar a cabo estudios de índole ecológica y/o evolutiva: por un lado, poseen una alta dependencia del hábitat que explotan (hospedador), lo que favorece la aparición de adaptaciones por parte de los parásitos para explotar de forma óptima a su hospedador u hospedadores. Por otro lado, los parásitos obtienen un beneficio en detrimento del hospedador, lo que

reduce la supervivencia y/o la eficacia biológica de éste. Esto implica que el hospedador debe desarrollar diversas estrategias para minimizar el daño del parásito y, que a su vez, el parásito tendrá que generar contradefensas para evitar la disminución de la eficacia biológica que las defensas del hospedador pudieran causarle. Este mecanismo de selección recíproca entre antagonistas, conocido como carrera de armamentos coevolutiva, puede considerarse el extremo de una extensa lista de adaptaciones a las que podrían conducir las interacciones mutuas a las que continuamente son sometidas los sistemas parásito-hospedador, desde los parásitos generalistas con muy baja especificidad y adaptaciones no específicas para una o varias especies de hospedadores concretas (Gandon & Van Zandt 1998), hasta los parásitos con un grado de especialización o adaptación local extrema (ver, por ejemplo, Atyeo & Windingstad 1979, Cullins et al. 1996, Elmes et al. 1999), pasando por un sinfín de estrategias ecológicas intermedias a menudo muy poco conocidas y estudiadas, con grados de especificidad y/o diversificación variables tanto a escala temporal como espacial. Es por tanto necesario aclarar que la aparición de fenómenos de adaptación y/o especiación en parásitos y/o hospedadores no tiene por qué derivar siempre de un proceso coevolutivo y viceversa (Ridley 1993; Paterson & Banks 2001, Soler 2002).

Procesos de diversificación en parásitos

Independientemente de la intensidad de las fuerzas evolutivas que rijan las continuas interacciones entre parásito y hospedador, un producto de esas interacciones puede ser la aparición de un proceso de divergencia y/o diversificación que eventualmente puede conducir a un proceso de especiación en una o varias poblaciones del parásito (Drès & Mallet 2002, Coyne & Orr 2004). Debido a la íntima relación que los parásitos mantienen con su hospedador, dichos procesos de diversificación son esperables y ocurrirán frecuentemente; sin embargo la capacidad y potencialidad dentro de cada población, linaje y/o taxón para experimentar un proceso de diversificación puede ser muy variable y dependerá principalmente de (i) las estrategias vitales y características ecológicas del parásito y de sus hospedadores, (ii) la interacción de estos rasgos entre sí y con las condiciones ambientales que los circundan, y (iii) del grado de variación

temporal y espacial del conjunto de estos rasgos, sus interacciones y el ambiente que los rodea. A lo largo de la historia del estudio de las relaciones parásito-hospedador se han identificado diversas características de los parásitos y hospedadores que pueden actuar como agentes moduladores de los procesos de diversificación entre parásitos tales como (a) habilidad del parásito para dispersarse entre varios hospedadores (Johnson et al. 2002), (b) predecibilidad y abundancia de los hospedadores en el tiempo y el espacio (Combes 1997, Vázquez et al. 2005), (c) método de transmisión del parásito (Barret et al. 2008), (d) complejidad y características del ciclo de vida del parásito (Blouin et al. 1998, Criscione & Blouin 2004, Barret et al. 2008), o el grado de especificidad o especialización de los parásitos (Norton & Carpenter 1998, Criscione et al. 2005, Šimkovà et al. 2006), entre otras. Como puede observarse, muchos de estos agentes no hacen sino determinar el grado de flujo génico que puede haber entre linajes o grupos de individuos que se encuentran en pleno proceso de diversificación o que ya han divergido. Dicho de otra forma, los eventos de divergencia y especiación son íntimamente dependientes de cualquier tipo de barrera que disminuya el flujo génico y fomente el aislamiento entre grupos de individuos (Coyne & Orr 2004), hasta tal punto que los procesos de diversificación pueden verse constreñidos por el flujo genético. De forma análoga, los procesos de divergencia y especiación, a través del aislamiento reproductivo de los individuos implicados en éstos, ya sea desencadenado por procesos ecológicos o no, pueden constreñir el flujo genético entre individuos (Mayr 1976, Coyne & Orr 2004; Kozak et al. 2006; Räsänen & Hendry 2008; Schluter 2009). Por tanto, para desentrañar y comprender con eficacia los procesos de diversificación y/o especiación que pueden experimentar los parásitos y el resto de seres vivos de la biosfera, es esencial determinar la **naturaleza y el origen de los agentes** (ecológicos o no) sobre los que actúan los **mecanismos y fuerzas evolutivas** que modulan el flujo génico y el aislamiento entre conjuntos de individuos, produciendo y/o manteniendo los procesos de creación de biodiversidad (Coyne & Orr 2004).

Parásitos generalistas y diversificación

Existen multitud de sistemas que demuestran que los parásitos son candidatos idóneos para estudiar los procesos de divergencia y especiación. Tradicionalmente los procesos de diversificación se han estudiados en sistemas altamente especializados, (por ejemplo, ácaros hematófagos, piojos, nematodos, o insectos fitófagos, ver ejemplos en Jaenike 1993, Proctor & Owens 2000, Drés & Mallet 2002, Johnson *et al*. 2002, Haas 2003, Giorgi et al. 2004, Tilmon 2008) en los cuales se predice que el flujo génico entre parásitos de distintas especies de hospedadores será muy limitado, promoviendo una fuerte diferenciación genética y una mayor probabilidad de apareamiento entre parásitos conespecíficos (Feder et al. 1988, Carroll & Boyd 1992). Además, en estos sistemas altamente especializados, los distintos hospedadores tienden a estar más discontinuamente distribuidos, lo que incrementa la probabilidad de diferenciación entre las poblaciones de parásitos geográficamente aisladas (Peterson & Denon 1998). Los procesos de diversificación también se han estudiado en sistemas donde el proceso de divergencia ya se ha completado y cuyas interacciones parásito-hospedador son conocidas y relativamente estables (Schluter 2001). Sin embargo cuando estudiamos especies ya separadas hace miles o millones de años es difícil conocer el origen de su divergencia o los agentes y mecanismos que la iniciaron y mantuvieron (Coyne & Orr 2004); por ejemplo, si empezaron a diferenciarse por un simple proceso de aislamiento en el que pudo actuar la selección natural o no, o por el contrario fueron realmente agentes selectivos los que desencadenaron el proceso de diversificación y su mantenimiento. Es por esto que se hace muy recomendable el estudio de estos procesos cuando están actuando en sus etapas más tempranas (Schluter 2001), en organismos que estén en sus primeras fases de especiación o en especies generalistas y/o polífagas que pudieran tener cierto grado de diferenciación y variabilidad entre subpoblaciones (por ejemplo, ecotipos, razas de hospedadores, poblaciones polifénicas y/o linajes con un grado variable de diferenciación genética, ver McCoy et al. 2001, 2005, Drés & Mallet 2002, Berlocher & Feder 2002, Tilmon 2008, Drummond et al. 2010), aunque siempre teniendo en cuenta que estas especies podrían ser candidatas a futuras diferenciaciones más intensas o quizá son simplemente el resultado de procesos

de diversificación fallidos (Via 2001). Independientemente de esto último, los parásitos generalistas se alzan como un sistema idóneo, tradicionalmente olvidado, para estudiar procesos de diversificación, ya que pueden tener una gran importancia en las etapas previas a los eventos de especialización, especiación y radiación tanto a nivel micro como macroevolutivo (Janz & Nylin 2008).

A pesar del amplio espectro de hospedadores a los que un parásito generalista puede infectar, cabe la posibilidad de que dentro de esta estrategia ecológica, algunas especies pudieran estar sometidas a algún tipo de diferenciación o barrera que impidiera el flujo genético normal entre individuos asociados a los distintos hospedadores. En los últimos años se está produciendo un incremento en el interés de estudios focalizados en desentrañar los mecanismos y agentes de diversificación en organismos parásitos generalistas de animales (McCoy et al. 2001, 2005, Bouzid et al. 2008, Elsasser et al. 2009, Haukisalmi et al. 2009, Rudge et al. 2009, Archie & Enzewa 2011). Incluso en insectos fitófagos, que son considerados un modelo idóneo para estudiar procesos de divergencia y especiación ecológica, debido al relativo predominio de la especialización y especificidad de hospedador entre linajes dentro de este grupo funcional (Futuyma & Moreno, Jaenike 1990, 1988, Singer 2008, aunque algunos recientes trabajos discuten esta afirmación - ver Nosil 2002, Futuyma 2005, Nyman et al. 2010-), los estudios ecológicos y evolutivos sobre divergencia y especiación en fitófagos generalistas son relativamente menos abundantes que los estudios sobre fitófagos especialistas (ver ejemplos de sistemas de especiación y diversificación en fitófagos polífagos y/o generalistas en Kelley et al. 2000, Nason et al. 2002, Blair et al. 2005, Morse & Farrell 2005, Weingartner et al. 2006, Nyman 2002, 2010).

Por tanto, el estudio de los parásitos generalistas tiene gran importancia debido al interés de éstos como precursores y desencadenantes de procesos incipientes de divergencia y posterior especialización y especiación (Kawecki 1998). Aquellos parásitos que, además de un rango amplio de hospedadores, poseen una amplía distribución geográfica están sometidos a una amplia variedad de agentes selectivos con un grado de intensidad muy diverso. Las condiciones locales que puedan experimentar los parásitos, los rasgos particulares de las

estrategias vitales de cada especie de parásito y/o hospedador, así como el rango y disponibilidad de hospedadores en cada población y/o área geográfica serán muy variables a lo largo del área de distribución del parásito (Janz & Nylin 2008). Por tanto, el efecto de la selección sobre los parásitos y hospedadores y la interacción entre ellos puede ser muy importante y activo en algunas localizaciones pero no en otras (Lajeunesse & Forbes 2002, Thomson 2005, Poulin et al. 2008). Esto influye sobre la variabilidad de los diversos mecanismos asociados a la diversificación, así como en el efecto e intensidad que los distintos tipos de barreras tendrán sobre el flujo genético de este tipo de organismos. Incluso si al estudiar un sistema generalista no se encuentran rastros de diversificación o especiación, es igualmente útil su estudio, puesto que la ausencia de divergencia nos puede ayudar a comprender cuáles son los agentes y mecanismos naturales que están impidiendo la diversificación en una especie concreta, para poder luego ser extrapolado a otros sistemas análogos.

La plasticidad fenotípica como motor de cambio evolutivo en parásitos generalistas

Los parásitos generalistas, por último, pueden ser organismos muy útiles para contrastar la importancia de la plasticidad fenotípica en los procesos de especiación. West-Eberhard (2003), en su influyente obra "Developmental Plasticity and Evolution", propone la idea de que los rasgos y/o estrategias vitales que presentan alta variabilidad en la expresión de su fenotipo (rasgos polifénicos) pueden promover la especiación. Si los distintos fenotipos existentes acaban seleccionando de forma continua los hábitats que les permiten una eficacia biológica máxima, y a su vez estos hábitats se mantienen estables y predecibles a lo largo del tiempo, la selección puede actuar promoviendo la diversificación y divergencia en un organismo para cada uno de los hábitats colonizados, partiendo de la especie polifénica ancestral (West-Eberhard 2003). En este contexto, el estudio de la plasticidad en parásitos generalistas, los cuales normalmente poseen multitud de respuestas alternativas para adaptarse al amplio rango de recursos (hospedadores) que explotan, nos brinda una oportunidad única para profundizar en los mecanismos incipientes de este tipo de diversificación que podría ser de

gran importancia en especies generalistas sometidas a un "escenario plástico" (Janz & Nylin 2008).

Diapausa y sincronización parásito-hospedador: papel en los procesos de diversificación en parásitos

La diapausa que experimentan durante su desarrollo multitud de animales (Tauber et al. 1986), es un rasgo idóneo para ser utilizado en estudios que impliquen variabilidad, plasticidad fenotípica, diversificación y/o especiación en organismos invertebrados, ya sean parásitos o no. Este mecanismo de dormancia, que permite a los individuos sobrevivir cuando las condiciones son desfavorables para el desarrollo y la reproducción y asegura la sincronización del ciclo de vida de los organismos con la estacionalidad de los recursos que utilizan (Tauber et al. 1986, Danks 1987) se caracteriza por una regulación altamente dependiente de las condiciones ambientales. Un número ingente de trabajos sobre cientos de especies diferentes de invertebrados, ha demostrado que tanto el inicio, como la duración y la terminación de la diapausa responden a factores ambientales y/o ecológicos entre los que destacan la temperatura, fotoperiodo, humedad, disponibilidad de alimento o densidad poblacional (Tauber et al. 1986). Por otra parte, la diapausa también tiene un fuerte componente genético que varía geográficamente a diversas escalas. Multitud de estudios han demostrado que la variabilidad intraespecífica y el polimorfismo genético de la diapausa es una característica general y extendida en cientos de organismos (Tauber et al. 1986, Leather et al. 1993). Por tanto, de todo lo anteriormente explicado, es fácil entender que la expresión y la variación fenotípica de la diapausa que podemos observar en un organismo cualquiera, puede ser bien el resultado de un polimorfismo genético ya fijado entre linajes, bien debido a la respuesta plástica y regulación promovida por las condiciones ambientales y/o ecológicas experimentadas (actuando de forma directa o indirecta), o bien por una combinación de las anteriores (polimorfismos genéticos con fenotipos individuales respondiendo en diverso grado a la regulación ejercida por los factores ambientales; Tauber et al. 1986).

Para muchos parásitos, la diapausa y otras adaptaciones temporales para sincronizar su ciclo de vida con la de sus hospedadores, son de vital importancia

para su supervivencia y futuras reproducciones. Los parásitos son especialmente sensibles a la variación en la sincronización de sus ciclos de vida con la disponibilidad de los organismos que explotan porque, en general, se alimentan de recursos efímeros que pueden estar muy irregularmente distribuidos en el espacio y en el tiempo, por lo que están sometidos a grandes presiones selectivas que conducen a la aparición de adaptaciones y estrategias varias para asegurar que sus ciclos infectivos estén sincronizados con el periodo en que sus hospedadores proveen los recursos más apropiados que maximicen su eficacia biológica (Tauber et al. 1986). La sincronización de los ciclos de vida parásito-hospedador está sujeta a una fuerte variabilidad, debido a la complejidad de la interacción, sujeta a variaciones espaciotemporales que pueden afectar no sólo al parásito, sino también al hospedador, y a las fuentes de alimento del hospedador (Tauber et al. 1986). Tradicionalmente se ha reconocido que la respuesta frente a la variación de los recursos en especies parásitas, se puede realizar o bien ajustando el ciclo de vida y reproducción con el ciclo de vida de los hospedadores, principalmente a través de la diapausa (dispersión temporal) o mediante el uso de hospedadores alternativos (dispersiones espaciales) (Tauber et al. 1986). Sin embargo, algunos trabajos recientes argumentan que algunas estrategias de regulación estocástica que dan lugar a una cierta asincronía parásito - hospedador (estrategias bet-hedging, coin flipping u otras estrategias evolutivamente estables reguladas de forma estocástica, ver West-Eberhard 2003) podrían ser una respuesta alternativa y eficiente frente a la impredecibilidad e irregularidad extrema de los hospedadores (Singer & Parmesan 2010). Los parásitos generalistas ampliamente distribuidos de nuevo se alzan como modelos idóneos para estudiar sincronización parásito – hospedador puesto que la variedad de ciclos de vida de hospedadores y condiciones ambientales a las que pueden estar sometidos a lo largo de su rango de distribución, permite estudiar el grado de ajuste y estrategias de optimización de sincronización a diversas escalas tanto geográficas como temporales.

La variabilidad en la diapausa u otros tipos de dormancia, así como la influencia de ésta sobre la sincronía parásito-hospedador ha sido propuesta como un importante mecanismo de aislamiento implicado en procesos de diversificación y especiación de muchos invertebrados parásitos polífagos (Via 2001, Rundle &

Nossil 2005) a través de la sincronización de éstos con el ciclo de vida particular de los distintos hospedadores que explotan (ver ejemplos y revisiones en Drés & Mallet 2002, West-Eberhard 2003, Tilmon et al. 2008). Este tipo de proceso de aislamiento temporal debido a la sincronización fenológica diferencial de un organismo con la fenología de los recursos a los que explota es un caso particular del denominado aislamiento temporal o alócronico, en el que la barrera que impide el flujo genético es de naturaleza temporal en lugar de física (como contraposición a una barrera alopátrica) y que puede desencadenar un proceso de especiación entre especies simpátricas o alopátricas (Coyne & Orr 2004). A pesar de la importancia que el aislamiento alocrónico podría tener en los parásitos generalistas (Price 1980, McCoy 2003) y en los procesos de especiación y diversificación de éstos, muy pocos casos de divergencia debido a la sincronización fenológica diferencial entre linajes asociados a distintos hospedadores han sido constatados en parásitos de animales (Theron & Combes 1995, McCoy et al. 2001, 2005).

En este contexto, la búsqueda de **evidencias** de la existencia de procesos de diversificación en sistemas parásitos generalistas-hospedador, así como el estudio de las estrategias vitales y características ecológicas del parásito, sus hospedadores, y el resultado de la interacción de estos rasgos entre sí, se hace todavía más necesario si cabe. El conocimiento adecuado de cualquier sistema parásito-hospedador permite aumentar la probabilidad de detección de potenciales **agentes** y **mecanismos** (ecológicos o no), que pudieran desencadenar y mantener procesos de diversificación en esos organismos. Por tanto, el trabajo desarrollado en esta tesis pretende ayudar a clarificar y desentrañar algunas de las lagunas todavía existentes en los procesos de divergencia en parásitos y, por ende, en el origen de la ingente biodiversidad que atesora nuestro planeta.

INTRODUCCION AL SISTEMA DE ESTUDIO

Con el ánimo de profundizar sobre la variabilidad y el grado de diversificación de los sistemas de parásitos generalistas, la presente tesis se centra en el estudio de la interacción entre el ectoparásito *Carnus hemapterus* y sus diversos hospedadores. Debido a que se trata de un sistema poco conocido para la comunidad investigadora en general, consideramos pertinente realizar una breve introducción sobre la ecología e historia natural de esta especie, así como de los hospedadores con los que trabajamos en los distintos capítulos expuestos en esta tesis. Para no saturar de información al lector, nos centraremos en aquellos rasgos ecológicos y estrategias vitales de cada organismo necesarias para comprender los objetivos y metodología de esta tesis.

Carnus hemapterus Nitzsch 1818 (Diptera: Carnidae) es un pequeño díptero muscomórfido de aproximadamente dos milímetros de longitud, ectoparásito de pollos de diversas especies de aves. El género *Carnus* comprende alrededor de 5 especies, la mayoría de distribución holártica (Brake 2011), aunque *Carnus hemapterus* es la única especie presente en Europa. La mayor parte de miembros de esta familia son moscas de hábitos saprófitos, normalmente asociados a carroña, heces o nidos de diversas especies de aves (Papp 1998, Brake 2011), siendo *Carnus* el único género parásito dentro de esta familia (Brake 2011). Se le describe como un parásito generalista ya que tiene un alto número de hospedadores aviares (Capelle & Whitworth 1973, Kirkpatrick & Colvin 1989, Dawson & Bortolotti 1997, Grimaldi 1997, Roulin 1998, Iwasa et al. 2008, Brake 2011) y un amplio rango geográfico (se distribuye por todo el Paleártico y gran parte de Norteamérica, Grimaldi 1997, Papp 1998, Brake 2011).

Se sabe poco de su ciclo de vida. Los adultos de esta especie parasitan a los pollos (preferentemente no emplumados) de sus hospedadores, ponen huevos, los cuales eclosionan dando larvas que se alimentan de la materia orgánica que hay en el nido. Hay tres estadios de desarrollo que duran aproximadamente 21 días (Guigen et al. 1983), pupando en el mismo nido. Durante la fase de pupa sufren una diapausa, usualmente de varios meses, emergiendo la primavera siguiente. Se ha descrito cierto grado de sincronía con la aparición de los pollos de sus hospedadores (Liker et al. 2001, Valera et al. 2003), para comenzar un nuevo

ciclo. Tras la emergencia los adultos tienen alas y son capaces de dispersarse activamente (Grimaldi 1997, Roulin 1998, 1999); no hay dispersión forética conocida sobre las aves adultas, y una vez han localizado a un hospedador adecuado, pierden las alas. Tras la emergencia, los adultos pueden sobrevivir entre 2-3 días sin alimentarse (Calero-Torralbo & Valera, datos no publicados). El apareamiento tiene lugar sobre el hospedador (Giguen et al. 1983). El abdomen de las hembras, una vez fecundadas, se hincha hasta tres veces su tamaño (fisogastria), y adquiere un tono blanquecino debido a los huevos que lleva dentro. *Carnus hemapterus* se alimenta de la sangre de sus hospedadores (Kirkpatrick & Colvin 1989, Dawson & Bortolotti 1997), y posiblemente de otro tipo de secreciones cutáneas, pudiendo influir negativamente en el desarrollo y condición de los pollos (Whitworth 1976, Cannings 1986, Schulz 1986, 1990, Wiebe 2009, Avilés et al. 2009, Hoi et al. 2010), aunque algunos estudios no han encontrado efecto alguno sobre los hospedadores (Kirkpatrick & Colvin 1989, Dawson & Bortolotti 1997, Liker et al. 2001).

En cuanto a sus hospedadores, éstos son muy variables aunque parece que tiene preferencia por aves nidificantes en huecos (trogloditas), así como rapaces diurnas y nocturnas y diversas especies de córvidos (Grimaldi 1997, Brake 2011). Sólo en nuestra zona de estudio en la provincia de Almería, existen registros de *Carnus* parasitando a 10 especies distintas (Ver tabla I.1), aunque es posible que algunas de estas citas sean de hospedadores eventuales.

Especie	Familia	Referencias
Clamator glandarius	Cuculidae	Calero-Torralbo, datos no publicados
Otus stops	Strigidae	Calero-Torrabo, datos no publicados
Athene noctua	Strigidae	Presente tesis
Coracias garrulus	Coracidae	Václav et al. 2011. Presente tesis.
Merops apiaster	Meropidae	Valera et al. 2003. Presente tesis
Upupa epops	Upupidae	Valera et al. 2006
Falco tinnunculus	Falconidae	Presente tesis
Sturnus vulgaris	Sturnidae	Calero-Torralbo, datos no publicados
Petronia petronia	Passeridae	Valera et al. 2003
Corvus monedula	Corvidae	Calero-Torralbo, datos no publicados

Tabla I.1 Hospedadores registrados para *Carnus hemapterus* en la provincia de Almería (España).

Los parásitos que se utilizaron para el desarrollo de esta tesis provienen de un rango diverso de hospedadores y localizaciones (ver Tabla I.2), con el fin de responder a distintas cuestiones planteadas en este estudio. En muchos casos los parásitos fueron recogidos de muestras de nidos o fueron amablemente proporcionados por parte de otros investigadores. Para algunos de los trabajos expuestos en esta tesis, se manejaron directamente algunos de los hospedadores habituales de este ectoparásito: concretamente, la Carraca Europea (*Coracias garrulus*) y la Abubilla (*Upupa epops*). A continuación ofrecemos algunos datos relativos a la biología reproductora y ecología de estas especies:

La Carraca es un Coraciforme migrador que se reproduce en nuestra área de estudio entre mayo y julio (Václav et al. 2011). Se alimenta preferentemente de grandes artrópodos (Avilés & Parejo 2002), aunque puede capturar pequeños reptiles y roedores (Calero-Torralbo, observación personal). Prefiere zonas abiertas de clima mediterráneo con temperaturas cálidas en verano para criar (Avilés et al. 1999, Václav et al. 2011). Es una especie monógama que usa huecos en árboles, agujeros en acantilados y ramblas producidos por abejarucos y también agujeros y huecos en construcciones humanas (puentes, cajas nido, casas de campo, etc,) para criar (Avilés & Sánchez 1997, Václav et al. 2011). No incluye ningún tipo de material en el nido, aunque limpia y prepara la cámara de incubación cada año (Calero-Torralbo, observación personal). Suele reutilizar los nidos usados por carracas u otras especies de hábitos reproductores similares

(Parejo et al. 2003, Václav et al. 2011). Es una especie territorial (aunque puede formar colonias laxas) y probablemente filopátrica (Cramp 1985). Realiza una puesta asincrónica de entre 4 a 6 huevos que empieza a incubar a partir del tercer huevo (Cramp 1985), lo que resulta en una gran diferencia de tamaños entre los pollos de una misma nidada (Parejo et al. 2007); la incubación dura entre 17 y 19 días. Los pollos nacen desnudos y suelen permanecer en el nido hasta los 20-22 días de vida (Cramp 1985).

La Abubilla es un Bucerotiforme residente, que se reproduce prácticamente a lo largo de toda la estación reproductora en nuestras latitudes (Martín-Vivaldi et al. 1999), desde febrero a junio. Especie ampliamente extendida por el Paleártico, tiene una dieta insectívora, incluyendo larvas y adultos de diversos tipos de artrópodos (Cramp 1985). Cría en agujeros de árboles, muros y otras construcciones humanas (Martín-Vivaldi et al. 1999). Es una especie monógama y territorial, que puede sacar adelante más de una nidada al año (Martín-Vivaldi et al. 1999); pueden reutilizar los nidos entre años y/o entre puestas dentro del mismo año y suelen hacer puestas de alrededor de 6 huevos de media (rango muy variable entre 4 y 12, Martín-Vivaldi et al. 1999). La incubación dura alrededor de 17 días, los pollos nacen de forma asincrónica, desnudos y suelen dejar el nido entre los 25 y 30 días de vida (Martin-Vivaldi et al. 1999).

Áreas de trabajo y muestreo	**Coordenadas geográficas**	**Hospedadores**	**Capítulos**
Tabernas, Almería	37.066 N -2.218 E 37.066 N -2.354 E 37.083 N -2.350 E	*Athene noctua, Coracias garrulus, Merops apiaster, Falco tinnunculus*	I, II, IV, V, VI
Doña María, Almería	37.135 N -2.711 E	*Upupa epops*	II, VI
Guadix, Granada	37.306 N -3.183 E 37.306 N -3.135 E	*Upupa epops*	II, IV, VI
Andujar, Jaén	37.976 N -4.122 E	*Merops apiaster*	VI
La Palma del Condado, Huelva	37.583 N -6.750 E	*Merops apiaster*	III
Cáceres	39.050 N -5.140 E	*Coracias garrulus, Falco tinnunculus, Sturnus unicolor*	II
Daganzo, Madrid	40.547 N -3.567 E	*Merops apiaster*	II, VI
El Espinar, Segovia	40.686 N -4.350 E	*Falco tinnunculus*	VI
Balmaseda, Vizcaya	43.198 N -3.181 E	*Falco peregrinus*	VI
Castro Verde, Portugal	37.706 N -8.071 E	*Coracias garrulus*	IV, VI
La Flotte, Francia	46.189 N -1.329 E	*Buteo buteo*	VI
Paradou, Francia	43.690 N 4.787 E	*Coracias garrulus*	VI
Parma, Italia	44.848 N 10.173 E	*Falco tinnunculus*	VI
Gosing, Austria	48.469 N 15.812 E	*Upupa epops*	VI
Pavlova, Eslovaquia	47.907 N 18.672 E	*Merops apiaster*	VI
Dunajska Streda, Eslovaquia	47.994 N 17.617 E	*Falco cherrug*	VI
Piestany, Eslovaquia	48.595 N 17.834 E	*Falco cherrug*	VI
Somotor, Eslovaquia	48.400 N 21.816 E	*Merops apiaster*	VI
Albertirsa, Hungría	47.252 N 19.608 E	*Merops apiaster*	VI
Riskecreek, Canadá	52.066 N -122.519 E	*Colaptes auratus*	VI
Lake Besnard, Canadá	55.436 N -105.784 E	*Falco sparverius*	VI

Tabla I.2 Localización y hospedadores de los parásitos utilizados en la presente tesis, y capítulos donde se incluyen individuos obtenidos de cada área de muestreo.

JUSTIFICACION DEL SISTEMA DE ESTUDIO

Las principales características que hacen a *Carnus hemapterus* y sus hospedadores especialmente útiles como modelo de estudio de procesos de diversificación en organismos parásitos generalistas son:

Taxonomía

Ciertos taxones tienen tendencia a desarrollar conductas parasíticas, debido a la existencia de rasgos comunes que les permiten luchar con ventaja en la carrera de armamentos parásito-hospedador (Van Valen 1973, de Meeus 1998). Entre ellos se encuentran los insectos y, particularmente, ciertos órdenes como los dípteros (Waage 1979), que poseen una riqueza y diversidad de especies con conductas parasíticas distintas sin parangón en la clase Insecta (Grimaldi & Engel 2005). Por otra parte, la mayoría de estudios sobre diversificación en insectos parásitos se han realizado en especies especialistas fitófagas (ver apartado *Parásitos generalistas y diversificación*), aunque en los últimos años se está prestando cada vez más atención a la necesidad de identificar adecuadamente la naturaleza y el origen de los procesos de diversificación y divergencia en sistemas parásitos de animales (ver apartado *Parásitos generalistas y diversificación*). El presente trabajo, además de contribuir al estudio de la ecología y evolución de sistemas parásitos generalistas de animales, ayuda a cubrir parcialmente la laguna existente en estudios sobre divergencia y estructura de las poblaciones de dípteros ectoparásitos de aves, donde la información que existe es escasa y de reciente aparición (Whiteman et al. 2007, Dudaniec et al. 2007), a pesar de la abundancia e importancia que este grupo funcional tiene como agente selectivo y modelador de estrategias vitales de los hospedadores (por ejemplo, comunidades aviares) de todo el mundo (Grimaldi & Engel 2005).

Rango de hospedadores

Como ya indicamos anteriormente, *Carnus hemapterus* parasita a un amplio rango de hospedadores, en muchas ocasiones bastante separados taxonómicamente y con una serie de características fenológicas, fisiológicas, ecológicas y conductuales bien diferenciadas. Estas características diferenciales podrían actuar como agentes selectivos y/o barreras que podrían interrumpir el flujo génico entre linajes divergentes asociados a los distintos tipos de hospedadores.

Predecibilidad y estabilidad de los hospedadores

Carnus hemapterus parece preferir especies que proporcionan un alto grado de predecibilidad y homogeneidad ambiental, como son las aves troglodita (crían en agujeros, frecuentemente reutilizados, y que poseen unas condiciones microclimáticas más estables que las del exterior, Ellis 1982, Lill & Fell 2007). La estabilidad y predecibilidad de los recursos que un organismo generalista puede explotar en el tiempo y el espacio se considera una condición necesaria para iniciar un proceso de especiación en especies generalistas polifénicas (West-Eberhard 2003). Por otra parte es destacable también que *Carnus hemapterus* completa su ciclo vital dentro del nido, pudiendo perpetuarse en el mismo lugar durante varios años si un hospedador cría también en él con cierta periodicidad. La fidelidad por los lugares de cría de muchas de las especies a las que parasita podría actuar como un mecanismo de reforzamiento y barrera a través de apareamientos concordantes entre linajes conespecíficos de cada hospedador (Coyne & Orr 2004).

Selección de hospedador

En el caso de que se diera cierto grado de especialización en *Carnus hemapterus*, es posible que aparezca algún tipo de compromiso o "trade-off" entre los distintos linajes de parásitos asociados a los distintos hospedadores, por lo que la elección de un hospedador erróneo podría traducirse en una pérdida de eficacia biológica para el parásito (Futuyma & Moreno 1988). Para evitar este problema, pueden aparecer diversos mecanismos que sirvan para seleccionar diferencialmente al

hospedador que le reporte una eficacia biológica óptima, como ya se ha visto que ocurre en otros parásitos (Jaenike 1993, Krasnov et al. 2002, Ulloa et al. 2004, Giorgi et al. 2004, Mikheev et al. 2004).

Ciclo de vida. Sincronía parásito-hospedador

El rasgo principal que regula el ciclo de vida en *Carnus hemapterus* es la diapausa, que se caracteriza por su enorme variabilidad intraespecífica (Tauber et al. 1986). Estudios previos (Liker et al. 2001, Valera et al. 2003) han señalado que puede existir una sincronía entre la eclosión de los pollos hospedadores y la terminación de la diapausa y posterior emergencia de los adultos de *Carnus hemapterus.* La variabilidad en la fenología de emergencia del parásito asociada a la fenología de cría de algunos de sus hospedadores podría ser el resultado de una respuesta plástica a las condiciones experimentadas durante el inicio, desarrollo o terminación del periodo de diapausa, lo que eventualmente podría conducir a un proceso de aislamiento en el tiempo (alocrónico) entre linajes asociados a hospedadores con fenología de cría distinta, como ya se ha visto que ocurre en otros organismos (véanse ejemplos en West-Eberhard 2003, Coyne & Orr 2004, Tilmon 2008).

OBJETIVOS

Una vez aclarado el atractivo y la utilidad del sistema de estudio de esta tesis, se hace indispensable concretar los objetivos propuestos para contribuir al avance del conocimiento de los procesos de diversificación en sistemas parásito – hospedador.

Como ya indicamos anteriormente este trabajo de investigación se ha centrado en los siguientes objetivos:

1. Estudio de diversas características ecológicas y rasgos de estrategias vitales del parásito *Carnus hemapterus* y de la interacción de éstas con sus hospedadores y el ambiente que los circunda con el fin de identificar potenciales factores y mecanismos evolutivos que pudieran desencadenar y mantener un proceso de diversificación en este sistema.
2. Estudio de la variación y plasticidad en la fenología de emergencia y diapausa de este ectopárasito y las consecuencias de esta plasticidad en la sincronización parásito-hospedador.
3. Examinar mediante análisis moleculares la estructura genética de las poblaciones de *Carnus hemapterus*, con el fin de encontrar evidencias de procesos de divergencia y diferenciación a diversas escalas geográficas.

1. Identificación de factores y mecanismos de diversificación: estrategias vitales y ecológicas de *Carnus hemapterus* y sincronización parásito-hospedador.

Como ya indicamos anteriormente, para que se de un proceso de diversificación en una especie parásita, han de existir agentes selectivos diferenciales sobre los que puedan actuar la fuerzas evolutivas para desencadenar un proceso de divergencia entre grupos de individuos. Por otra parte, para que un proceso de diversificación se mantenga, es necesario que los linajes previamente divergidos tengan algún tipo de barrera (espacial, temporal y/o reproductiva) que impida el libre flujo genético entre ellos (Coyne & Orr 2004). Es necesario, por tanto,

indagar sobre los rasgos, estrategias vitales e historia natural, tanto de los parásitos como de los hospedadores, que pudieran influir sobre el aislamiento y flujo genético de la especie a estudiar. Nos centraremos en estudiar rasgos claves de parásitos y hospedadores y las interacciones entre ellos, que se consideran fundamentales a la hora de desencadenar procesos de diversificación, así como en controlar y dirigir la intensidad del flujo genético entre grupos de individuos divergiendo. También abordaremos la variación de algunos de estos rasgos a lo largo de diversos gradientes espaciotemporales.

1.a. Ecología de la selección de hospedador en *Carnus hemapterus* (**Capítulo I**).

La preferencia de *Carnus hemapterus* por un tipo de hospedador u hospedadores concretos es una estrategia comportamental que puede aumentar el aislamiento entre conjuntos de poblaciones que estén divergiendo, y puede disminuir la probabilidad de contacto y flujo genético de ese grupo con otros (Räsanen & Henry 2008). Por tanto, esclarecer los criterios de selección de hospedador a nivel intraespecífico en esta especie sería un factor clave como paso previo para entender los mecanismos implicados en un hipotético proceso de selección a nivel interespecífico que pudiera influir sobre el aislamiento y diversificación de individuos asociados a un tipo de hospedador concreto. El tamaño y otros caracteres morfológicos de los hospedadores, así como diversas características fisiológicas diferenciales de éstos, podrían ser factores clave para desencadenar el proceso de selección preferente en *Carnus hemapterus*. La preferencia por un tipo de hospedador con unas características concretas puede ocurrir a nivel intraespecífico, en ésta u otra especie que parasite sobre pollos de aves (Christe et al. 1998, Roulin et al. 2003, Valera et al. 2006), ya que una elección no adecuada podría afectar a su eficacia biológica (Tschirren et al. 2007). Partiendo de la hipótesis de que *Carnus hemapterus* puede parasitar preferentemente a hospedadores con unas características ecológicas, fisiológicas y/o ontogénicas determinadas, se realizó el seguimiento de la carga parásita a nivel individual y de nidada sobre pollos de carraca en Tabernas (Almería) durante varias estaciones de cría, y se relacionó ésta con datos biométricos, inmunológicos y fisiológicos (temperatura) de los hospedadores.

1.b. Dispersión temporal, estructura filogeográfica y eventos históricos de colonización **(Capítulos II y VI) .**

La capacidad de dispersión de un parásito es un factor fundamental que puede explicar su distribución actual y pasada, la capacidad de colonizar nuevos hábitats o el potencial de los individuos emigrantes para comunicar poblaciones previamente aisladas (Begon et al. 1986, Perfectti 2002). Individuos con baja capacidad de dispersión tendrán mayores dificultades para comunicar poblaciones previamente aisladas o colonizar nuevos territorios. Se trabajará en los siguientes aspectos de la dispersión y movilidad de *Carnus hemapterus*:

Dispersión temporal. Diapausa prolongada **(Capítulo II)**

La diapausa es una estrategia extendida entre los invertebrados cuya función es combatir periodos adversos y asegurar el éxito reproductivo apareciendo cuando las condiciones son favorables y los recursos son los adecuados (Enright 1970, Tauber et al. 1986, Leather et al. 1993). Por tanto la diapausa puede verse como un tipo de dispersión temporal frente a condiciones ambientales no favorables, funcionalmente equivalente a la dispersión espacial (Hairston 2000, Bohonak and Jenkins 2003). En parásitos la diapausa podría servir para evitar periodos donde los hospedadores no se encuentran presentes y sincronizar las fases infectivas de aquellos con los periodos en los que los recursos disponibles (hospedadores) son más abundantes y de mayor calidad. La diapausa prolongada es una estrategia que consiste en una diapausa más larga de lo habitual que experimentan algunos individuos de una población y que les impide aprovechar una o más oportunidades de reproducción (Danks 1987, Menu et al. 2000). Este tipo de diapausa se suele interpretar como una adaptación a ambientes impredecibles. Para *Carnus hemapterus*, podría ser una buena estrategia alternativa a la dispersión espacial que una proporción de los individuos de un nido no emergiera durante dos años o más, evitando así los problemas derivados de la ausencia del hospedador en el nido, emergiendo cuando haya de nuevo un hospedador disponible en el nido. Para comprobar si se da el fenómeno de diapausa prolongada en esta especie y con qué frecuencia y periodicidad se produce, se recogieron muestras de pupas de

Carnus hemapterus de distintas especies y se realizó el seguimiento de la fenología de emergencia durante varios años.

Dispersion espacial, estructura filogeográfica y colonización **(Capítulo VI)**.

Puesto que *Carnus hemapterus* no se ha encontrado en aves adultas fuera de la época de reproducción, es de suponer que los medios de dispersión que este parásito posee son el vuelo activo durante la fase adulta de vida libre. Según Matyukhin y Krivosheina (2008) *Carnus* es incapaz de realizar vuelos de larga distancia. Una hipótesis que se deriva de esta información y que pretendemos testar es que poblaciones separadas de *Carnus hemapterus* a diversas escalas geográficas poseerán una estructura filogeográfica acusada. Con el fin de conocer la capacidad de dispersión y colonización de esta especie a una escala espacial y temporal más amplia, se muestrearon nidos parasitados en diversas localidades de la Península Ibérica y parte de Europa y Norteamérica, intentando cubrir el máximo posible de su área de distribución actual. Los individuos muestreados se sometieron a un proceso de extracción y amplificación de su ADN mitocondrial. El ADN mitocondrial es un marcador idóneo para llevar a cabo inferencias históricas, filogeográficas y evolutivas, puesto que no sufre recombinación y se transmite matrilinealmente, por lo que se pueden rastrear fácilmente eventos que nos ayuden a entender la estructura genética y los procesos de dispersión y/o colonización en las distintas poblaciones a estudiar (Avise 2004). Adicionalmente, hemos rastreado en busca de eventos de colonización a larga distancia por parte de este parásito. En caso de no encontrarlos, ayudaría a confirmar la idea de que esta especie no usa individuos adultos de hospedadores para dispersarse, como sí hacen otras especies de ectoparásitos conocidos, como piojos, moscas hipobóscidas o garrapatas (Marshall 1981).

1.c. Polifenismo y emergencia alocrónica en *Carnus hemapterus* asociada a la fenología reproductiva de sus hospedadores (**Capítulos IV y V**).

Para *Carnus hemapterus* los recursos tróficos (pollos no emplumados) dentro del nido en el que pasan el invierno están disponibles solo unas cuantas semanas durante todo el año. Planteamos la hipótesis de que el final de la dormancia y la emergencia de los adultos de este parásito deben coincidir lo más exactamente posible con el periodo de eclosión de los huevos de su hospedador y las primeras semanas de vida del pollo. Se sabe que muchas especies fitófagas ajustan su emergencia con la fenología de aparición de la planta o de partes de las que se alimentan (Craig et al. 1993, Feder et al. 1993, Tikkanen & Lyytikainen-Saarenmaa 2002). Por tanto es esperable que pueda ocurrir lo mismo en un sistema análogo parásito-animal como *Carnus hemapterus* y sus hospedadores. La sincronización con las distintas fenologías reproductivas de los hospedadores a los que parasita podría actuar como factor desencadenante de diversificación a través de un proceso de aislamiento temporal. Se trabajará a dos escalas diferentes:

Sincronización interespecífica **(capítulo IV).**

Carnus hemapterus posee un amplio espectro de hospedadores que pueden empezar a criar desde febrero-marzo (ej. búho real o abubilla) hasta junio-julio (ej. carraca o abejaruco). Esto significa que existe un periodo continuo en que teóricamente *Carnus hemapterus* podría sobrevivir parasitando a cualquiera de las especies habituales en un área dada. Sin embargo, como ya explicamos anteriormente, es probable que aparezcan linajes con una fenología de emergencia sincronizada con la de un hospedador en particular. *Carnus hemapterus* puede realizar todo su ciclo en el hospedador o en sus inmediaciones (nido), por lo que si la especie de la que se alimenta cría en el mismo sitio de forma continua, la predecibilidad y disponibilidad de ésta será alta, por lo que es esperable que *Carnus* haga coincidir al máximo su emergencia con la de la especie que parasita año tras año, promoviendo el aislamiento entre linajes asociados a distintos hospedadores con fenología de cría temporalmente diversa. Es lo que se denomina un proceso de emergencia alocrónico, que puede preceder a un proceso de

diversificación del mismo tipo. Para comprobar si existe una emergencia alocrónica y sincronización de *Carnus* asociada con la fenología de cría de algunos de sus hospedadores habituales, se realizó un experimento en nuestra área de estudio, en el que se obtuvieron muestras de tres hospedadores habituales de *Carnus hemapterus* (abubilla, mochuelo y carraca) con fenologías de cría distintas y parcialmente separadas en el tiempo. Se formaron grupos de tres muestras (una de cada hospedador), y cada grupo se colocó en una oquedad natural similar a las usadas habitualmente por estos hospedadores para criar. Durante la primavera siguiente se registró la fenología de emergencia así como la abundancia de parásitos en cada muestra y se comparó ésta con la fenología de cría de dos de las especies hospedadoras estudiadas mediante el uso de modelos nulos. El diseño de "jardín común" nos permitirá aislar la contribución de los diferentes hospedadores sobre la fenología de los parásitos y minimizar la contribución de la variación ambiental sobre la fenología y la diapausa de *Carnus hemapterus*, permitiendo establecer la existencia o no de emergencia alocrónica en función de la fenología de los hospedadores. Los modelos nulos permiten comparar la distribución fenólogica observada de los hospedadores y cada uno de los linajes del parásito asociados a cada hospedador (y su correspondiente sincronía) con la distribución esperada de ambos en el caso de que ésta fuera completamente al azar.

Sincronización intraespecífica y variabilidad de la fenología parásito – hospedador. **(Capítulo V).**

La sincronización parásito – hospedador dentro de una misma especie, y cómo varía ésta en función de las condiciones ambientales o de diversos rasgos ecológicos de los hospedadores es un tema raramente estudiado. Sin embargo podría ser determinante para explicar cómo los procesos de diversificación pueden variar entre regiones o a lo largo del tiempo. El sistema *Carnus hemapterus* – Carraca provee una excelente oportunidad para ver cómo afecta la variación espaciotemporal de diversos factores en el resultado de la interacción y la sincronía entre estas dos especies. Específicamente nos planteamos si (1) las variaciones ambientales interanuales pueden afectar similarmente a parásitos y hospedadores y a la sincronización de sus ciclos de vida de igual forma, (2) si los

hospedadores pueden considerarse un recurso estable y predecible en el espacio y en el tiempo y (3) si *Carnus hemapterus* usa la presencia y/o fenología de cría del hospedador como pista para emerger y sincronizar su ciclo de vida con el del hospedador. A tal fin se realizó el seguimiento de nidos concretos de una población de carraca en el campo de Tabernas (Almería), durante varios años, y se registró la fenología de cría de los hospedadores, fenología de emergencia de los parásitos, abundancia y periodo de infección en cada nido, con el fin de conocer el grado de sincronía parásito-hospedador y la variación de ésta a dos escalas diferentes (población de carraca en el área de estudio y nido).

2. Variabilidad y plasticidad de la diapausa y la fenología de emergencia en *Carnus hemapterus.*

Cuando observamos un proceso de sincronización alocrónica en un sistema parásito-hospedador, éste no tiene porque deberse a un polimorfismo genético entre linajes asociados a los distintos hospedadores. Una hipótesis alternativa que explicaría igualmente el fenómeno observado sería la plasticidad fenotípica o polifenismo en los rasgos que controlan la sincronización en esa especie (West-Eberhard 2003). Centrándonos en nuestro sistema, podría ocurrir que la duración de la diapausa estuviera controlada por estímulos ambientales (por ejemplo temperatura, como se ha visto en otros muchos sistemas de invertebrados que experimentan diapausa, Tauber et al. 1986, Leather et al. 1993) y que la simple presencia del hospedador o su temperatura corporal fueran condición suficiente para terminar la diapausa, produciéndose una emergencia sincronizada pero independiente de la especie parasitada. En este caso, la sincronización sería el resultado de la adaptación por parte del parásito generalista frente a la impredicibilidad o irregularidad del recurso a explotar. Sin embargo, como ya indicamos anteriormente, las hipótesis sobre especiación en especies generalistas con expresión de rasgos polifénicos aplicadas a este parásito indican que si existen respuestas plásticas en la duración de la diapausa asociadas a la fenología de los distintos hospedadores que *Carnus* parasita, y a su vez, la fenología de los hospedadores se mantiene estable y predecible localmente a lo largo del tiempo, la selección podría actuar promoviendo la diversificación y divergencia de *Carnus*

hemapterus en los distintos hospedadores, partiendo de la especie polifénica de respuesta plástica ancestral (West-Eberhard 2003). Por tanto, como primer paso para comprobar si esta hipótesis es plausible en este sistema parasítico generalista es esencial conocer si la variabilidad y duración de la diapausa y la fenología de emergencia en *Carnus hemapterus* es un rasgo plástico y en qué grado. Para comprobar este punto, realizamos dos aproximaciones: la primera de las cuales implica el efecto y el grado de plasticidad de la fenología de emergencia a un cambio de hospedador a través de las diferencias en la fenología de incubación del nuevo hospedador **(Capítulo III)** y la segunda, a través de un experimento de translocación de parásitos (**Capítulo IV**).

2.a. Regulación y control de la diapausa en *Carnus hemapterus*. Efecto de la temperatura **(Capítulo III)**.

Como dijimos anteriormente, la variabilidad en la duración de la diapausa podría ser uno de los factores claves que podrían desencadenar un proceso de diversificación en esta especie. No existe información acerca de los mecanismos de control de la diapausa en *Carnus hemapterus*, de qué depende la duración e intensidad de ésta, o del grado de dependencia de ésta de estímulos y pistas ambientales para su inicio, terminación o desarrollo. Entre los posibles estímulos ambientales, la temperatura aparece como un candidato de peso entre especies de climas templados (Leather et al. 1993). En el caso de parásitos de vertebrados homeotermos no sólo la temperatura ambiental podría ser importante, sino que el efecto de la temperatura del hospedador podría servir como pista para saber cuando van a eclosionar los pollos de los que se alimenta. En nuestro caso, la temperatura de incubación del hospedador durante la época de cría podría regular y controlar la emergencia y ruptura de la diapausa invernal de *Carnus hemapterus*, y de esta forma ayudar a sincronizar la emergencia de los parásitos con la fenología de cría de cada tipo de hospedador, lo que eventualmente podría reforzar el aislamiento temporal y disminuir el flujo genético entre linajes asociados a hospedadores con fenologías de cría distinta. Para testar la hipótesis acerca de si la temperatura del hospedador a través de la incubación de éste durante la estación de cría pudiera afectar a la duración y regulación de la

diapausa en *Carnus hemapterus* y cómo afectaría un cambio de hospedador con una fenología de cría distinta sobre la emergencia y fenología del parásito, se realizaron experimentos sobre muestras conteniendo pupas de *Carnus* en diapausa. Las pupas se sometieron al efecto de la fenología de cría de varios de sus hospedadores más habituales reproduciendo los periodos de incubación en cámaras climáticas en laboratorio. Se estudió el efecto que un cambio de hospedador con un perfil de temperatura distinto del hospedador origen podría tener sobre la plasticidad y duración de la diapausa, simulando los fenómenos naturales de reocupación interespecífica de nidos que ocurren en la naturaleza (Casas-Crivillé & Valera 2005).

2.b. Plasticidad en la fenología de emergencia de *Carnus hemapterus* debido a la variación de las condiciones locales experimentadas. **(Capítulo IV)**.

Los experimentos de translocación son muy útiles en el estudio de adaptaciones locales y plasticidad fenotípica, puesto que permiten obtener la respuesta a las condiciones ambientales experimentadas en el área translocada, aislándola del efecto de las condiciones de origen (Nuismer & Gandon 2008). Si la sincronización entre *Carnus* y su hospedador es principalmente un rasgo plástico esperaremos que no haya diferencias patentes en la sincronización y emergencia de los parásitos translocados de Portugal y los parásitos control de Almería (ambos experimentando el mismo ambiente) y viceversa. Por el contrario si la sincronización está localmente adaptada a las condiciones de origen, esperaremos un nulo o mínimo efecto de las condiciones climáticas del lugar de translocación por lo que no serán esperables diferencias entre las muestras control y las muestras traslocadas de cada una de las localidades implicadas en el experimento. Se utilizaron muestras de parásitos en diapausa de una misma especie (carraca) que cría en dos localidades sometidas a condiciones ambientales (ej. temperatura) diferentes: el desierto de Tabernas, en Almería (clima mediterráneo semiárido) y Castro Verde, en Portugal (estepa de clima mediterráneo con influencia atlántica). Las muestras recogidas en ambas localidades se dividieron en dos partes, una de esas partes se traslocó y la otra quedó como control en la localidad origen, almacenándose en oquedades semejantes a las utilizadas por la carraca para criar.

Se estudió la fenología de emergencia y abundancia de los parásitos en ambas localidades.

3. Búsqueda de evidencias de procesos de diversificación en *Carnus hemapterus* (Capitulo VI)

El estudio de la estructura genética de las poblaciones de cualquier organismo, nos puede ayudar a conocer cual es el grado de diversificación de éstas y cuales son los principales factores que han dado forma a la estructura genética observada. La estructura y diferenciación genética de una especie a lo largo de su área de distribución va a cambiar debido a variaciones espaciales, temporales y/o ecológicas del ambiente que le rodea y que están influyendo sobre el grado de conexión y flujo genético entre los individuos y/o poblaciones. Por tanto para una correcta exploración de la estructura genética de un organismo concreto, se hace necesario abordar estudios que incluyan el mayor número posible de escalas tanto temporales, como geográficas. Los parásitos, particularmente, debido a su íntima relación con los recursos que explotan (hospedadores) están sujetos a presiones selectivas muy intensas, que pueden dar lugar a estructuras genéticas acusadas, muy ligadas al hospedador u hospedadores que parasitan. Si se está produciendo un proceso de diversificación en una especie, es de esperar un cierto aislamiento y reducción del flujo genético entre los distintos linajes, poblaciones o individuos (Coyne & Orr 2004). Con el fin de conocer el grado de diferenciación genética y la influencia de las distintas fuerzas que pueden conformar la estructura genética de esta especie a diversas escalas geográficas, se trabajó con ADN mitocondrial proveniente de moscas de distintos hospedadores y emplazamientos geográficos diversos. Nuestra hipótesis de partida es que la estructura genética de este parásito y las fuerzas que modelan la diferenciación génica en esta especie, pueden cambiar en función de la escala empleada. Mientras que a escalas geográficas amplias los eventos históricos, barreras geográficas o el mero aislamiento producido por la distancia que separa dos poblaciones remotas pueden ser los principales responsables de la estructura genética observada, a escalas menores es posible que la fenología de los distintos hospedadores simpátricos de una misma región puedan ejercer como barreras temporales en *Carnus hemapterus*, aislando

las distintas cepas asociadas a la fenología de cría de cada uno de los hospedadores. Esperamos por tanto encontrar diversas estructuras genéticas y filogeográficas en función del área y de la escala espacial empleada, que expliquen y sugieran cuáles podrían ser los principales eventos y barrreras (selectivas o no) que han modelado la estructura y diversificación genética en este díptero ectoparásito.

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CAPÍTULO I

Ectoparasite intensity is linked to ontogeny and cell-mediated immunity in an avian host system with pronounced hatching asynchrony

Abstract: Several contrasting hypotheses have been proposed to account for host age-biased parasite distribution, some of them suggesting a key role of ectoparasites in the evolution and maintenance of weight hierarchies within broods. We examined parasite distribution among individual hosts across the whole period of host exposure to the parasite in a host system that shows distinct within-brood differences in age and age-related mortality. In contrast to previous hypothesis, we found that the abundance of a haematophagous, mobile ectoparasite *Carnus hemapterus* on nestling European rollers (*Coracias garrulus*) was highest approximately during the mid-nestling stage of their host, coinciding with the inflection point of the host growth phase. Parasite load increased neither with absolute resource availability (i.e. body size) nor body condition index. By contrast to previous evidence, higher parasite load under natural conditions was associated with a stronger cell-mediated immune response. However, this association was moderated by low parasite densities, as well as a better brood body condition index. Overall, although we revealed remarkable host ontogenetic effects on parasite distribution, the present study suggests that a highly mobile ectoparasite generally prefers healthier hosts. We propose that, in host systems with a marked asynchrony of hatching and background mortality within the brood, parasites favour persistence rather than nutritional attractiveness of the host.

Keywords: density dependence - ectoparasites - host choice - immunocompetence – ontogenetic effects – phytohemagglutinin - tasty chick hypothesis.

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Introduction

Knowledge of host-choice strategies by parasites is basic to understanding the evolution of host-parasite associations, the epidemiological significance of such associations, and the fitness consequences for hosts (Christe et al. 1998, Lively 2001, Galvani 2003, Giorgi et al. 2004). Whereas recent work reports host preferences by a variety of parasite systems (Roulin et al. 2003, Mikheev et al. 2004, Hawlena et al. 2005, Krasnov et al. 2005), establishing the general rules of host-choice strategies by parasites has proved to be difficult (Roulin et al. 2003, Hawlena et al. 2005). One such area of controversy exists over the host preference by the parasite in relation to host age.

Several hypotheses have been proposed to account for host age-biased parasite distribution (Table 1). Evidence in favour and against some of the predictions and/or assumptions of these hypotheses have been reported (e.g. Descamps et al. 2002, Simon et al. 2003, Roulin et al. 2003, Valera et al. 2004). The contradictory evidence obtained to date could be explained by particular aspects of the host–parasite systems studied (Roulin et al. 2003, Krasnov et al. 2006), by the effect of confounding variables (e.g. host size, Valera et al. 2004, parasite density, Hawlena et al. 2005, or seasonal effects, Roulin et al. 2007), or because the basic assumptions of the hypotheses were not fulfilled. The results obtained hitherto suggest that parasite host-selection criteria are more complex and dynamic than previously thought and our approaches to the problem may have been insufficient. Several approaches have been followed to advance knowledge in this issue. Some authors have focused on individual species (Christe et al. 1998, 2003, Descamps et al. 2002, Simon et al. 2003, Valera et al. 2004), others have addressed the problem using a multi-species approach (Roulin et al. 2003), or novel host–parasite system (Hawlena et al. 2005).

Though parasite distribution has become increasingly studied across different host–parasite systems, surprisingly little information exists about the key assumptions that are necessary to establish predictions of parasite distribution (e.g. ontogenetic variation in host immunity and body condition, Bize et al. 2003), the applicability of predictions to different host systems (e.g. host systems with a variable predictability of background mortality), or the flexibility of parasite

decision criteria in relation to changing ecological factors (Hawlena et al. 2005). Thus, a detailed examination of age-biased parasite distribution models under natural conditions, with careful consideration of underlying assumptions and confounding variables, would be opportune to further advance our understanding of parasite distribution.

In the present study, we examine current age-biased parasitism hypotheses (see Table 1), using a novel study system involving an ectoparasitic fly *Carnus hemapterus* (Nitzsch, 1818; Diptera: Carnidae) and the nestlings of an avian host, the European roller (*Coracias garrulus*, Linnaeus, 1758). This system is relevant for an investigation into age-biased parasite distribution because *C. hemapterus* only parasitizes nestlings (Grimaldi 1997), it does not need a host for transmission (Grimaldi 1997), it is haematophagous and therefore it may likely be affected by host immune defence (Wakelin & Apanius 1997), and nestling rollers show distinct within-brood differences in age and age-related mortality. Thus, considering their good chemosensory and locomotory abilities and the close spatial proximity of their potential hosts, *Carnus* flies are excellent candidates for exhibiting host preferences at an individual level.

Hypothesis	Assumption	Prediction	Reference
Tasty chick	Junior nestlings are less immunocompetent than their senior siblings	Within broods parasites prefer junior rather than senior nestlings	Christe *et al.* (1998)
Well-fed host	Older hosts represent better nutritional resources than younger hosts	Parasites prefer older (well-fed) rather than younger (poorly-fed) hosts	Christe *et al.* (2003)
Context-dependent	Quality of adult and young hosts is relative	Parasites change their preference for adult (well-fed) and juvenile (poorly-fed) hosts depending on host, parasite, and environment-related factors	Hawlena *et al.* (2005)

Table 1. Hypotheses on host age-dependent variation in parasitism intensity

Material and Methods

Study area and species

The study area was located at the Desert of Tabernas (Almería, South-east Spain, 37º05' N, 2º21' W). The landscape mostly consists of badlands and wadis with olive and almond groves interspersed among numerous dry riverbeds. Climate in this area is semi-arid with long, hot summers and high annual and seasonal variability of rainfall (mean annual rainfall: 218 mm). There were strong inter-annual differences in weather during the study period of two years (2005 and 2006), with the winter in 2005 being the coldest one in the last 20 years (range of minimum temperatures from 1 Jan to 31 Mar, 2005 = −7.7ºC to 13.4ºC; 2006 = −0.7ºC to 10.0ºC). This, together with an extremely dry breeding season of 2005 (total precipitation from 1 Apr to 15 Jul: 2005 = 6.6 mm; 2006 = 129.8 mm) reduced primary and secondary productivity in 2005 and likely accounted for the poorer breeding performance of rollers. Both clutch size and brood size were significantly lower in 2005 than in 2006 (clutch size ± SE, 2005 = 4.28 ± 0.17, N = 39; 2006 = 5.13 ± 0.12, N = 45; separate variance t-test: t = 4.15, $P < 0.001$; brood size at the time of *Carnus* parasitism assessment ± SE, 2005 = 3.35 ± 0.35, N = 23; 2006 = 4.52 ± 0.20, N = 25; t-test: t = 2.98, $P < 0.005$).

The European roller (hereafter just roller) is a common avian breeder in the study area, occupying natural holes excavated in sandy cliffs as well as cavities in human constructions. Because laying occurs at 2-day intervals and incubation begins before the clutch is complete, egg hatching in rollers is distinctly asynchronous (within-brood nestling ages are in the range 2-10 days; R. Václav, unpublished data). We detected prominent age-biased mortality in nestling rollers. In particular, the mortality of the youngest and second youngest nestlings amounted to 82% (51/62 nests) and 39% (24/62 nests), respectively. The actual mortality rates of junior nestlings are likely even higher because we estimated mortality during the mid-nestling stage. Nestlings are naked at hatching, but by the age of 13 days their body is almost completely covered with closed feather sheaths. The sheaths open from around 15–17 days, with flight feathers appearing first, followed by the feathers of the throat, belly, and rump (Cramp

1998, R. Václav, personal observation). Nestling rollers fledge approximately 20–22 days after hatching (R. Václav, personal observation).

Carnus hemapterus (hereafter just *Carnus*) is a 2 mm-long, highly mobile ectoparasitic fly that colonizes nestling birds (Grimaldi 1997), usually concentrating on a few specific regions of the host, such as inguinal and axillary areas and the skin at the base of wing axillae (Marshall 1981). This haematophagous parasite (Kirkpatrick & Colvin 1989, Dawson & Bortolotti 1997) can have detrimental effects on nestling health (Whitworth 1976, Cannings 1986, Soler et al. 1999), even though some studies found no evidence that *Carnus* infestation adversely affects hosts (Kirkpatrick & Colvin 1989, Dawson & Bortolotti 1997, Liker et al. 2001). Adult flies have a winged and wingless phase. After their emergence, adults are initially winged, but lose their wings as soon as they locate a suitable host (Roulin 1998). Carnid flies do not need a host for transmission because they actively colonize hosts' nests during the winged phase of their life cycle (Grimaldi 1997). Therefore, it is unlikely that the need for successful transmission could influence *Carnus* host choice. *Carnus* emergence is usually synchronized with the occurrence of the host and persists continuously throughout the whole nestling period of the host (see Valera et al. 2003). Other aspects of the life cycle of this parasite, like the life span of adult flies, or the rate of feeding are, to our knowledge, unknown.

Field methods

Fieldwork was carried out in 2005 and 2006. From the first observations of rollers in early April, a population holding about 40 pairs was visited at least five times per week. After sighting first copulations, potential nest cavities were inspected every other day until the day of hatching of the last chick of the study population. During regular nest inspections, we monitored progress in egg laying and hatching. We took measurements of wing length (maximum flattened chord measured to the nearest 1 mm with a wing rule), body mass (with 0.1 g accuracy), and tarsus length (with 0.1 mm accuracy) after all chicks in a brood hatched and then always on both days of the nestling immunity test (see below).

The relationship between wing length and body mass is based on the values taken at the day of phytohemagglutinin (PHA) administration for all the nestlings involved in our analyses (N = 180). Based on the logistic term including wing length and body mass, we calculated residual body mass, which served as a body condition index (Schulte-Hostedde et al. 2005). Within-nest (brood) body condition refers to the mean body condition index of all nestlings in the brood.

To estimate the size of habitat patch available for *Carnus* flies, we calculated nestling body surface area by raising nestling body mass to the 2/3 power (Heusner 1985). In 2006, nestling body temperature (cloacal) for 22 broods was measured with a thermometer immediately before PHA administration. As body temperature gradually increased with nestling age, in the analysis of *Carnus* parasitism, we used the residuals of body temperature from the linear regression between body temperature and wing length.

Host immunity assessment

We estimated nestling cell-mediated immunity (CMI) by administering to each nestling 0.4 mg of PHA (PHA-P; Sigma L8754) in 40 µl of phosphate-buffered saline, injecting PHA solution subcutaneously into the nestling's left wing patagium. PHA is a mitogen stimulating the proliferation of T-lymphocytes, but also other leukocyte types, with a resulting inflammatory response and a swelling at the site of PHA inoculation (Elgert 1996). Wing web thickness was measured five times at the injection site with a digital thickness gauge (Mitutoyo 7/547) before and 24 h after PHA administration. In analyses we used mean values of wing web thickness. The PHA skin-swelling response was estimated as the difference between mean initial and final left wing web thickness (Smits et al. 1999, Martin et al. 2006). PHA treatment was usually carried out when the oldest nestlings in the brood reached 8–12 d of age. This period was chosen because it coincided with the most intensive brood infestation by *Carnus* flies. All nestlings in the nest were injected with PHA solution at the same day and only once during the nestling period.

Carnus hemapterus sampling

Within-brood variation in *Carnus* load was studied in 2005 (23 nests) and 2006 (25 nests) at the day of PHA administration. Roller broods were carefully taken from the nest and placed in a cotton bag. Subsequently each nestling was separated and placed in an individual cotton bag and the number of carnid flies on the body surface of each chick was counted twice. This visual census method has been found to be reliable (Roulin 1998, Roulin et al. 2001). The number of flies recorded in both counts for 195 nestlings was highly repeatable (r_I = 0.98, *df* = 194, 195, $P < 0.001$). Thus, we used means calculated from the two counts. Every nest was sampled once. Additionally, to study the relationship between the occurrence of the flies on a nestling and actual *Carnus* parasitism, we recorded the occurrence of bites by *Carnus* flies (conspicuous bloody marks distributed mainly under the wings and along feather sheaths, see Soler et al. 1999) for all the nestlings sampled at the day of PHA administration in 2006 (N = 114). We found a significant positive association between the occurrence of flies and the occurrence of skin lesions (of 101 nestlings with *Carnus* flies, 101 nestlings had wounds, whereas of 13 nestlings without *Carnus* flies, 5 had and 8 did not have wounds; Yates corrected χ^2 = 57.75, *df* = 1, $P < 0.001$), suggesting that *Carnus* flies used the nestlings on which they were found for feeding. In most cases the only ectoparasite found on nestling rollers was *C. hemapterus*. Exceptionally, Ixodes ticks and haematophagous mites infested two roller nests in 2005, and four nests had haematophagous mites in 2006.

Data analysis

We studied *Carnus* parasitism across all developmental stages of nestling rollers. Therefore, the first step was to scale nestling *Carnus* load intensity, immune response, body temperature, and body mass to a measure of nestling age. We used wing length as a measure of nestling age (see Lessells & Avery 1988 for a similar avian system). The relationships between the studied variables and wing length were examined with quadratic (parasite load; see Results), linear [body temperature (°C) = 3799 + 0.25 (wing length); r^2 = 0.19, $F_{1,93}$ = 21.83, $P < 0.001$],

sigmoidal (logistic: body mass = $a/[1 + be^{-r(\text{wing length})}]$; Weibull, Gompertz, and Richards) and rational models (including polynomials; rational: CMI response; see Results). The most successful model (explaining most of observed variance) for each relationship was used to calculate residuals.

In 2006, 20 nestlings from four nests showed more than three times higher *Carnus* infestation rates than usual for nestlings of their age. Also, unlike for nestlings from other nests, the nestlings in the four nests were clearly apathetic and lethargic. Therefore, to avoid skewing the regression computation toward the 20 nestlings, we established the regression curve between *Carnus* load and wing length without the 20 nestlings. However, we calculated residual *Carnus* load and examined *Carnus* parasitism for all nestlings. Including the 20 nestlings into analysis on *Carnus* load does not change main results.

The predictors of *Carnus* infestation intensity were examined with general linear models; we checked the assumptions of normality (Shapiro-Wilks *W* test), homoscedascity (Cochran test), and linearity (residual plots inspections). Also, we tested the assumption that there is no interaction between categorical factors and continuous predictors with homogeneity-of-slopes generalized linear model (GLM). To overcome the problem of intercorrelation between continuous predictors, we included in the model the interaction term between significantly correlated predictors (Neter et al. 1996). To achieve normality, *Carnus* load data (counts) and nestling body surface area were square-root and square-transformed, respectively, before analysis.

Because we detected significant inter-annual differences in CMI, body condition index, and *Carnus* load intensity (see Results), we controlled for the effect of year by calculating residuals from a linear regression of a respective variable on year, entering year as a dummy variable.

We studied host-parasite relationships at nestling and brood levels. In order to avoid the problem of pseudo-replication when using nestlings as data points and to control for parental effects (e.g. laying date), we used general linear mixed models with nest as a random effect. Based on colour ringing, we estimate that about 30% (14/46) of adults bred in the study population in both years. In order to see whether pseudo-replication could affect our results, we randomly

subsampled (10 times) the original data set with a sampling rate of 70%. Because subsampling did not affect our main findings, the complete data set was used for analyses. Unless otherwise stated, means ± 1SE are presented throughout the article.

Results

Patterns of Carnus parasitism

Infestation of nestling rollers by carnid flies was widespread among nests in both study years, with 100% prevalence of *Carnus* parasitism found both in 2005 (range: 5–120 flies/nest, $N = 23$) and 2006 (3–458 flies/nest, $N = 25$). The *Carnus* load of examined nests was considerable in both study periods (2005: 14.42 ± 4.28 carnid flies/nest, $N = 23$; 2006: 29.62 ± 4.11, $N = 25$), but it was significantly higher in 2006 than 2005 (separate variance *t*-test: $t = 2.54$, df = 40.9, $P = 0.015$). *Carnus* load varied extensively within nests (2005, range: 0–63 flies/nestling, $N = 72$; 2006: 0–179 flies/nestling, $N = 108$). Of 14 (of 180) nestlings showing no *Carnus* infestation, nine nestlings were youngest and five second-youngest in the brood age hierarchy.

Overall, there was a quadratic relationship between *Carnus* load and roller wing length [Sqrt-transformed *Carnus* load = −3.88 + 2.29(wing length) − 0.16(wing length)^2; $F_{3,157} = 239.44$, $P < 0.001$], so that the former increased sharply until the nestling's wing length reached 7–8 cm (~13-14 d of age), after which it declined rapidly (Fig. 1). *Carnus* load was not influenced by habitat availability (i.e. nestling body surface area) because the relationship between the nestling's body surface area and *Carnus* load was quadratic [sqrt-transformed *Carnus* load = −2.51 + 0.02(square-transformed body surface) − 0.000022(square-transformed body surface)^2, $F_{3, 157} = 207.51$, $P < 0.001$)]. That is to say, nestlings weighing around 100–120 g carried more flies than smaller and bigger nestlings.

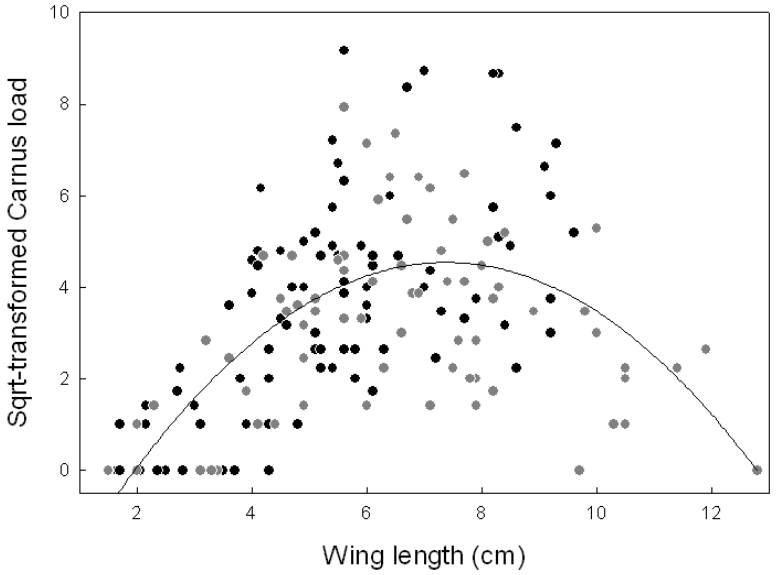

Figure 1. Relationship between nestling wing length (cm), age and *Carnus* load. Except for four nests with extremely *Carnus*-infested nestlings (N = 20 chicks), the *Carnus* plot shows data for both study years (2005: grey circles; 2006: black circles).

Patterns of host body condition and immunocompetence

The relationship between body mass and wing length followed a logistic function [body mass (g) = $138.58/(1 + 5.61e^{-0.46(\text{wing length})})$, $F_{3,177} = 5441.85$, $P < 0.001$; Fig. 2]. Nestlings and broods in 2006 showed higher body condition index than in 2005 (nestlings: separate variance t-test: $t = 4.05$, $P < 0.001$, $N = 180$; broods: t-test: $t = 2.27$, $P = 0.028$, $N = 48$). A body condition index did not show any clear relationship with wing length (linear model: $r^2 = \sim 0$, $F_{1, 178} < 0.01$, $P = \sim 1$).

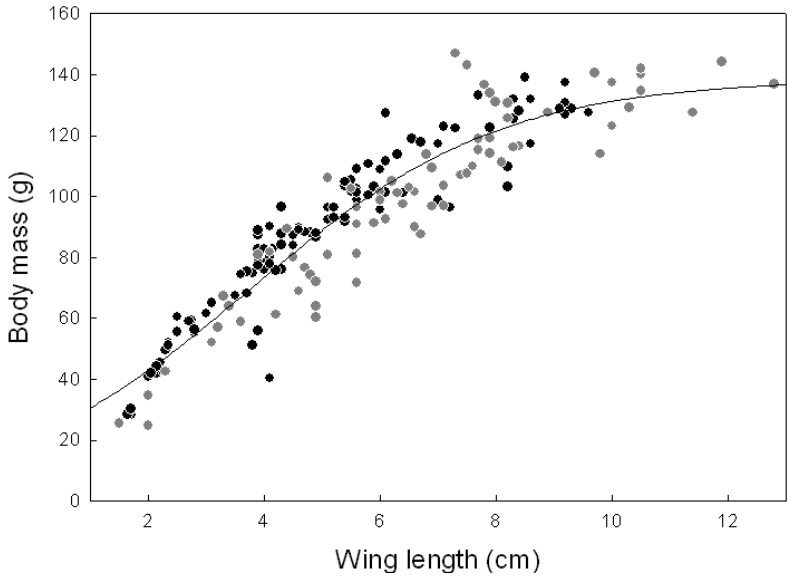

Figure 2. Relationship between nestling wing length (cm) and body mass (g). The graph includes data for both years (2005: grey circles; 2006: black circles).

The CMI response peaked when the nestling's wing length reached 6–7 cm (i.e. ~12 d of age), and a gradual fall continued thereafter until the fledging date [rational function, CMI response (mm) = (0.07 + 0.27[wing length])/(1 − 0.17[wing length] + 0.02[wing length]^2), $F_{4,176}$ = 294.87, P < 0.001; Fig. 3]. The relationship between CMI and wing length was not confounded by *Carnus* load (CMI response corrected for *Carnus* load = (0.80 + 0.35[wing length])/(1 − 0.06[wing length] + 0.01[wing length]^2, $F_{4,176}$ = 516.05, P < 0.001]. The CMI response was higher in 2006 compared to 2005 both at an individual (t-test: t = 2.85, P < 0.005, N = 180) and, marginally, at a brood (t-test: t = 1.82, P = 0.075, N = 48) level.

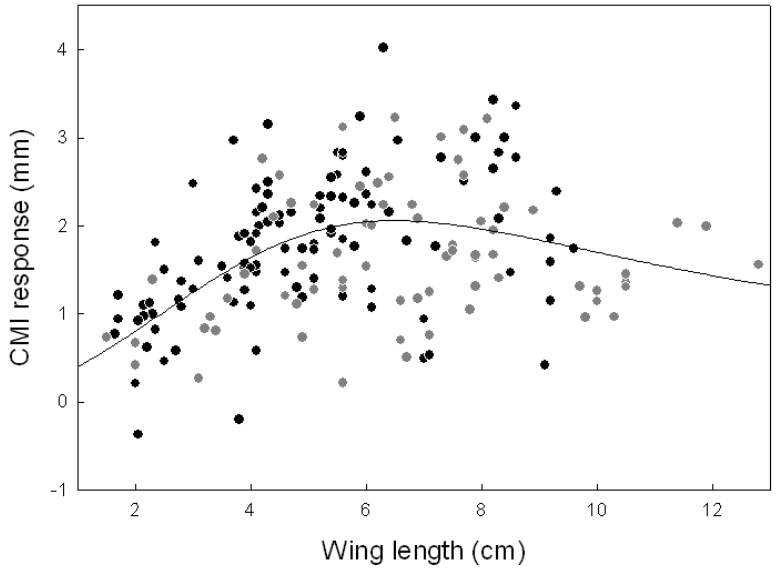

Figure 3. Relationship between nestling wing length (cm) and CMI response (mm). The graph includes data for both years (2005: grey circles; 2006: black circles).

General predictors of Carnus parasitism

The nestling's CMI response was a significant predictor of *Carnus* load (Table 2); *Carnus* load was positively related to the CMI response (slope = 0.13, $t_{129} = 2.12$, $P = 0.036$), though this relationship seemed to have been mediated through interaction with body condition (Table 2).

	SS	*df*	*F*	*P*
Nest	42.83	47	6.79	0.001
CMI response	0.60	1	4.49	0.036
Body condition index	0.27	1	2.00	0.16
CMI response × Body condition index	0.52	1	3.84	0.052
Error	17.32	129		

Table 2. Predictors of nestling Carnus hemapterus load. The nestling's CMI response was standardised for wing length through non-linear (rational) regression. The nestling's body condition index was calculated by scaling body mass to wing length using a logistic regression model. Both predictors were corrected for year. Nest was entered as a random factor.

When comparing the pairs of siblings showing similar wing lengths but contrasting CMI responses (wing length: high-CMI response chicks = 6.31 ± 0.31 cm; low-CMI response chicks = 6.03 ± 0.33 cm; paired *t*-test, $t = 1.71$, $P = 0.094$, $N = 45$ sibling pairs; wing length- and year-corrected CMI response: high-CMI response chicks = 0.58 ± 0.09 mm; low-CMI response chicks = −0.46 ± 0.08 mm; $t = 10.23$, $P < 0.001$, $N = 45$), we found that siblings with a stronger CMI response showed heavier *Carnus* infestation than their siblings with a lower CMI response ($t = 2.37$, $P = 0.022$, $N = 45$ pairs; Fig. 4).

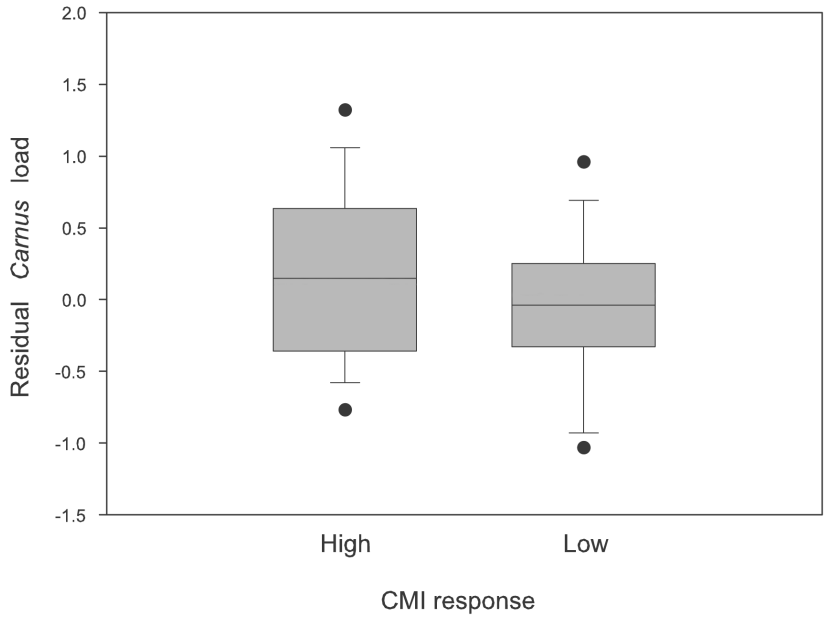

Figure 4. Difference in *Carnus* load between pairs of siblings showing a contrasting CMI response but comparable size. *Carnus* load was sqrt-transformed, wing length-corrected and then corrected for inter-annual differences. The plot shows means (solid lines), lower and upper quartiles (boxes), 10th and 90th percentiles (whiskers), and outliers (dots).

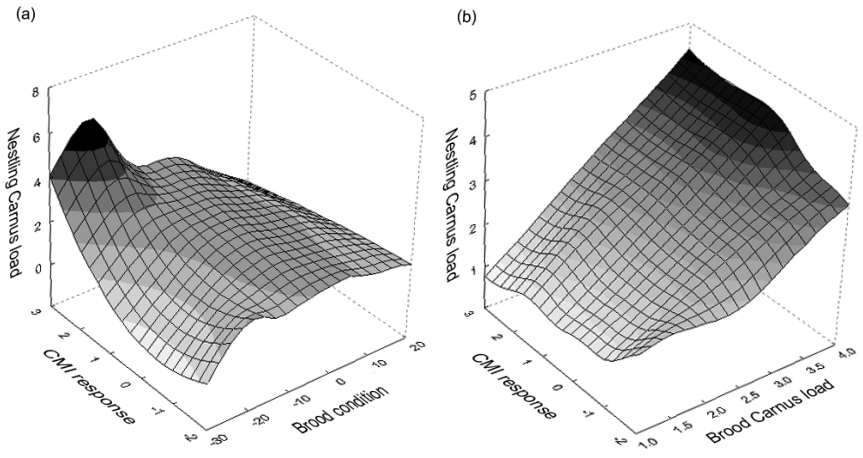

Figure 5. Relationship between nestling cell-mediated immunity (CMI) response, *Carnus* load and brood (a) body condition index and (b) *Carnus* load. Brood body condition was calculated by averaging nestling body condition indexes within broods. Overall, brood *Carnus* load was calculated by averaging wing length-corrected and sqrt-transformed nestling *Carnus* loads within broods. Nestling *Carnus* load and phytohemagglutinin response were corrected for wing length.

Context-dependent Carnus parasitism

Examining *Carnus* parasitism at a brood level, we found that there was a significant interaction effect of the within-nest CMI response and year on nest *Carnus* load (homogeneity of slopes GLM: year × within-nest CMI response, $F_{1,40} = 5.61$, $P = 0.023$). Consequently, analysing the two years separately, we found that *Carnus* parasitism within nests was a positive function of the within-nest CMI response in 2005 (multiple linear regression: $r^2 = 0.29$, $F_{2,20} = 4.06$, $P = 0.033$; within-nest CMI response, slope = 0.55, $t_{20} = 2.83$, $P = 0.010$, within-nest body condition index, slope = −0.19, $t_{20} = -1.00$, $P = 0.33$), but not in 2006 ($r^2 = 0.05$, $F_{2,22} = 0.57$, $P = 0.57$; within-nest CMI response, slope = −0.18, $t_{22} = -0.85$, $P = 0.40$; within-nest body condition index, slope = 0.15, $t_{22} = 0.73$, $P = 0.47$).

Similarly, as for the within-nest *Carnus* load, we found a tendency for the interaction effect of year and nestling CMI response (homogeneity of slopes GLM, including year, nestling CMI response and body condition index: $F_{1,172} = 3.81$, $P = 0.052$) but also year, nestling CMI response and body condition index ($F_{1,172} = 3.55$, $P = 0.061$) on nestling *Carnus* load. Year effects could be attributed

to the inter-annual differences in brood body condition and *Carnus* load (see above). Thus, to test whether there is a relationship between a nestling's *Carnus* parasitic load and its CMI response while controlling for the year effects, we examined the interaction effects of the nestling's CMI response and brood body condition index and brood *Carnus* abundance on nestling *Carnus* loads (Table 3). We found that when brood condition was low, the nestlings with the strongest CMI response were most highly parasitized, but if brood condition was high, the link betwen the nestling's CMI response and *Carnus* load was largely absent (Fig. 5a). In turn, if broods were more highly parasitized, the most highly parasitized chicks were those with the highest CMI response, but if brood *Carnus* abundance was low, the association between CMI and *Carnus* load disappeared (Fig. 5b).

	SS	*df*	*F*	*P*
Nest	48.92	47	7.96	0.001
Nestling CMI response	0.28	1	2.10	0.149
Brood condition × Nestling CMI response	0.66	1	5.02	0.027
Brood *Carnus* load × Nestling CMI response	0.52	1	3.94	0.049
Error	16.88	129		

Table 3. Predictors of nestling *Carnus hemapterus* load, assuming that the form of the relationship between the nestling's CMI response and *Carnus* load is dependent on the within-nest body condition index and *Carnus* load, (i.e. the two traits that differed between years). The nestling's CMI response was standardised for wing length through non-linear (rational) regression. Nest was entered as a random factor.

Phenotypic cues to host immunocompetence

Apart from ontogenetic effects (see Fig. 3), nestling CMI could also covary with body condition or body temperature. There was no interaction effect of year and body condition index on CMI response (homogeneity of slopes GLM: year × body condition index, $F_{1,176} = 0.11$, $P = 0.74$). Consequently, utilizing pooled data from both years, we found that the nestling's CMI response was weakly, but significantly, related to body condition index (linear regression: $r^2 = 0.06$, slope =

0.25, $t_{178} = 3.41$, $P < 0.001$). After examining nestling body temperature data from 2006, wing-length corrected body temperature turned out to be a more sensitive predictor of nestling CMI than body condition index (multiple linear regression: $r^2 = 0.10$, $F_{2,92} = 4.92$, $P < 0.01$; size-corrected body temperature, slope = −0.23, $t_{92} = -2.33$, $P = 0.022$; body condition index, slope = 0.19, $t_{92} = 1.92$, $P = 0.059$). Despite this fact, the nestling's CMI response remains the most important predictor of *Carnus* load (Table 4).

	SS	*df*	*F*	*P*
Nest	453.91	21	6.95	0.001
CMI response	13.91	1	4.44	0.039
Body condition index	0.13	1	0.04	0.84
Body temperature	0.42	1	0.14	0.71
CMI response × Body condition index	8.63	1	2.77	0.10
CMI response × Body temperature	0.58	1	0.19	0.67
Error	211.60	68		

Table 4. Predictors of nestling *Carnus hemapterus* load including body temperature. The CMI response and body temperature were standardised for wing length through non-linear (rational) and linear regression, respectively. A body condition index was calculated by scaling body mass to wing length using a logistic regression model. Nest was entered as a random factor. The results are based only on data from 2006.

Discussion

Ontogenetic patterns of Carnus parasitism and host immunity and condition

Under natural conditions *Carnus* loads on nestling rollers peaked approximately during the mid-nestling stage when nestlings were just at the inflection point of their growth phase. *Carnus* load did not increase with increasing absolute resource availability because host body mass increased sinusoidally during the nestling period. *Carnus* flies did not have a propensity to aggregate on older or younger hatchlings regardless of their position in the brood hierarchy.

The immune activity of nestling rollers was non-linearly related to a measure of age; the immune response to a T-cell mitogen (PHA) increased sharply until ca 12 d of age when it started to decline (see also Tella et al. 2002). Because the inclusion of nestlings of the same age but with a variable position in the brood age hierarchy did not distort the sigmoidal CMI–wing length relationship, the ontogeny of the immune system appears to play a vital role in variation in the immune function of nestling rollers, with mid-aged nestlings showing a relatively stronger CMI response compared to that of older and, particularly, younger nestlings.

By contrast to nestling immunity or *Carnus* load, an index of body condition did not clearly change during the nestling phase. Nevertheless, a body condition index still represents a relevant biological measure for nestling rollers because it correlated positively with CMI.

Within-brood Carnus distribution

Three hypotheses were proposed about how parasites should distribute themselves among available hosts in relation to host age. The present study does lend direct support to neither of them but instead stresses the importance of the time at which parasite load is estimated. Given that the parasite infestation of an individual host changes with its age, parasite loads for the same brood could be higher in senior or junior siblings (i.e. in older or younger half of a brood) depending on the time of parasite load estimation. For example, Valera et al. (2004) showed that *Carnus* flies preferred bigger hosts in a similar host system (European bee-eaters, *Merops apiaster*) to that used in the present study. However, while Valera et al. (2004) reported that the highest *Carnus* load occurs in bee-eater nestlings at the age of 15 days (note that bee-eaters fledge at approximately 30 days of age), Valera et al. (2004) only examined the parasite choice for 5-15-day-old nestlings. We propose that the ontogenetic pattern of parasite infestation intensity and host immunity be known before making any inference about the host exploitation strategy in relation to host age.

We found that, irrespective of hatching order, nestling rollers until about 5 d of age were free or carried minimal numbers of *Carnus* flies. After controlling

for year and nest-related effects and for host age and body condition index, the highest *Carnus* infestation rates were consistently found on the nestlings mounting the strongest CMI. Thus, our results disagree with the idea of Christe et al. (1998) that parasites should distribute themselves on the most vulnerable host within a brood. Roulin et al. (2003) suggested that parasites might not show a consistent preference for the least resistant host in all host systems due to the low nutritional profitability of "vulnerable" hosts or due to the high costs of sampling and switching among potential hosts. Our study supports the findings of Roulin et al. (2003) demostrating that *Carnus* ectoparasites avoid certain host phenotypes, particularly feathered nestlings. There is evidence that *C. hemapterus* feeds on the basis of growing feathers (Marshall 1981), and thus the avoidance of older chicks by *Carnus* flies may be due to the retraction of blood vessels nourishing feathers as well as the keratinization of feather sheaths.

The present study is apparently in agreement with the "well-fed host strategy" proposed by Christe et al. (2003) because we found that the highest *Carnus* infestation coincided with the time when both nestling growth and body mass culminated. Nevertheless, when examined together, nestling CMI turned out to explain significantly more of the variance in *Carnus* infestation intensity than body condition. Nutritional status and immunity are usually tightly positively correlated (for birds see Alonso-Alvarez & Tella 2001). Nevertheless, these two parameters may provide different cues to host quality. Although nestling body condition can be a proxy for the quantity and/or quality of food resources, nestling immune function also is a reliable predictor of nestling survival prospects (e.g. Christe et al. 1998, Gonzalez et al. 1999, Christe et al. 2001, see Møller & Saino 2004). Moreover, the development of an immune function can be traded off with other important functions such as growth (Saino et al. 1998; see Norris & Evans 2000). Therefore, immunity and condition may not always covary. For example, Dubiec & Cichoń (2005) showed that although condition in late and early hatched nestling great tits *Parus mayor* did not differ, the nestlings hatching later developed a less competent immune system. We propose that in host systems with age-biased background mortality parasites preferring fitter nestlings might not seek abundant but persistent food resources (Jovani & Serrano 2002).

The present study suggests that, under high brood conditions, *Carnus* flies moderated their preference for immunocompetent nestling rollers. Christe et al. (2003) proposed that parasites can exploit either vulnerable or well-fed hosts depending on between-host differences in physiological status. In particular, it was suggested that parasites switch between adult and juvenile hosts depending on differences in host nutritional status. However, adults and juveniles differ profoundly in more than just nutritional status. For example, juveniles commonly have lower survival prospects than adults (see Gaillard et al. 1998), which has been attributed to the lower capacity of the immune system in juveniles (Gonzalez et al. 1999, Tella et al. 2000).

Independent of host-related effects, Hawlena et al. (2005) found that the switch in parasite preference occurs with changing parasite density. The present study supports the result of Hawlena et al. (2005) that under high intra-specific parasite density parasites prefer a less vulnerable host. The Hawlena et al. (2005) were not able to discriminate whether the switch occurred due to differences in host mortality or nutritional status. By contrast, our results suggest that parasites prefer a less vulnerable host due to its better survival prospects rather than nutritional quality because under high parasite densities parasites seemed to enhance their preference for the immunocompetent host. Overall, we revealed a high degree of flexibility in host-selection criteria with respect to host- (ontogenetic) and parasite-related (socio-ecological) factors.

Why should host immunity be important?

Immune function is an important life history trait that has evolved to increase fitness through longevity; yet immune function is continuously traded off with other bodily functions that are immediately more important (Sheldon & Verhulst 1996). Thus, immunocompetence can be a sensitive marker for the physiological burden placed upon an animal, indicating whether the animal trades survival in immediately unfavourable conditions with a less-well developed immune defence mechanism (Lochmiller & Deerenberg 2000). The positive and negative relationships of CMI with body condition index and size-corrected body temperature, respectively, imply that the PHA-induced immune response did not

simply follow trends in parasite loads but instead reflected health status because nestling rollers mounting a weaker CMI response had lower body mass index and elevated internal body temperature (Roulin et al. 2007).

Nestling rollers represent a host system with differential within-brood mortality where last-hatched nestlings face very small chances of survival until fledging. Therefore, ectoparasites infesting nestling rollers such as *Carnus* flies could be under selection to aggregate on a more persistent host because host recolonization might imply switching to lower quality hosts that are likely to perish or to higher quality individuals that are likely already settled by conspecifics. Provided that intraspecific competition among unrelated parasites further increases parasite virulence and host mortality (Frank 1996, Galvani 2003), the preference for a less vulnerable host should be particularly important under high parasite densities. Our results stress the fact that *Carnus* flies favoured the most immunocompetent hosts consistently across broods of all ages. However, under closer examination, the preference for healthy nestlings turned out to be strongest particularly under conditions that heighten the risk of host mortality, (i.e. low brood condition and high parasite density). Therefore, we propose that host mortality can affect the preference for relatively healthy hosts in systems with high host background mortality.

Experimental work has shown that parasitisation usually negatively affects host CMI (Christe et al. 2000, Martin et al. 2006). The apparent lethargy and apathy of heavily infested broods of rollers suggests that high *Carnus* abundance can be detrimental to nestling rollers. However, under natural conditions more infested nestling rollers showed better CMI than their less infested siblings. Therefore, The present stody implies that *Carnus* flies may flexibly switch among hosts and select those with immediately better health status.

In summary, after taking into account strong ontogenetic effects on parasite distribution, our study strongly suggests that in a host system with heterogeneous within-brood background mortality, a haematophagous ectoparasite preferred healthier hosts. This implies, on one hand, outstanding discrimination abilities of the parasite because host health changes dynamically during host ontogeny. On the other hand, the ability of *Carnus* to moderate its

preference according to ecological circumstances implies some degree of flexibility in host selection criteria. Finally, our study suggests that the host's health may not always be a consequence of parasite abundance, but that parasites might cue on hosts' health status and colonize them accordingly.

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CAPÍTULO II

Prolonged diapause in the ectoparasite *Carnus hemapterus* (Diptera: Cyclorrhapha, Acalyptratae) - how frequent is it in parasites?

Abstract: Prolonged diapause is usually interpreted as an adaptation to unpredictable environmental conditions and resource availability. Many parasites usually face highly unpredictable environments; therefore prolonged diapause should be common among these organisms. Here we examine the occurrence and frequency of prolonged diapause in the ectoparasite *Carnus hemapterus* (Diptera: Cyclorrhapha, Acalyptratae). We found that the studied population is polymorphic with respect to diapause duration. Emergence of carnid flies after two and three wintering seasons was therefore detected in around 17% and 21% of the samples respectively. The number of flies with prolonged diapause ranked 0.88%-50% with respect to the number of flies emerging the first spring. Both the occurrence of prolonged diapause and the number of flies with long life cycle are related to the number of flies emerging the first spring. The emergence pattern of flies with prolonged diapause was very similar to the one observed for flies with short cycle and occurred in synchrony with the occurrence of hosts. Prolonged diapause has been frequently reported in plant-feeding insects and in some host-parasitoid systems, but this is, to our knowledge, the second report ever on prolonged diapause in true parasites of animals. We discuss the reasons for the apparent rarity of prolonged diapause among these organisms.

Keywords: Bet hedging, *Carnus hemapterus*, life history, long life cycles, prolonged diapause.

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Introduction

Life cycles of parasites may provide important information on the pathogenic importance of each particular parasite, its epidemiological significance in relation to the development of effective control programs, and the evolutionary potential of parasites. An important determinant of the cycle of parasites and insect herbivores is resource location. For these organisms, resource detection has a double dimension: they must find an appropriate host but also ensure that their host-feeding stages are synchronised with the times when those hosts provide the appropriate food resource. Therefore, these animals have evolved dispersal and developmental mechanisms (especially diapause) to safeguard their success in finding an appropriate host at the right moment (Enright 1970, Danks 1987, Tauber et al. 1986, Jones 2001). Diapause is a form of dormancy determined both by genetic and environmental factors that allows individuals to survive when conditions are unfavourable for development and reproduction, and ensures synchronization of active stages with favourable conditions (Tauber et al. 1986, Danks 1987, 1999, Soula & Menu 2003). Diapause and dispersal have been considered as two alternative responses to unfavourable environmental conditions (Southwood 1977, Hanski 1988, Bohonak & Jenkins 2003), so that temporal dispersal via developmental mechanisms (especially diapause) is considered to be functionally equivalent to spatial dispersal (Hairston 2000, Hairston & Kearns 2002, Bohonak & Jenkins 2003).

In many insect (Danks 1987, 1992, Tauber & Tauber 1981, Hanski 1988, Menu et al. 2000) and plant species (Philippi, 1993a, b, Clauss & Venable 2000) life cycle duration varies within the population. Some individuals of the same generation may miss one or more breeding opportunities by remaining longer in diapause than others, a phenomenon called prolonged diapause (Hanski 1988). Prolonged diapause is usually interpreted as an adaptation to unpredictable environments (Hopper 1999) and has been investigated primarily in plant-feeding insects and their parasitoids (Danks 1987, 1992; Hanski 1988, Soula & Menu 2005). Parasites, probably more than any other group of organisms, face a high degree of variability and unpredictability in their environment. Since unpredictability in the temporal availability of susceptible hosts is a likely

selective pressure affecting the life history strategies of parasites (Hakalahti et al. 2004), life cycle adaptations to such unpredictability, like prolonged diapause, would be expected. Long life cycles that include prolonged diapauses are especially common in plant-feeding insects dependent on potentially unreliable food supplies (Danks 1987, 1992, Soula & Menu 2005), but few studies report on prolonged diapause in "true" parasites (i.e. parasites that usually do not kill their hosts) of animals (but see Baird 1975).

Here we report on intrapopulation variation in the diapause pattern and prolonged diapause of a widespread ectoparasite of a large number of bird species, *Carnus hemapterus* (Diptera: Cyclorrhapha, Acalyptratae) (Capelle & Whitworth 1973, Kirkpatrick & Colvin 1989, Dawson & Bortolotti, 1997, Grimaldi 1997). We aim to contribute to the knowledge of life cycles of parasites by providing evidence of prolonged diapause in a parasite species and by addressing the question of how frequent is prolonged diapause in true parasites of animals and the reasons for its apparent low frequency in this group.

Material and methods

Study species

Carnus hemapterus is a 2-mm long blood-sucking fly that parasitises nestling of a variety of bird species (Grimaldi 1997). Neither adult carnid flies nor larvae have been found on adult birds. The term host therefore refers in this system exclusively to the nestlings of the attacked species. *Carnus hemapterus* is distributed throughout the Palearctic region and eastern and northern North America. Its life cycle comprises an adult stage, three larval phases encompassing around 21 days at 22 ºC and 95% relative humidity and a nymphal stage. After a diapause usually lasting several months (Guiguen et al. 1983) imagines emerge the following spring at the time after birds have reoccupied nesting sites, thus allowing the perpetuation of *Carnus* in the nest. Adults are initially winged and capable of flying, but they typically lose their wings once they have found a suitable host (Roulin 1998, 1999). Flies are assumed to colonise new host nests actively during the winged phase of their life cycle (Grimaldi 1997, Roulin 1998,

1999). Adults are short-lived (less than 2 days, M.A. Calero-Torralbo, unpublished data) and copulations take place on the hosts (Guiguen et al. 1983, pers. obs.).

Hoopoes *Upupa epops*, Little owls *Athene noctua*, Eurasian kestrels *Falco tinnunculus*, European bee-eaters *Merops apiaster* and European rollers *Coracias garrulus* are cavity nesting bird species widespread in Spain where they frequently occur in sympatry in arid areas. The former three are resident species, whereas the bee-eater and the roller are migrant birds. In our study area bee-eaters dig their burrows in sandy cliffs and hoopoes breed in natural holes in olive trees and stone piles. Rollers, kestrels and little owls breed in holes in sandy cliffs as well as in crevices and cavities in human constructions. Bee-eaters seldom re-use their nests but they usually re-occupy breeding colonies for several years. Hoopoes' nests are used exclusively by this species. Rollers, little owls and kestrels frequently re-use the same hole in successive years, although they exchange their nests not infrequently. All bird species lay a single clutch (although replacement clutches occur) with the exception of the hoopoe, which may lay a second clutch after raising a successful brood (Martín-Vivaldi et al. 1999).

Data collection

In the framework of a broader study on the relationships between *Carnus hemapterus* and its hosts we collected nest material from several host species and locations. Emergence of carnid flies did not occur in some samples, probably because the nests were not infested by *Carnus hemapterus*. Here we report on the samples on which the estimate of the frequency of prolonged diapause is based on (Table 1). Most samples were collected from natural nests but some nest boxes (probably used by rollers, kestrels, starlings *Sturnus unicolor* and sparrows *Passer* spp.) were also sampled. All samples but 5 come from different nests. Five cavities were used for breeding in successive years by the same bird species (2 by rollers and 3 by little owls). These nests were sampled twice (in January 2003 and in March 2004, Table 1) and each sample was stored apart from the others and subsequently monitored for several periods (see below and Table 1). Since

environmental factors influencing the larval and pupal stage and diapause of *Carnus hemapterus* are likely to vary among years we consider the sample as the unit of replication.

Sampling consisted in removing part of the material from the nest bottom using a spoon attached to a stick long enough to reach the nest chamber or by hand. Whenever the nest was made of vegetable material, the whole structure (i.e., the nest cup) and the earth below the nest were taken. The amount of nest material collected varied among nests.

Emergence patterns are ideally studied in the field. Nevertheless this was not possible since this would necessitate the periodical sampling of nests occupied by birds during the breeding season (i.e. when carnid flies emerge), which would result in the disturbance of adults and nestlings. Therefore, the emergence of flies was recorded in the laboratory. This approach, in turn, ensured homogeneous conditions (i.e., the same temperature) for the samples, which it is not possible in the field, where local conditions and the timing or length of use of nests by birds could influence emergence time.

After collection, the samples were kept in plastic bags and stored in the Estación Experimental de Zonas Áridas (Almería, around 35 km from the location where most samples were collected). The samples were placed in a room with open windows and drawn curtains to resemble the conditions experienced by pupae in cavities in the wild (i.e. ambient temperature moderated by partial enclosure and semidarkness). All samples were stored in the same place, except for two roller samples collected in January 2003, which were kept throughout the winter season 2003-2004 in a cellar at a lower temperature than the rest of the samples. These two samples were moved the following wintering season to the same room where most samples were stored.

Our initial sampling schedule contemplated monitoring of emerging flies (approximately every 3-4 days until insect emergence ceased) during the first spring after collection (Table 1). Thus, the study period lasted from 31 January 2003 until 8 July 2003 for the samples collected in 2002-2003, from 9 March until 13 July 2004 for those samples collected in 2004 in Tabernas and from 4 March until 13 July 2004 for the samples collected in 2004 from nest-boxes in western

Spain. However, the 2 roller nests collected in January 2003 were checked by chance in mid June 2004 (i.e. the second spring after collection) and recently dead flies and emergence of fresh flies were detected, thus supplying evidence for prolonged diapause. From then onwards, emerged flies in these samples were counted but the flies that could have emerged before we started our observations were most probably missing by then. The information about flies with prolonged diapause in these two nests is therefore conservative. As it was too late to monitor emergence in the remaining samples from different hosts and localities, all the samples were controlled again the next spring from 2 March 2005 until 15 July 2005 (Table 1).

In summary, our data set consisted of 2 samples (roller) studied for 3 consecutive springs (2003 to 2005), 22 samples (1 kestrel, 4 little owl, 9 roller, 8 from nest boxes) for 2 consecutive springs (2004 and 2005), and 22 samples (11 bee-eater, 8 hoopoe, 3 little owl) for the first and the third spring after sampling (2003 and 2005) (Table 1). Therefore, the number of controlled samples for prolonged diapause after at least two consecutive springs is 24.

Host species and no. samples	Location	Collection date	Monitoring period for short cycle/long cycle emergence
Bee-eater 10	Madrid, Central Spain (40º32'N 03º27'W)	July 2002	Spring 2003/Spring 2005
1	Almería, Southern Spain (37º09'N 2º13'W)	March 2003	
Hoopoe 6	Granada, Southern Spain (37º18'N 3º11'W)	January 2003	Spring 2003/Spring 2005
2	Almería (37º08'N 02º43'W)	January 2003	
Roller 2	Almería (37º05'N 2º21'W)	January 2003	Spring 2003/June 2004/Spring 2005
9		March 2004	Spring 2004/Spring 2005
Little owl 3	Almería (37º05'N 2º21'W)	January 2003	Spring 2003/Spring 2005
4		March 2004	Spring 2004/Spring 2005
Kestrel 1	Almería (37º09'N 2º13'W)	March 2004	Spring 2004/Spring 2005
Nest-boxes 8	Cáceres, Western Spain (39º03'N 5º14'W)	January 2004	Spring 2004/Spring 2005

Table 1. Location, collection date and monitoring period for emergence of short and long-cycle *Carnus hemapterus* flies from samples collected from different bird species.

Estimation of the frequency of prolonged diapause

The frequency of prolonged diapause was estimated with respect to the number of flies emerging the first spring only on the samples with prolonged diapause. We refer to the samples in which we have not recorded prolonged diapause only to estimate the proportion of samples with prolonged diapause.

The samples collected in this study probably contain pupae with different diapause history. Flies emerging the first spring can therefore include individuals that pupated more than one year before the first spring. Our data on frequency of prolonged diapause are thus conservative both regarding the number of flies with a long-cycle and the duration (i.e. years) in prolonged diapause.

Statistical analyses

Parasite distributions are known to be aggregated, which makes their quantification and comparison difficult (Rózsa et al. 2000). Following Rózsa et al. (2000) prevalence (proportion of infected samples) and mean intensity (mean number of individuals found in the infected samples) of flies were used for quantification purposes. We compared median intensity (median number of individuals found in the infected samples) of flies by using Mood's median test. Statistical tests were done using the program Quantitative Parasitology 2.0 (Reiczigel & Rózsa, 2001) and the STATISTICA 6.0 package (StatSoft, Inc. 2001).

Results

Frequency of prolonged diapause in Carnus hemapterus

Emergence of carnid flies 1 year after the first emergence was detected in 4 samples (Table 2) out of the 24 samples controlled for prolonged diapause after at least two consecutive springs (Table 1), that is 16.7%. Compared to the number of flies emerged during the first spring, we calculated an average of 3.3% (S.E. = 0.91, range = 0.9%-5.3%) individuals with long cycle.

	Roller		Little owl			Hoopoe		Kestrel
Nest number	**1**	**2**	**1**	**2**	**3**	**1**	**2**	**1**
Emergence in the first spring	1416 (2003)	684 (2003)	194 (2003)	4 (2003)	78 (2004)	65 (2003)	83 (2003)	19 (2004)
prolonged diapause (min. 1 year)	45 (3.2%)	6 (0.9%)	-	-	3 (3.8%)	-	-	1 (5.3%)
prolonged diapause (min. 2 years)	25 (1.8%)	0 (0.0%)	29 (14.9%)	2 (50.0%)		1 (1.5%)	5 (6.0%)	

Table 2. Emergence of *Carnus hemapterus* in nests where prolonged diapause was recorded. The number of flies emerging in successive springs in nests of different hosts is shown (in brackets the percentage of individuals compared to the number of flies emerging the first spring). Years in brackets refer to the year when emergence is monitored for the first time. Dashes refer to years when emergence was not monitored whereas empty cells indicate that 2-year prolonged diapause should eventually occur in spring 2006.

Results obtained from a different set of samples (see Table 1) show that emergence of carnid flies 2 years after the first emergence bout occurred in at least 5 (20.8%) out of 24 samples (Table 2). Monitoring of the 2 roller samples studied for 3 consecutive springs reveal that carnid flies emerged from both samples the second spring, and that emergence was recorded in only 1 sample the third spring (Table 2). Considering only the samples with prolonged diapause, an average of 14.8% flies (S.E. = 9.11, range = 1.5%-50%, the latter being of a nest where emergence was low the first spring, Table 2) emerged after more than two years of diapause with respect to the flies that emerged the first spring.

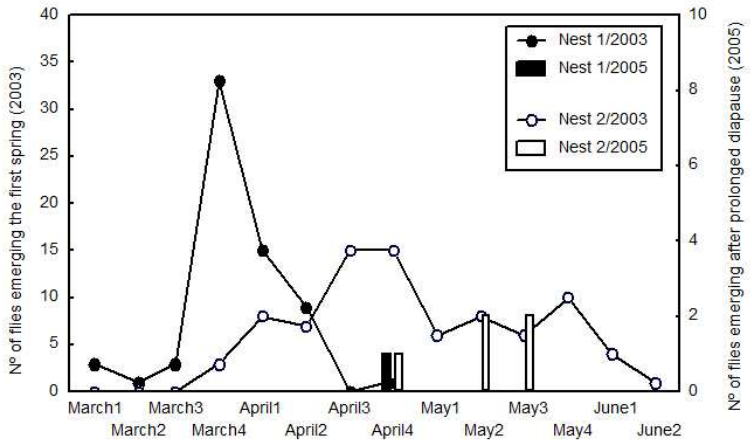
The median number of flies emerging the first spring was significantly higher in samples where prolonged diapause was recorded than in samples where it was not (median values: 80.5 and 5 respectively, Mood's median test, P = 0.018). The number of emerged flies after prolonged diapause (all cases pooled) was positively correlated with the number of flies emerging the first spring (Spearman rank correlation, $r_s = 0.89$, $P = 0.002$, $n = 8$).

Phenology of emergence of Carnus hemapterus

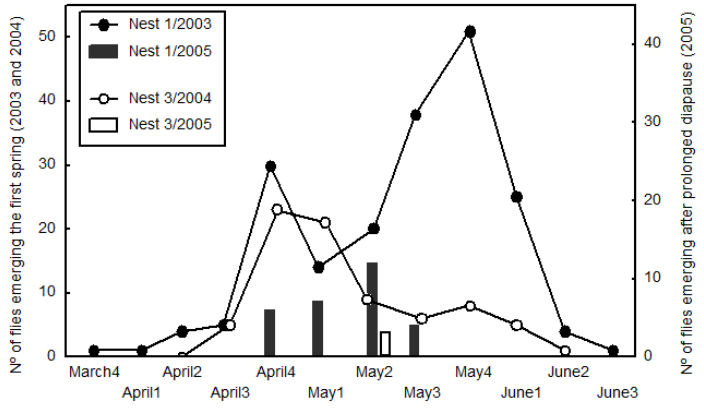
The pattern of appearance of flies with protracted emergence resembled very much the one observed for flies emerging the first spring. Flies with prolonged diapause in hoopoe nests 1 and 2 (Table 2) emerged within the period observed for flies emerging the first spring (Fig. 1a). This is also the case for flies emerging from little owl nests 1 and 3 (Fig. 1b, Table 2). The only 2 flies with delayed

diapause found in nest 2 (not represented in the figure) emerged the first week of May, well within the period observed for flies emerging the first spring in the same nest (first week of April to second week of May).

1a.



1b.



1c.

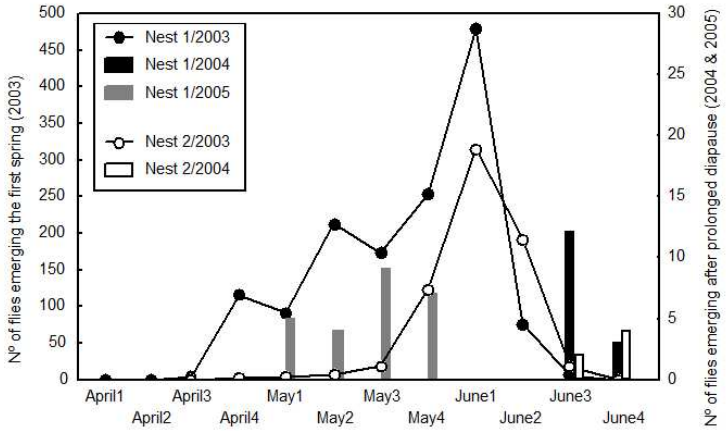


Figure 1. Phenology of emergence of short cycle (lines) and long cycle (bars) *Carnus hemapterus* flies from nests of a) hoopoes, b) little owls, and c) rollers. For each species the emergence of flies the first spring (left ordinate) and flies with prolonged diapause (right ordinate) in several nests in successive springs is shown. Data are referred to emergence per week from early March to late June.

Concerning flies from rollers' nests, we found a similar pattern for individuals emerging in 2003 and 2005 (Fig. 1c). The apparently late emergence of flies in 2004 could be due to our missing the first flies emerging (see methods) (Fig. 1c only includes the flies whose emergence could be dated accurately) and/or to the lower storage temperature the winter season 2003-2004 (see methods and discussion).

The only fly with prolonged diapause found in a kestrel nest (Table 2) emerged 2 weeks later (fourth week of May) than the latest ones observed the first spring in this nest (emergence period from the second week of April to the first week of May).

Discussion

We recorded emergence of *C. hemapterus* after at least 2 and 3 wintering seasons in around 17% and 21% of the samples respectively. In 1 sample emergence of carnid flies was recorded for 3 consecutive springs. The number of flies emerging in those samples ranked 0.9%-50% with respect to the number of flies emerging the first spring. Several factors suggest that the frequency of prolonged diapause

in *C. hemapterus* can be more frequent than these figures show. First, given that both the occurrence and the number of flies with long life cycle are related to the number of flies emerging the first spring, the likelihood of detection of individuals with prolonged diapause in nests where little nest material was collected and/or with a low parasitic load (the range of emergence was 1 - 1416 flies) is low. Second, we cannot discard that some of the flies emerging the first spring may in fact be individuals with long cycle that started prolonged diapause years before.

It is well known that many insect species can extend their life cycle over several years (Tauber & Tauber 1981, Danks 1987, 1992, Hanski 1988, Debouzie & Menu 1992). Long life- cycles are usually viewed as resulting from prolonged diapause caused by extension of the usual winter diapause. However, in contrast to the usual hypothesis, Soula and Menu (2005) showed that the long cycles in the Chestnut weevil *Curculio elephas* is due to a prolonged diapause occurring secondarily to a developmental phase. Therefore, we interpret the emergence of carnid flies in samples stored two or more adverse seasons as the result of prolonged diapause without information on the exact mechanism. An alternative explanation is that some flies emerging after our last control in the first spring could have mated in the bags and produce larvae and pupae which would overwinter and result in adults the next spring, but this is unlikely as carnid flies need to feed on a host to survive (Kirkpatrick & Colvin 1989, Dawson & Bortolotti 1997, Grimaldi 1997), mating usually occurs on the host (Guiguen et al. 1983, pers. obs) and adult flies live for less than 2 days in absence of the host (Calero-Torralbo et al unpublish data). Thus, we maintain that *C. hemapterus* has prolonged diapause.

A within-generation variation in life cycle duration (mixing of short and long cycles) occurs in many insects, crustaceans, and plants (see Soula & Menu 2005 and references therein). However, the occurrence of prolonged diapause in parasites is much less common. It does exist in plant-feeding insects (Danks 1987, 1992, Soula & Menu 2005) and some host-parasitoid systems (see Danks 1987, Hanski 1988), but a thorough literature review yielded one study reporting prolonged diapause in a “true” parasite (i.e. not a parasitoid) of animals, the rodent botfly *Cuterebra tenebrosa* (Baird 1975). Danks (1992) reviewed the

occurrence of long life cycles in ectoparasitic insects like human fleas (*Pulex irritans*), sand martin fleas (*Ceratophyllus styx jordani*) or bugs (*Rhodnius prolixus*). He concluded that such long cycles were in accord with the unreliable availability of hosts. However, these cases cannot be considered prolonged diapause because individuals "do not skip an opportunity for breeding and opt for survival" (Hanski 1988), rather they are the result of prolonged dormancy until host availability is detected.

Several hypotheses for the adaptive value of long cycles have been proposed (Tauber et al. 1986, Danks 1987, Hanski 1988, Menu et al. 2000, Soula & Menu 2003). Long life cycles are correlated with environmental adversities, such as cold or unpredictable temperatures, patchy, unreliable or low quality food supplies (Danks 1992). Prolonged diapause, usually resulting in long life cycles, is interpreted as a local adaptation to multiannual fluctuations in resource availability (Debouzie & Menu 1992, Hopper 1999, Menu et al. 2000, Soula & Menu 2003, but see Menu 1993, Menu & Desouhant 2002 for other selective factors for variability in life cycle duration). In some species it has been considered a bet-hedging or risk-spreading strategy, because it prevents extinction when resources are lacking (Menu & Debouzie 1993; Menu et al. 2000). For parasitic organisms that infect via free-living stages actively seeking for a host, the probability of host encounter is likely to be highly unpredictable (Hakalahti et al. 2004). It is therefore not surprising that the infection strategies of such parasites may be one of the most obvious traits in which bet-hedging life-history traits are likely to exist (Fenton & Hudson 2002, Hakalahti et al. 2004). Since *C. hemapterus* can perpetuate by itself in a given nest, one could assume that dispersal is not necessary for this species. However, factors like alterations in the nest or its surroundings (making it unattractive for birds), interannual nest-site change of the host to avoid parasitism (Loye & Carroll 1998) or predation before hatching may result in vacant nests, which seriously jeopardises the future of the lineage of parasites inhabiting that nest. In that case, freshly emerged flies should disperse and look for an occupied nest.

Dispersal is perhaps the most dangerous part of most parasites' life cycles (Ward et al. 1998). Several factors suggest that dispersal in time (i.e. prolonged

diapause) can be better for *C. hemapterus* than spatial dispersal. Even though *C. hemapterus* is a vagile insect, its flight range is probably short and it is unclear whether it can control its own flight direction. Moreover, its dispersal ability is probably limited by the short life span of adult flies (less than 2 days). If a suitable host is not found rapidly, adult flies die. In the case where no dispersing fly succeeds in finding a host, the survival of the lineage is ensured by the pupae in prolonged diapause. They give rise to adults in succeeding years when the nest can be occupied again by the same or other host species. In agreement with this, it is remarkable that the emergence pattern of flies with prolonged diapause was similar to the one observed for flies with short cycle. Most flies with prolonged diapause emerged between mid April and late May, when chicks of most of the host species have already hatched, so that flies seem to synchronize their emergence dates with host occurrence (Liker et al. 2001, Valera et al. 2003, Valera et al. 2006). Breaking of diapause and activation of metabolic processes commonly requires an exposure to one or several cues (e.g. changes in temperature or photoperiod) (Tauber et al. 1986). Powell (1989, 2001) showed that environmental factors (i.e. temperature) promoted variation in diapause patterns in plant-feeding moth larvae. Observational and experimental data (manipulating temperature during the pupal stage) suggest that the emergence time of *C. hemapterus* is determined by an endogenous annual timing mechanism that can be modified, within certain limits, by temperature (Chapter III), as is the case for many insects in temperate climates (Tauber & Tauber 1981, Smith & McIver 1984, Leather et al. 1993). The late emergence observed in flies from rollers' nests in 2004 could be explained by the lower temperature experimented by pupae during the winter season 2003-2004. In this study system, appropriate environmental cues are thus likely to favour diapause development.

Whereas we cannot discard other selective factors (like predation, larval attack by entomopathogenic fungi or climate) for within-generation variability in life cycle duration (see, for instance, Menu 1993, Menu & Debouzie 1993, Menu & Desouhant 2002), our current knowledge suggests that host finding is an important selective pressure for this study species. Further work should focus on whether the unpredictability of host encounter could produce bet-hedging for

variability in life cycle duration in *C. hemapterus*. Within-generation variability in diapause duration can result from diversified bet-hedging (*sensu* Seger & Brockmann, 1987), from a mixed evolutionary stable strategy or a genetic polymorphism of pure strategies (Soula & Menu 2003). Although Hopper's review (1999) suggests that genetic variation and conditional responses to environmental cues explain most phenotypic variation in diapause, without invoking bet-hedging, he pointed out that diapause is an appropriate trait to increase our knowledge of risk-spreading in insect populations. Recently, Menu & Desouhant (2002) and Soula & Menu (2003) provided evidences of diversified bet-hedging for variability in life cycle duration in the chestnut weevil.

This being, to our knowledge, the second report on prolonged diapause in true parasites of animals, the question arises whether this mechanism is rare among these organisms or whether it has passed unnoticed. Diapause and dispersal are considered alternative ways of escaping unfavourable conditions so that the frequency of prolonged diapause is negatively correlated with dispersal rate (Hanski 1988, Bohonak & Jenkins 2003). The short life-span of adult carnid flies could account for the occurrence of prolonged diapause in this parasite. Thus, *C. hemapterus* could be considered a peculiar case in the "parasite community". Alternatively, the occurrence of prolonged diapause may have been overlooked in parasites for different reasons (e.g. the long period needed to detect it, the complexity of host-parasite systems). Hanski (1988) stated, "Prolonged diapause will rarely be detected where it is not looked for, and it is generally only looked for where it is expected to occur". Since environmental unpredictability is the rule for many parasitic organisms we suggest that prolonged diapause could occur in other parasites. Here we stress the need of long-term studies to fully understand the life cycles of parasites.

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CAPÍTULO III

Synchronization of host-parasite cycles by means of diapause: host influence and parasite response to involuntary host switching.

Abstract: Parasites need synchronizing their infective phases with the appearance of the fittest resource (host) and, for many species; diapause is a mechanism contributing to such coincidence. A variety of ecological factors, like changes in host temperature profiles produced by involuntary host switching (substitution of the usual host by an infrequent one), can modify host-parasite synchronization of diapausing ectoparasites of endothermic species. To understand the influence of host switching on the mechanisms of parasite synchronization, we conducted experiments using the system formed by the ectoparasitic fly *Carnus hemapterus* and its avian hosts. We simulated the occurrence of the usual host and natural cases of host switching by exposing overwintering carnid pupae from Bee-eater nests (*Merops apiaster*) to the earlier incubation periods of two *Carnus* host species that frequently reoccupy Bee-eater nests. Pupae exposed to host switching treatments advanced the mean date of emergence and produced an earlier an faster rate of emergence in comparison with pupae exposed both to the control (absence of any host) and Bee-eater treatments. The effect was more evident for the treatment resembling the host with the most dissimilar phenology to the one of the usual host. Our results show that host temperature profile is an environmental cue used by this parasite and reveal that *Carnus hemapterus* has some potential to react to involuntary host switching by means of plasticity in the termination of diapause.

Keywords: *Carnus hemapterus*, diapause, ectoparasite, host temperature, host-switching, life cycle, phenotypic plasticity.

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Introduction

Synchronization of life cycles with the availability of resources is basic for many organisms among which parasites are not an exception (Poulin 1988). Parasitic species, particularly those that feed on ephemeral resources, must ensure that their host-feeding stages are synchronized with the times when those hosts provide the appropriate food resource. Therefore, many parasites have evolved dispersal and developmental mechanisms (especially diapause) to safeguard their success in finding an appropriate host at the right moment (Tauber et al. 1986, Danks 1987). Diapause is a form of dormancy that allows individuals to survive when conditions are unfavourable and ensures synchronization of active stages with favourable conditions (Danks 1987, Masaki 2002). Diapause regulation mechanisms must achieve that induction, development and diapause completion are realized in most suited periods for every individual, responding to reliable external stimuli and cues that modulate direct or indirectly different physiological events during the phases of diapause (Kostal 2006), regulating its duration and intensity (Hodek 2002, Masaki 2002). Although these environmental factors are diverse, numerous studies distinguish temperature and photoperiod (single or combined) as the most important controlling signals (see references in Tauber et al. 1986, Danks 1987).

Temperature has been acknowledged since long as an influential variable for insects and development thermal responses in diapause are well understood in parasitic insects (Feder et al. 1997, Wharton 1999, Randolph 2004). Ambient temperature is a reliable signal specially in long winter diapauses (several months), due to the annual periodicity at regional level; or in places where photoperiodic changes are small (tropical areas) or not appreciable and daily temperature fluctuations are buffered (e.g. species in which some phases of the diapause take place in holes, caves or into the soil) (Danks 1987, 2006b). Several authors have pointed out that, in ectoparasites of endothermic hosts, temperature of the host or its surroundings (burrows or nests where they reside or breed) can control and modify parasite life-cycle duration and intensity (Marshall 1981, Danks 1992). Whereas some examples exist (e.g. some flea and bug species use host temperature to break their dormancy and ensure host availability, Marshall

1981, Danks 1992), to our knowledge there is no study focusing specifically on the effect of host body temperature on insect parasites life cycles. The influence of host temperature on insect biology is likely to depend on the ecological scenario, being probably stronger in cold climates than in warmer ones (see Wetzel & Weigl 1994). Our wide knowledge on thermal response patterns in insects could probably forecast the response of insects to host temperature. However there are other scenarios where insect response to host temperature is probable less intuitive and with deeper implications. The effect of host temperature and host switching on synchronization between trophic levels is a largely unexplored topic (but see Marshall 1981, Wetzel & Weigl 1994) that is important for a better understanding of the evolution of host-parasite interactions. Host bodies (and their intimate environment like nests) of polyphagous parasites can show distinct temperature profiles determined by physiological mechanisms and/or behavioural features (e.g. breeding phenology, roosting behaviour, type of nest with varying insulation characteristics…) that can vary remarkably among potential hosts. Moreover, for those parasites that do not actively choose their host, changes in the habitual host (i.e. host switching) are likely to represent a significant challenge for the parasite's ability to exploit the new host. Variation in key features of the host, like temperature, is likely to influence diapausing or quiescent parasites that should synchronize their infection phase with the new resource. Nest parasites can be particularly the case. Given that some nests can be reused by different bird species, successive generations of a parasite may be exposed to different host species, which may have similar or very different breeding biology and vary in important features (e.g. temperature profiles). This raises the question of whether the parasite is able to modify its life cycle in relation to the host species (i.e. degree of host specificity, Roulin 1998, Valera et al. 2003). In summary, the influence of host temperature and its variation on parasites life cycle is largely unknown and experiments resembling natural circumstances are required to better understand the manner in which environmental factors may affect host-parasite relationships via diapause in an ecological context (Danks 2007).

The system formed by the ectoparasite fly *Carnus hemapterus* Nitzsch and its avian host species provides us an excellent opportunity to study parasite

responses to host switching and the influence of host temperature on diapause regulation. *Carnus hemapterus* parasitizes nestlings of bird species with very different breeding phenologies, with some preference for birds nesting in cavities (Grimaldi 1997). This fly overwinters as pupae in the nest and the emergence of the infecting phase is partly synchronized with the occurrence of their hosts (i.e. hatching of nestlings) (Liker et al. 2001, Valera et al. 2003). Involuntary host switching can occur if a different host from the one that used it in previous breeding seasons reuses the nest. In this study we aim to contribute to a better understanding of the mechanisms involved in the synchronization of host-parasite cycles by studying the influence of host temperature on termination of diapause in an ectoparasitic fly and by examining the response of diapausing parasites to changes in its intimate environment as a result of involuntary host switching. To achieve these goals, we try to answer the following questions: i) What are the environmental signals used by *Carnus hemapterus* to resume metabolic arrest and thus ensure the availability of resources? ii) How does host temperature profile influence parasite diapause? iii) How does the parasite respond to host switching and to what extent can *Carnus* adapt its life cycle to different host species? We hypothesise that differences in breeding phenology among alternative host species will influence on *Carnus* diapause traits and that the parasite will respond to changing temperature profiles. To answer these questions we experimentally simulate natural cases of host switching by exposing overwintering pupae of carnid flies parasitizing European Bee-eaters (*Merops apiaster*) to the incubation periods of two common *Carnus* hosts (the Little Owl *Athene noctua*, and the Hoopoe *Upupa epops*) that frequently reoccupy bee-eater nests.

Material and methods

Study area and species

The study area is located at La Palma del Condado, (Huelva, South-west Spain 37º 35’ N, 6º 45’ W) where Bee-eaters breed in several colonies. The colony from which samples were collected is situated in an old sand quarry and has been occupied by Bee-eaters for years. More than 40 pairs bred during both study years 2005 and 2006. Climate in this area is typically Mediterranean with Atlantic

influence, with long hot and dry summers and mild winters. Precipitations are abundant mainly in autumn/spring (mean annual rainfall during 2001-2006 period = 588.2 mm) (Junta de Andalucía meteorological data).

Carnus hemapterus is a 2 mm long blood-sucking fly that parasitizes nestlings of a variety of bird species (Grimaldi 1997). *Carnus* is distributed throughout the Palaearctic region and North America. Its life-cycle comprises an adult stage, 3 larval phases encompassing around 21 days at 22ºC and 95% relative humidity and a pupal stage (Guiguen et al. 1983). The puparia are black, short barrel shaped and very cryptic, simulating nest remains of chitinous parts of arthropods consumed by the hosts. After a several months winter diapause (Guiguen et al. 1983) adult flies emerge in the following spring approximately when their nestling hosts hatch (Valera et al. 2003). Adult flies are initially winged, but typically lose their wings once they locate a suitable host (Roulin 1998). Since neither the adults nor the larvae have been found on adult birds, flies are assumed to colonise new host nests actively during the winged phase of their life cycle (Grimaldi 1997, Roulin 1998). Nonetheless, *Carnus* can perpetuate by itself in the nest for several years since prolonged diapause has been recorded for this species (Valera et al. 2006a). Adult flies are short lived during dispersion (around 2 days, MACT personal observation).

The European Bee-eater *Merops apiaster* is a single-brooded, migrant bird that nests in cavities at the end of long burrows. It usually forms breeding colonies (up to 80 pairs in the study area; MACT personal observation) that can be used for many years becoming traditional breeding areas. Eggs are laid directly on the sandy soil of the incubation chamber. Incubation lasts around 20 days starting before the clutch is complete (Cramp 1985). The female usually sleeps in the nest (Cramp 1985). Ar & Piontkewitz (1992) estimated that the mean temperature in a Bee-eater incubation chamber during the day was of 27.8 ± 1.6 ºC.

Bee-eater nests are commonly used by a variety of birds for breeding among which Hoopoes *Upupa epops* and Little Owls *Athene noctua* have been reported (Casas-Crivillé & Valera 2005). The latter species are resident, cavity nesting birds commonly parasitized by *Carnus hemapterus* (Valera et al. 2006a). They frequently occur in sympatry with the Bee-eater in Spain. In southern Spain

Hoopoes breed from February to June, with about 20% of pairs laying a second clutch (Martín-Vivaldi et al. 1999). Incubation lasts around 17 days (Martín-Vivaldi et al. 1999). Little Owls lay a single clutch although replacement clutches occur. Incubation lasts around 27-28 days and the breeding period is usually from April to June (Cramp 1985). Thus, in our study area Hoopoes start breeding first, followed by Little Owls and Bee-eaters being the latest breeders.

Data collection

During February 21st-22nd 2005 and February 16th 2006 nests with evident cues of having been used the previous breeding season (abundance of arthropods remains and bird pellets in the nest), and therefore more likely to contain *Carnus* pupae, were sampled. Twenty five samples were collected each year from the breeding chamber by using a spoon attached to a stick. The amount of nest material collected varied among nests (range: 417-1623 g). Nonetheless, the number of emerged flies is not related to the amount of nest material collected (Spearman rank correlations for each treatment and year, $P > 0.1$ for all cases; see also Valera et al. 2006b). After collection, samples were kept in transparent plastic bags, carried to the Estación Experimental de Zonas Áridas (Almería, South-east Spain, 36º 50'N 02º 28'W) and stored in a dark room with open windows to resemble natural conditions (i.e. ambient temperature moderated by partial enclosure and semi-darkness).

Experimental design

The aim of our study was to evaluate the effect of the habitual host and of host switching on the emergence of *Carnus hemapterus*, simulating situations occurring in nature. Thus, our experimental design consists of exposing carnid pupae to three different situations: occurrence of the usual host (the Bee-eater), occurrence of a different host (Little Owl in 2005, Hoopoe in 2006) and control (resembling pupae in unoccupied nests). In 2005 our experiment included a Little Owl, a Bee-eater and a control treatment whereas in 2006 it comprised a Hoopoe, a Bee-eater and a control treatment (Figure 1).

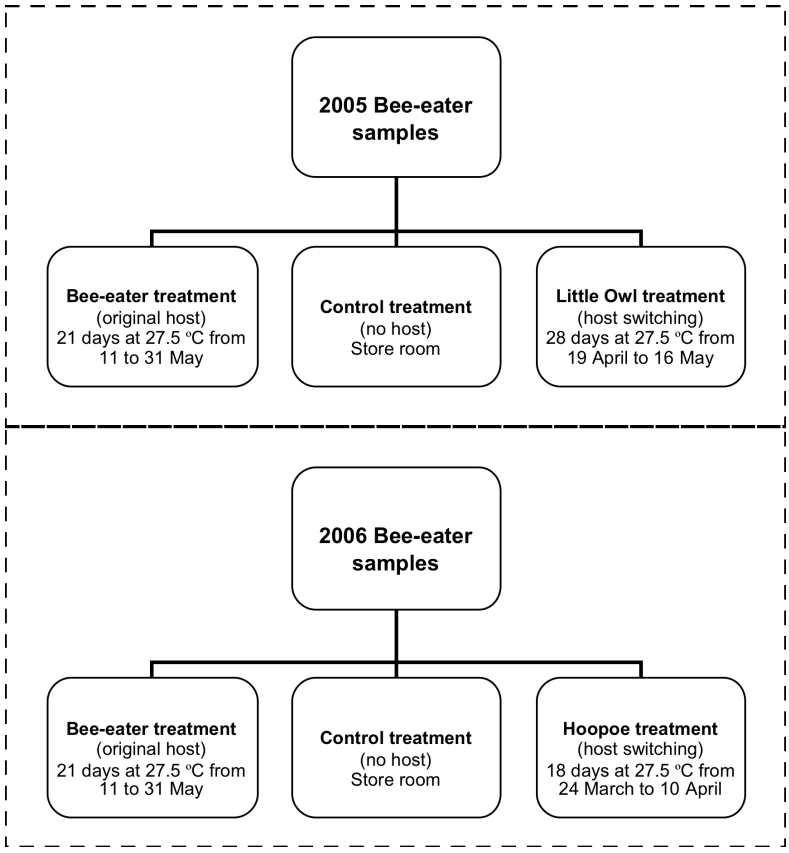


Figure 1. Experimental design diagram for simulations of host occurrence and host switching in 2005 and 2006.

Samples of Bee-eater nests were thoroughly mingled, randomly split in three subsamples of the same mass and arbitrarily subjected to the following three treatments: i) incubation by the original host: we artificially reproduced the Bee-eater incubation period, keeping the subsamples during 21 days at 27.5ºC (JP Selecta, model Incubat 150, ref. 2000994). Following the Bee-eater breeding period in our latitude (Cramp 1985, pers. obs.), the treatment began from 11 May and lasted until 31 May (both in 2005 and 2006). From then onwards the subsamples remained in the store room; ii) host-switching incubation: to test whether a host change (with different breeding dates and/or duration than the habitual host), can affect *Carnus* emergence, we artificially reproduced the Little Owl and the Hoopoe incubation periods. Given the narrow range of incubation

temperature reported for very different bird species (Webb 1987) and that thermal microenvironment of burrows is very constant (White et al. 1978, Ar & Piontkewitz 1992, Lill & Fell 2007) we assume that temperature in the breeding chamber in nests occupied by these two species is similar to the one in Bee-eater nests. Following the breeding dates and duration in our latitudes (Cramp 1985, Martín-Vivaldi et al. 1999), we kept the samples during 28 days at 27.5º C, from 19 April to 16 May 2005 (Little Owl treatment), and for the Hoopoe treatment during 18 days at 27.5º C, from 24 March to 10 April 2006. From the end of the treatment onwards subsamples remained in the store room; iii) control treatment: subsamples remained the whole time in the storeroom.

Samples were periodically monitored (each 2-3 days) for *Carnus* emergence from 14 March 2005 until 9 July 2005 and from 14 March 2006 until 23 June 2006 (in both cases 10 days after the last emerged fly was recorded). Flies emerging from each subsample and date were separately preserved in 99% ethanol and subsequently counted and identified with the aid of a binocular microscope. Emergence dates were grouped into weeks starting from the first week of April (when earliest emergence was recorded). During 2006, the temperature in the storeroom was checked every three hours by means of a temperature data logger (Maxim/Dallas Integrated Products, Inc.). At the time of the bee-eater treatment it averaged 25.5ºC (range: 23.5 - 28.0ºC).

Carnus hemapterus emergence was registered in 12 out of 25 nests sampled in 2005. In 8 nests emergence was recorded in all treatments, in 1 nest emergence was recorded only in the Little Owl and control treatment and in 3 nest emergence was recorded in just a single treatment. During 2006, 15 nests had emergence in all three treatments. In one nest emergence was recorded only in the Hoopoe and control treatments and in 2 nests emergence was recorded in just a single treatment.

A subsample of flies emerged during the study period was deposited in the Zoological Collection of the Estación Experimental de Zonas Áridas (Almería, Spain) (reference numbers 6488 to 6496).

Statistics

Except for the calculation of prevalence, analyses were restricted to those nests where emergence was recorded in all three treatments since the number of emerged flies in the remaining nests (i.e. in those where emergence occurred in one or two treatments) was very low. Prevalence (proportion of infected samples among all the samples examined) of parasites and mean intensity (number of individuals found in the infected samples) of flies was calculated and χ^2 tests were used for comparing prevalences. One-way within-subjects (repeated measures) ANOVAs were used to test the effect of treatments on the number of flies emerged per sample, length of the emergence period and mean date of emergence.

For the analysis of the effect of treatments on the emergence pattern of flies our experimental design encompasses two within-subject factors: i) emergence time and ii) subsamples of the same nest exposed to different treatments. Thus, to analyse the effect of the treatments on *Carnus* emergence along the emergence period we used multi-way within-subjects repeated measure ANOVA tests (von Ende 2001). The dependent variable was the cumulative percentage of emerged flies per week after checking for normality. Given that the earliest emergences occurred intermittently, only in some treatments and at a low number (see results) we focused on the main period of emergence and excluded from the analyses the first week of emergence in 2005 and the three first weeks in 2006. Anyway results did not change when including such weeks. Weeks when most subsamples had reached 100% emergence were also discarded since they do not influence the overall emergence pattern. Thus, seven weeks (from the fourth week of April until the second week of June) and five weeks (from the fourth week of April until the last week of May) were included in the 2005 and 2006 analyses respectively.

We used Mauchly's test to check the assumption of sphericity and, when the latter was not met, we adjusted the degrees of freedom by using the Greenhouse-Geisser and Huynh and Feldt estimators (see von Ende 2001). Here we provide the conservative Greenhouse-Geisser corrected probability. We followed both the univariate and the multivariate approach when possible (i.e.

sample size did not allow testing the effect of the interaction between time and treatment in 2005). Since both approaches gave the same results here we report the results obtained with the more powerful univariate approach (von Ende 2001). Given that the sample size in 2005 is low in comparison to the number of dependent variables (i.e. seven weeks and three treatments; see von Ende 2001) we first analyzed our data pooled into three periods (two first weeks, the main emergence period of three weeks, and the latest two weeks). Since the results do not change when compared with the ones obtained when considering seven periods (i.e. seven weeks) and since a more accurate view of the effect of treatments on *Carnus* emergence is gained in this way we prefer showing the latter results.

Since we hypothesise that both experimental treatments have a differential effect on *Carnus* emergence both when compared to each other and when compared with the control, we performed univariate tests of significance for planned comparisons when opportune.

Results

Effect of the incubation treatments on prevalence and abundance of flies

Prevalence of *Carnus hemapterus* was not affected by the treatments either in 2005 or in 2006 (Chi-square tests, χ^2 = 0.11, $P > 0.10$; χ^2 = 0.11, $P > 0.10$, respectively) (Table 1).

Treatment	2005		2006	
	Prevalence	Mean nº flies (range)	Prevalence	Mean nº flies (range)
Control	40% (25)	87.4 (1 – 281)	64% (25)	13.8 (2 – 57)
Bee-eater	36% (25)	71.4 (2 – 248)	64% (25)	14.8 (1 – 45)
Little-owl	40% (25)	56.6 (1 – 158)	-	-
Hoopoe	-	-	68% (25)	10.8 (1 – 49)

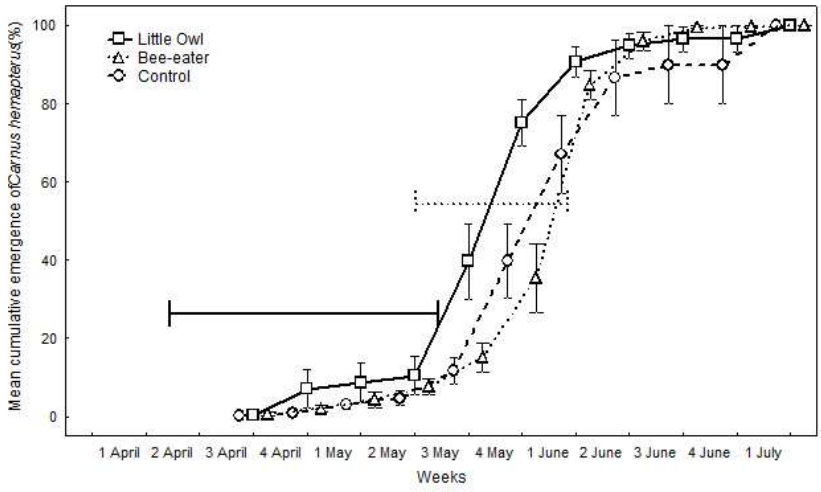
Table 1. Prevalence (sample size in brackets) and abundance of *Carnus hemapterus* flies in subsamples of each treatment in 2005 and 2006.

Experimental treatments performed in 2005 did influence the number of emerged flies (Repeated Measures ANOVA, $F_{2,14} = 4.70$, $P = 0.027$) since more flies emerged in the control subsamples than in the Little Owl and the Bee-eater subsamples (Univariate tests of significance for planned comparisons, $P = 0.045$ and $P = 0.024$, respectively) (Table 1). In contrast, treatments in 2006 did not influence the number of emerged flies per nest (Repeated Measures ANOVA, $F_{2,28} = 1.8$, $P = 0.18$) (Table 1).

Effect on the incubation treatments on the phenology of emergence of Carnus hemapterus

In 2005 emergence was first recorded during the third week of April in some subsamples of all treatments (Fig. 2a) and became common in all treatments during the fourth week of April. The earliest emergence in 2006, as early as the first week of April, was recorded in subsamples under the Hoopoe treatment.

2a.



2b.

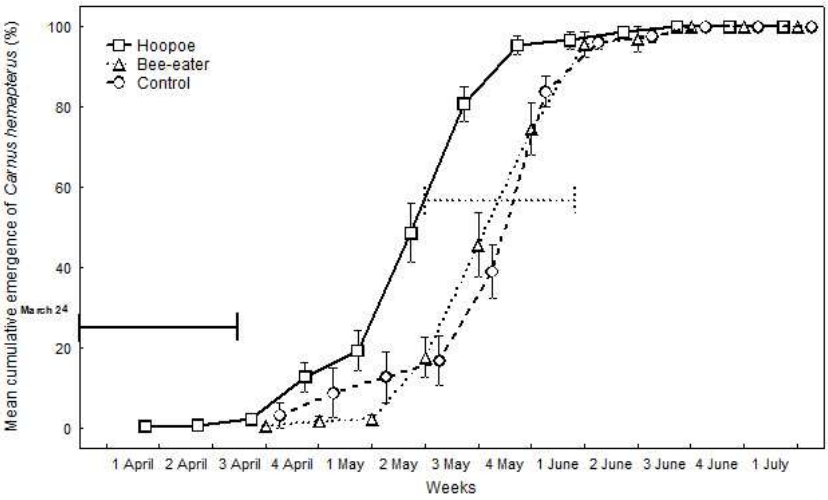
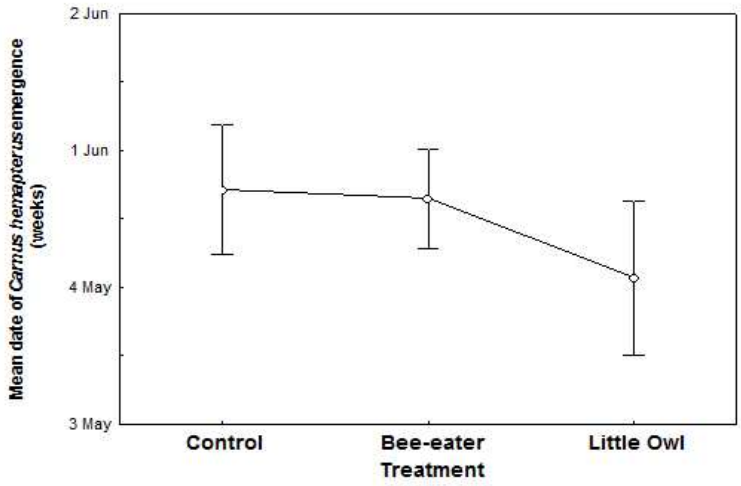


Figure 2. Mean cumulative emergence (±SE), of *Carnus hemapterus* under different treatments performed during 2005 (a), and 2006 (b). Horizontal dotted lines represent the duration of the habitual host (Bee-eater) incubation treatment in both 2005 and 2006; horizontal continuous lines represent the duration of the host-switching incubation treatment (Little Owl in 2005 and Hoopoe in 2006, the latter starting 24th March).

At the end of April it became common in the Hoopoe treatment and it was not until the second week of May when emergence occurred regularly in the Bee-eater and control treatments (Fig. 2b). Nonetheless, treatments did not influence the length of the emergence period (number of weeks when emergence occurred)

in any year (Repeated measures ANOVA, 2005: $F_{2,14} = 0.05$, $P = 0.94$; 2006: $F_{2,28} = 0.57$, $P = 0.57$). Most nests reached 100% emergence by mid June in 2005 and during the first week of June in 2006.

3a.



3b.

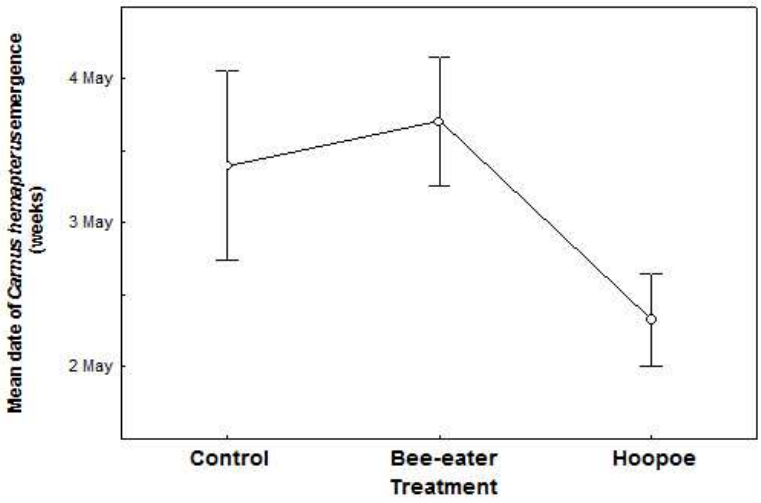


Figure 3. Mean emergence date (±SE), ranging from 26th March to 11th July (2 May = 8th – 14th) of *Carnus hemapterus* under different treatments during 2005 (a) and 2006 (b).

Treatments did influence the mean date of emergence both in 2005 and in 2006 (Repeated measures ANOVA, 2005: $F_{2,14} = 5.9$, $P = 0.013$, adjusted $P = 0.027$; 2006: $F_{2,28} = 12.1$, $P < 0.001$, adjusted $P < 0.001$, respectively) so that the mean emergence of flies from subsamples under the Little Owl treatment (in 2005) and the Hoopoe treatment (in 2006) occurred earlier in comparison to the one of flies under the control (Univariate tests of significance for planned comparisons, $P = 0.004$ and $P = 0.005$, respectively) and Bee-eater treatment ($P = 0.063$ and $P < 0.001$, respectively) (Figs. 3 a, b). The Hoopoe treatment had a stronger effect than the Little Owl treatment since Hoopoe-incubated flies emerged on average 1.07 weeks earlier in comparison to control flies (Fig. 3b) whereas Little Owl-incubated flies emerged just 0.64 weeks earlier than control flies (Fig. 3a).

2005	df	F	P	Adjusted p
Treatment	2, 14	8.4	0.004	0.005
Time	6, 42	277.4	< 0.001	< 0.001
Treatment x Time	12, 84	5.1	< 0.001	0.014

Table 2. Results of the multi-way within-subjects repeated measure ANOVA for the experiment in 2005. The dependent variable is the cumulative percentage of emerged flies per subsample, and treatment (Little Owl, Bee-eater and control) and time (seven weeks from 25 April until 11 June) are the predictors. Adjusted p values refer to the Greenhouse-Geisser corrected probability (see Methods).

Analysing the weekly emergence of flies in each treatment we found, both in 2005 and in 2006, a significant treatment x time interaction (Tables 2 and 3 respectively), indicating that treatments influenced the emergence pattern of adult flies. Specifically, in 2005 pupae started emerging at the same time regardless the treatment) but from mid May onwards pupae under the Little Owl incubation treatment emerged at a faster rate (Fig. 2a). In 2006, some Hoopoe-incubated pupae (two out of 176 emerged in this treatment, 1.13%) appeared two weeks earlier than pupae under the other treatments.

2006	Df	F	P	Adjusted p
Treatment	2, 28	14.9	< 0.001	< 0.001
Time	4,56	128.6	< 0.001	< 0.001
Treatment x Time	8, 112	4.8	< 0.001	0.002

Table 3. Results of the multi-way within-subjects repeated measure ANOVA for the experiment in 2006. The dependent variable is the cumulative percentage of emerged flies per subsample, and treatment (Hoopoe, Bee-eater and control) and time (five weeks from 24 April until 28 May) are the predictors. Adjusted p values refer to the Greenhouse-Geisser corrected probability (see Methods).

Nonetheless, the differential effect of the treatments is evident only from the end of April, when the Hoopoe treatment produced a faster emergence than the Bee-eater and the control treatments (Fig. 2b). The Bee-eater treatment had no significant effect on *Carnus* emergence when compared with the control treatment (Univariate test of significance for planned comparisons, 2005: $P = 0.21$, 2006: $P = 0.41$).

Discussion

Our study experimentally shows the ability of a diapausing ectoparasitic fly to respond to thermal changes in its intimate environment caused by the type of host. Pupae exposure to the incubation of the usual or a different host did not influence the prevalence, the length of the emergence period nor the number of emerged flies (with the exception of control subsamples in 2005). In contrast, experimental simulations of host switching resulted in significant changes in the phenology of emergence of the parasite, modifying both the mean date and the rate of emergence. As a result, flies under the new host emerged earlier and faster in comparison with flies under the habitual host and the control treatments. The effect was more evident for flies under the Hoopoe treatment, the host with the most dissimilar phenology to the usual one, the Bee-eater, since emergence of the parasite started some weeks earlier than in subsamples under the other treatments (even though only few flies emerged at that time). The treatment reproducing the occurrence of the usual host had no discernible effect when compared with the

results obtained with the control treatment (absence of host). This is not surprising since differences in temperature between both treatments (27.5ºC vs. an average temperature of 25.5ºC in the store room at the time of the bee-eater treatment in 2006) was small and, thus, they had a negligible effect on the general pattern of emergence of flies under both treatments. For comparison, differences in temperature between the Hoopoe treatment (27.5ºC) and the control one at the time when the former was applied were three-fold higher (mean temperature in the store room during the Hoopoe treatment = 21.6ºC). The question remains whether differences in temperature between the control treatment and an experimental one resembling the same host in a different (colder) climatic area (where ambient and nest temperature differences would be larger), or another usual host breeding earlier in the season (e.g. Hoopoe) (and thus causing larger differences between both treatments) would have resulted in different emergence patterns.

Thermal response of diapausing insects is well known since long and decreased diapause duration in many different arthropods as a consequence of gradual increase of environmental temperature has been experimentally shown (e.g. Broufas & Koveos 2000, Kemp & Bosch 2005, Teixeira & Polavarapu 2005). Surprisingly, the effect of host temperature on diapausing parasites has been less investigated. Wetzel & Weigl (1994) and Tripet & Richner (1999) showed the importance of host temperature profiles as an environmental factor that can determine differences in several parasite features but they did not pay specific attention on the effect of host temperature on the phenology of emergence of diapausing parasites and its ecological consequences in terms of host-parasite synchronization. The use of host temperature as a cue to break dormancy has been described for ectoparasites of endothermic hosts like some flea or bug species (Marshall 1981, Danks 1992). However, our results show that the rate of *Carnus* emergence increased just before the end of the Hoopoe and Little Owl treatments (Fig. 2), suggesting that the underlying mechanism is not the above-mentioned break dormancy, that is an aseasonal quiescence where duration of dormancy is highly variable (short or long cycle) and generally depends on the absence/presence of the host (Marshall 1981, Tauber et al. 1986). In contrast,

Carnus hemapterus dormancy is a long-cycle seasonal diapause (Guiguen et al. 1983, Valera et al. 2006b). Thus, differences observed among treatments are probably the result of faster heat accumulation (Tauber et al. 1986, Kostal 2006) rather than the result of sudden break dormancy.

What are the consequences of *Carnus* response to host temperature and host switching? Maximising the temporal overlap with the host is crucial to parasites (Poulin 1998). Thus synchronization of host-parasite cycles is expected and, in fact, it has been reported elsewhere (see, for instance, Andres & Cordero 1998, Rolff 2000). Valera et al. (2003) showed that the emergence of adult carnid flies in a bee-eater colony was synchronized with hatching of its usual host so that only 9.0% of the flies emerged before any bee-eater nestling hatched in the population (see also Liker et al. 2001). However, appearance of the fittest resource can vary according to the characteristics of the particular host exploited by the parasite (e.g. breeding phenology) and/or the geographic location. Moreover, generalist parasites like *Carnus hemapterus* can potentially feed upon a wide variety of hosts. For instance, Valera et al. (2003) found that second broods of rock sparrows coexisting with bee-eaters in the same colony were infected by *Carnus* whereas first broods (occurring earlier that bee-eater broods) were free of parasites. Spatial and/or temporal adaptations to different hosts' phenologies can help to diminish such sort of mismatch (Carroll & Boyd 1992, Filchak et al. 2000, Nyman 2002). Intraspecific clinal variation in the thermal regulation of the rate of diapause development has been demonstrated in a variety of organisms (see Tauber et al. 1986, Hoffmann et al. 2003) and, thus, selection for various *Carnus hemapterus* thermal phenotypes (Nijhout 1999, Weinig & Smichtt 2004) with rates of development at different optima of temperature adapted to the breeding phenology of different hosts could be possible.

Most insects in temperate climate use either photoperiod or temperature, or a combination of both, as cues for their timing decisions (Tauber & Tauber 1981, Smith & McIver 1984, Leather et al. 1993). Our data suggest that, by using the same signal (temperature) but from different sources (abiotic -ambient temperature- and biotic -host temperature-), *Carnus* is able to respond both to seasonal, predictable unsuitable periods (e.g. autumn-winter) as well as, at least

partially, to unpredictable short-cycle modifications (host switching involving differences in breeding phenologies), adjusting its emergence to the appearance of the nestlings of the new hosts and increasing its chances of survival and future reproduction.

It could be argued that the observed differences in emergence here revealed could be due to genetic variation within the fly population for emergence date. However, mixing of samples during collection and randomization of assignment to each treatment excludes this explanation. Rather, we propose that *Carnus hemapterus* displays some degree of phenotypic plasticity to adjust its emergence to different hosts. When appropriate food resource appearance is irregularly distributed along the season, polymorphism in diapause duration between individuals feeding on particular hosts can take place (Feder et al. 1993, Tikkanen & Lyytikainen-Saarenmaa 2002). Nonetheless, when sets of individuals are specialized to parasitize a particular resource, they can lose the ability to exploit others (Giorgi et al. 2004), jeopardizing their future reproductions when the preferred host is not present. Therefore, selection for ability to respond to eventual alternative host appearance by means of phenotypic plasticity in diapause traits could be advantageous. Moreover, it is known that the development system of insects is preadapted in many ways for the rapid evolution of phenotypic plasticity (Nijhout 1999). For generalist species that occupy a wide range of habitats, like *Carnus hemapterus*, a high degree of plasticity is advantageous and could consequently be a result of natural selection (Blanckenhorn 1998).

We cannot discriminate whether this species shows a "general-purpose genotype" (Baker 1965), capable to respond to any host, or whether the plastic response is limited either because there exists some physiological limit, or because there is a loss of specific thermal phenotypic responses in local populations (Kemp & Bosch 2005). Our results do suggest that there may exist some kind of limited thermal phenotypic plasticity (West-Eberhard 2003) because in the most strenuous case (the Hoopoe treatment) only a small fraction of flies (9.23% of the total number of flies emerged in that treatment) appeared during April, when most first Hoopoe clutches are hatching in our latitudes (Martín-Vivaldi et al. 1999). Thermal phenotypic plasticity may, on the other hand, be

costly since responses to changes in temperature, when combined with other factors affecting insect survival (e.g. heavy competition for resources during the larval stage due to high population density, see Gibbs et al. 2004; Koch et al. 2005), could jeopardize survival. This could be the case in 2005, when the temperature treatments together with the high density of larvae could result in the lower number of emerged flies in the experimental treatments in comparison to the control one.

Previous work on the interactions between host and parasites has focused primarily on the importance of host immunology, morphology or behaviour. Our study rather explores intimate mechanisms regulating the synchronization of host-parasite cycles and stresses the idea that host temperature is an ecological factor that must be considered when host-parasite relationships are studied. Differences in temperature profiles among hosts could be an important selective pressure driving ecological specialization processes via diapause adaptations to particular temperature profiles in parasites. Testing whether host temperature could be a mechanism of initial divergence of populations in *Carnus* and other parasites would require additional research on within-population variance and range of phenotypic plasticity in the emergence patterns of parasites.

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CAPÍTULO IV

Allochronic emergence and interspecific host-associated phenological synchronization in a generalist ectoparasite.

Abstract: Generalist parasites are exposed to a variety of hosts with marked degree of ecological and phenological differences. In diapausing parasites, where the arrest of diapause and the emergence of the infective phase are synchronized with the appearance of the resource (host), host-associated synchronization with the different host phenologies can led in a temporal isolation, that eventually could drive an allochronic process of diversification among the different host-associated parasite lineages. We examined the existence of temporal isolation, plasticity in the length of diapause and host-parasite synchronization in the generalist avian ectoparasite *Carnus hemapterus*, by means of two different experimental procedures, a common garden and a translocation experiment. Results of common garden experiment showed that there is a host-associated parasite emergence with three of the most habitual host species in our latitudes. By means of translocation procedure we found that development and arrest of the diapause is a plastic trait in this specie, confirming that phenology of emergence in *Carnus hemapterus* can be easily modified by environmental changes experienced during last phases of diapause development. By means of null models simulation of phenological co-occurrence, we also showed that phenological distribution of Roller and Hoopoe associated *Carnus* strains were synchronized with the breeding phenology of their respective hosts. As a whole, our results confirm the existence of temporally isolated and host-associated alternative phenotypes in this generalist specie. Consequences of this temporal barrier in the framework of an eventual process of allochronic diversification are discussed.

Keywords: *Coracias garrulus*, *Upupa Epops*, life-cycle synchronization, clinal speciation, polyphenism, phenotypic plasticity, diapause development.

Miguel A. Calero-Torralbo, Francisco Valera, Jordi Moya-Laraño

Introduction

Organisms are constantly developing strategies and adaptations to synchronize their life-cycles with the most favourable periods to ensure highest rates of survival and reproduction. Parasitic organisms are particularly prone to synchronize life-cycle with their resources because, in general, they feed on ephemeral and/or irregularly distributed individuals and they must ensure that their host-feeding stages become synchronized with the times when hosts provide the appropriate food resources. Most biotic or abiotic factors that fluctuate and influence over the organisms throughout their life span are either directly or indirectly related with seasonal changes (Tauber et al. 1986); and among invertebrates (including parasites, see for example Feder et al. 1997, Sommerville et al. 2002, Randolph 2004), dormancy, either by means of diapause or quiescence, is considered a main strategy to face seasonal and/or cyclic adversity and synchronize development with the period when conditions are ideal to achieve optimum values of fitness (Tauber et al. 1986, Danks 1987).

Intraspecific variability and genetic polymorphism in diapausing stages has been well documented in hundreds of species (see Brown & Hodek 1983, Tauber et al. 1986, Leather et al. 1993) and since diapause regulation and duration are strongly dependent of environmental factors like temperature, photoperiod, moisture, food availability, or population density (Kostal 2006, Tauber et al. 1986), plasticity and fixation of alternative phenotypes in the expression of diapause traits is a widespread feature in diapausing organisms (West-Eberhard 2003). Therefore, observed polyphenism and variability in diapause expression can be the result of a fixed genetic polymorphism, plasticity controlled by environmental cues (acting direct or indirectly over the diapausing stage) or a combination of both mechanisms (genetic polymorphism with individual phenotypes being partially regulated by environmental factors) (Tauber et al. 1986, West-Eberhard 2003, Kostal 2006).

Clinal variation both at spatial and temporal scale in diapause duration has been deeply documented and intraspecific variation in diapause has been proposed as an important isolation mechanism involved in many processes of speciation, race formation and/or specialization events of parasites and phytophagous

organisms (West-Eberhard 2003). Particularly allochronic speciation (divergence of species that are separated in time rather than in space, Alexander & Bigelow 1960) via host variation in phenology has been suggested as an important mechanism of temporal isolation and/or divergent selection (Via 2001, Rundle & Nossil 2005). Allochronic divergence in phytophagous arthropods is common (Craig et al. 1997, Filchak et al. 2000, Tikkanen & Julkunnen - Tiito 2003, Abbot & Withgott 2004, Feder et al. 2005, Santos et al. 2007, 2011, Ording et al. 2010); in contrast, very few cases of allochrony have been studied in animal parasites (see Therom & Combes 1995, McCoy et al. 2001), despite the importance that this diversifying mechanism may have in this guild (Price 1980, McCoy 2003). Particularly, generalist parasites with broad host range are ideal organisms to study allochrony, since parasites tend to specialize on their local environments (i.e. hosts) (Combes 1991, Thomson 1994, de Meeûs et al. 1998) and natural selection would favour parasite specialization on the most available host species (Haldane 1949) or increase the probability of multiple plastic phenotypes within parasite populations exposed to different hosts with marked differences in life-cycle phenologies. This could eventually drive selective divergence processes by means of fixation of alternative life-cycle, due to assortative mating between synchronic individuals, thereby turning incidental assortative mating into assortative mating by genotype. (West-Eberhard 2003, Schlichting 2004). Thus, some degree of synchronization between parasites and some of their hosts can be predicted even for generalist parasites.

By studying the generalist ectoparasitic fly *Carnus hemapterus*, and several of their avian host species, we investigate the existence of allochronic emergence associated with different host breeding phenologies. *Carnus hemapterus* mainly parasitizes unfeathered nestlings of a large number of species at a large geographical range (Brake 2011). Time of emergence of *Carnus hemapterus* is mainly controlled by diapause and post-diapause duration and temperature and host breeding phenology seems to play a main role as a regulatory and informative cue adjusting *Carnus* emergence (Calero-Torralbo & Valera 2008, Calero-Torralbo et al. in prep.). Intraspecific (at nest and population level) host-parasite synchronization has been reported by several authors (Liker

2001, Valera et al. 2003, Martin-Vivaldi et al. 2006, Václav et al. 2008, Calero-Torralbo et al. in prep.). Nevertheless, there is no work testing the existence of inter-specific host-parasite phenological co-occurrence and allochronic separation by means of different parasite host-associated emergence. Information about the extent of plastic responses in *Carnus hemapterus* phenology is also scarce (see Calero-Torralbo & Valera 2008) and the nature of the expression of *Carnus hemapterus* emergence (a plastic broad-range phenology, a specialized strategy and/or parasite adaptation to particular host phenologies or a combination of both) remains unsolved.

In this work we explore host-parasite synchronization in an avian-parasite system; we predict the existence of host-associated allochrony as a first stage in a hypothetical process of parasite divergence and try to define the degree of plasticity involved in *Carnus hemapterus* phenology and diapause duration. We specifically conduct three different approaches to answer the above questions:

1. Parasite allochrony: In order to minimize the contribution of environmental variation to parasite phenology during the last stages of *Carnus hemapterus* diapause (probably more dependent on environmental conditions than winter stages; see Calero-Torralbo & Valera 2008), and isolate the host-related effects over parasite phenologies, we conduct a common garden experiment with three host species using natural holes similar to those normally used by the hosts during the breeding period in our study area
2. Host-parasite phenological synchronization: We explore the degree of parasite matching with the different host breeding phenologies using null models (Gotelli & Graves 1996, Gotelli 2000). Specifically we asked if parasite temporal distribution in different hosts follows a non-random pattern.
3. Environmentally induced plasticity in parasite phenology. To detect local differences and incorporate environmental variation over *Carnus hemapterus* diapause duration and emergence, we conduct a translocation experiment using parasites from the same host (Roller, *Coracias garrulus*) but from two distant populations in the Iberian Peninsula. We analyse

patterns of phenotypic variation and try to detect and disentagle the contribution of local host phenology from the effect of environmental factors over parasite emergence during last stages of diapause development.

Material and methods

Study areas and species

The study areas are located at Tabernas (Almeria, South-eastern Spain 37.066 N, -2.354 E) and Castro Verde (Baixo Alentejo, Southern Portugal 37.706 N, -8.071 E). The landscape of Tabernas mostly consists of badlands and wadis with olive and almond groves interspersed among numerous dry riverbeds. The climate is semiarid with long hot summers and high annual and seasonal variability in both rainfall and temperature (average annual maximum temperature during the warmest month = 34.7 ºC, average annual minimum temperature during the coldest month = 4.1 ºC ; mean annual rainfall = 239.4 mm; cold desert climate under the Köpper-Geiger climate classification system; Peel et al. 2007, Lazaro et al. 2004). Castro Verde is a cereal pseudosteppe area comprising a mosaic landscape of cereal, stubble and fallows; the climate is of Mediterranean type, with hot dry summers and mild humid winters (average annual maximum temperature during the warmer month = 32.0 ºC, average annual minimum temperature during the coldest month = 5.0 ºC, mean annual rainfall = 589.4mm; warm Mediterranean climate under the Köpper-Geiger climate classification system; Peel et al. 2007, http://www.weather.com).

Carnus hemapterus (hereafter *Carnus*) is a 2-mm long polyphagous ectoparasitic fly that parasitizes nestlings of a variety of bird species (host range = 60 cited species; Brake 2011). Its life cycle comprises an adult stage, three larval phases lasting around 21 days at 22 ºC and a pupal stage (Guiguen et al. 1983). After a winter diapause usually lasting several months, imagines (infective stage) emerge the following spring approximately when most suitable hosts (unfeathered nestlings) are available. Adult flies are initially winged, but typically lose their wings once they locate a suitable host (Roulin 1998). Observational and recent

molecular surveys on *Carnus* phylogeography (Chapter VI), suggest that flies do not use adult birds as a vehicle of transmission and dispersal movements during the winged free-living stage are the main strategy to colonize new host nests. Adult flies are short lived during dispersion (around 2 days without feeding, Calero-Torralbo unpublished data).

We study flies from three different and usual host species exploited by *Carnus hemapterus* in the study areas (Martin-Vivaldi et al. 2006, Valera et al. 2006, Václav et al. 2008) and belonging to three different orders within the phylogenetic clade of the "land birds" (Hackett et al. 2008): the European Roller *Coracias garrulus* (Coraciiformes), Little Owl *Athene noctua* (Strigiformes) and Hoopoe *Upupa epops* (Bucerotiformes).

The European Roller is a single-brooded, migrant and territorial bird; in Tabernas area it breeds in burrows excavated by other birds in sandstone cliffs, in cavities in human constructions as well as in nest-boxes provided by the researchers from 2005 onwards; in Castro Verde area it breeds in cavities in human constructions (farms and abandoned structures) as well as in nest-boxes provided by the 'Liga para a Protecção da Natureza' (LPN - http://www.lpn.pt/) from the year 2002. The Little Owl is a single-brooded, resident and territorial bird; in our study area it breeds in burrows excavated by other birds in sandstone cliffs as well as in cavities in human constructions like bridges, stone walls or abandoned farm houses. The Hoopoe is a resident species in our study area. In a well-studied Hoopoe population (Martin-Vivaldi et al. 1999) only 19% of pairs star a second clutch from which 8% achieve two broods. Hoopoes breed in burrows as well as in nest-boxes and human constructions such as walls and other abandoned structures. Remarkable variation in egg hatching asynchrony, clutch and brood size has been documented for the three species, (Martín-Vivaldi et al. 1999, Cramp 1998, Václav et. al 2008, Václav et al. 2011). Nestlings of rollers and hoopoes are naked at hatching, but, by the age of 13 and 4-5 days, their body is almost completely covered with closed feather sheaths. The sheaths open from around 15-17 and 9-11 days respectively (Cramp 1998, R. Vàclav, J.M. Peralta - Sánchez pers. observ.). Little owl nestlings however are covered by a layer of white down at hatching and by the age of 2 weeks their body is completely

covered with the 2nd down (mesoptile, rather feather-like, Cramp 1998). In Southern Spain, hoopoes breed from February to June, little owls breed from April to June and rollers from May to July (Martín-Vivaldi et al. 1999, Cramp 1998, Václav et al. 2011). These three species breed in simpatry, with some pairs overlapping their breeding period, even though, in general, in our study area hoopoes start breeding first, followed by Little Owls and the Rollers.

Data Collection

(a) Host phenology

We recorded Hoopoe and Roller breeding phenology in order to compare it with the phenology of emergence of *Carnus*. We could not get data enough for Little Owl. For Rollers we recorded hatching dates for 6 years (2005-2010) in a well studied population at Tabernas area (see Václav et al. 2008, 2011). We used breeding data collected during 1991-1995 from a nearby Hoopoe population around Hoya de Guadix and Corvales Reserve (Granada, Southern Spain, 37.306 N, -3.135 E and 37.120 N, -3.584 E respectively; see Martin-Vivaldi et al. 1999) as well as data from Hoya de Guadix during 2002-2004 (Martin-Vivaldi, unpublished data). As a whole, we obtained data of 225 Roller nests and 136 Hoopoe nests. Hatching date of the first nestling of each brood (in Julian days of the calendar) was used as a measure of host phenology. Replacement clutches were disregarded.

(b) Parasite phenology

We controlled Carnus emergence in nests of the three different host species during 2006 and 2007. Nests that had been used the previous breeding season, and that were therefore more likely to contain overwintering pupae, were sampled. In 2006, we collected Hoopoe samples during 23 March 2006 from nest-boxes placed in Hoya de Guadix; samples from Rollers and Little Owl nests were collected during 28-30 March 2006 from natural holes and nest-boxes (only for Rollers) in Tabernas. As a whole, we collected samples from 33 nests (11 Hoopoe, 11 Little Owl and 11 Roller).

We also collected roller samples from Castro Verde and Tabernas during 5-7 December 2006 and 24 November 2006 respectively (17 samples from each location).

The amount of nest material collected varied among nests (range: 63-454 and 103-784 grams for samples collected in 2006 and 2007 respectively); nonetheless, the number of emerged flies was not related to the amount of nest material collected (Spearman rank correlations for each host/locality and year of emergence, $P>0.1$ for all cases, see also Valera et al. 2006, Calero-Torralbo et al. 2008). After collection, samples were kept in transparent plastic bags and stored in natural holes in both locations.

Experimental design

We conducted two different experiments (see figure 1 for experimental design diagrams in both years). With the aim of detecting host species-associated differences in *Carnus* phenology we designed a common garden experiment during 2006. Samples of the three host species were grouped together in different holes in sandy cliffs at Tabernas. We established 11 replicates, each of them including three different nest material samples coming from one Roller, one Little Owl and one Hoopoe nest. We cleaned and recovered collapsed Roller and Little Owl nests and placed the samples inside. In this way, samples with pupae from the different host species were under the same microclimatic conditions that resembled the ones commonly experienced by pupae during the overwintering phase.

During 2007 we conducted a translocation experiment in order to detect environmental associated differences and local effects in *Carnus* phenology within the same host species. Samples collected were thoroughly mingled, randomly split into 2 subsamples of the same mass and randomly assigned to the following 2 treatments: (i) Control treatment: subsamples remained in the same locality where they were collected (ii) Translocated treatment: subsamples were moved from Castro Verde to Almeria and vice versa. Control and Translocated subsamples from both localities were randomly grouped in pairs and were placed in old roller nests (burrows in sandy cliffs and holes in bridges in Tabernas, holes

in human abandoned structures in Castro Verde) (see Figure 1 for dates of establishment in each area). We established 17 replicates for each locality.

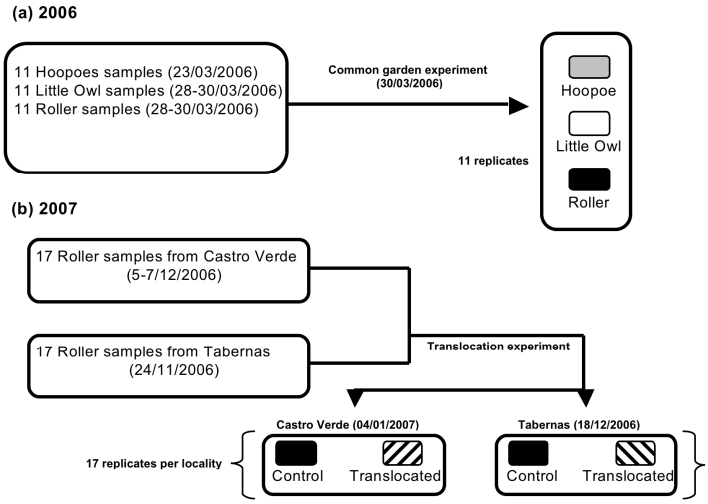


Figure 1. Experimental design diagram for (a) Common garden experiment in 2006 and (b) Translocation experiment in 2007. Dates when samples were collected and date of the onset of the experiments are in parentheses.

Samples were periodically monitored in the field (every 2-4 days) for *Carnus* emergence from 01 April 2006 until 20 July in 2006, from 10 March 2007 until 14 July 2007 in Tabernas and from 3 March 2007 until 17 July 2007 in Castro Verde. Flies emerging from each subsample and date were separately preserved in 99% ethanol and subsequently counted and identified with the aid of a binocular microscope.

In 2006, *Carnus* emergence was registered in 11 out of 11 Rollers nests, 8 out of 11 Hoopoe nests and 6 out of 11 Little Owl nests. We therefore recorded emergence from the three hosts in 6 out of 11 replicates. We also registered incomplete emergence (Hoopoe and Roller only) in 2 replicates and in 3 replicates emergence occurred only in roller samples. Concerning the Translocation experiment, emergence was registered in 32 out of 34 samples (15 samples from Castro Verde and 17 from Tabernas). Due to non-emergence in some subsamples

of 3 nests (2 from Tabernas and 1 from Castro Verde), emergence was recorded for both treatments in only 29 samples (14 from Castro Verde and 15 from Tabernas).

Statistical analysis

We calculated parasite prevalence (proportion of infected samples) and number of flies emerged in each sample (host/locality). Following Reiczigel and Rózsa (2005) we tested for differences in prevalence by means of Fisher's exact tests. To achieve normality, *Carnus* abundance (number of flies per sample) was log transformed. After checking ANOVA assumptions, we performed a one-way ANOVA to test for differences in abundance among hosts in 2006.

To study host (species)-related differences in parasite phenology we used repeated measures ANOVA analysis with log-transformed mean date of parasite emergence per sample as the dependent variable. Host species (i.e, three treatments) was the within-subject factor. Since the repeated measures ANOVA procedure does not allow the inclusion of empty cells (in our case, samples with no *Carnus* emergence), the analysis was restricted to those replicates where emergence was recorded in the three hosts (6 replicates, 18 samples). Sphericity was checked and Greenhouse-Geisser estimators (most conservative corrected probability; Von Ende 2001), were showed when appropriate. Additionally, univariate tests of significance for planned comparisons were performed to test for differential effects of *Carnus* phenology between pairs of hosts. All the pairwise paired t-tests across hosts gave the same qualitative results. However, the later procedure would not have provided an overall p-value for differences across hosts, as did the repeated measures ANOVA.

To study the effect of translocation over parasite abundance in both localities we performed a repeated measures ANOVA test with one within-subject factor (subsamples of the same nest exposed to different treatments). We used Maulchy's test to check the assumption of sphericity and, when the latter was not met, we adjusted the degrees of freedom by using the Greenhouse-Geisser estimator. The effect of translocation on parasite emergence date was assessed by running a Generalized Linear Mixed Model with normal errors (GLMM) (after

checking that data conform to the assumptions) followed by a Bayesian approach with Markov Chain Monte Carlo (MCMC) technique for accurate parameter estimation and to provide 95% Confidence Intervals for the random effects. The origin of the samples (Castro Verde/Tabernas) and Treatment (translocated samples versus non-translocated samples) were used as fixed effect variables. To avoid probable biases caused by pseudoreplication effects due to flies emerging from the same nest, and to test whether cavity differences could be substantial and significant, nest identity was introduced as a random effect in the GLMM.

We also tested host – parasite co-occurrence and phenological synchronization by means of null models. Null models analyse dates of parasite presence frequency across different host phenologies (data of species specific breeding phenologies) comparing with those parasite phenologies expected by chance. Null models have been used to detect temporal variation in abundance in host-parasite communities (Gotelli & Rohde 2002, Krasnov et al. 2006, Krasnov et al. 2010) but, to our knowledge, they have never been implemented to detect patterns of host-parasite phenological synchronization in polyphagous species, despite the usefulness of null models to detect structured patterns of temporal co-occurrence in interacting organisms that strongly depend on matching their phenologies with the fittest resources stage (i.e. hummingbirds and flower phenologies - Aizen & Vazquez 2006-, bat and fruit phenologies -Burns 2005, 2006-). Although more sophisticated null models for testing for phenological match between interacting groups of organisms have been recently developed (Aizen & Vázquez 2006), here we used a much simpler null model because we only had two species and two *Carnus* "strains" (i.e., flies coming from two different host species). Here, we merely ask whether in a common garden, the two *Carnus* "strains" tend to match, on average, their phenology of emergence to the breeding phenology of the host of origin (Hoopoe or Roller). The algorithm of the null model was as follows:

1) Take each of the 8 replicates for the 2006 experiment.
2) For each replicate calculate the mean emergence date ($\overline{p}_s$) for each of the two *Carnus* "strains".
3) Calculate the mean hatching date for each of the two hosts ($\overline{p}_h$).

4) Within each replicate and for each host/strain pair (Hoopoe and Roller), calculate the "pivotal" distance statistic for comparison as:

$$d_{obs} = \left|\overline{p}_s - \overline{p}_h\right| \qquad \text{(eq1)}$$

5) Within each replicate, randomly permute the emergence dates across flies regardless of "strain" and calculate the resulting (random) mean for each "strain" ($\overline{p}_r$).

6) Calculate the expected statistic with the permutated values:

$$d_{\exp} = \left|\overline{p}_r - \overline{p}_h\right| \qquad \text{(eq2)}$$

7) Compare d_{obs} to d_{exp} for each host/strain pair (Hoopoe and Roller).

8) Repeat steps 5-7 999 times for each replicate.

9) For each replicate and host/strain, compute the number of times that $d_{obs} > d_{exp}$. The proportion of failures will be the p-value for that replicate and host/strain.

10) Run a Fisher meta-analysis (Sokal & Rohlf 1995) to obtain an overall p-value across replicates and for each host/strain pair as:

$$\chi^2 = -2\sum_{i=1}^{N} \ln(p_i) \qquad \text{(eq3)}$$

Where χ^2 takes $2N$ degrees of freedom; N is the number of replicates and p_i the p-value of one of the two strains in the i-th replicate.

Using medians instead of means provided qualitatively the same results (not shown). Notice that the power to detect an effect will be less for the "strain" with more flies emerging, as less values of the other strain will be shuffled to dilute the potentially non-random effect. These differences will be more pronounced with more unbalanced replicates (see Table 3 for two extreme cases of lack of power in Roller). We therefore run a meta-analysis across replicates and obtained an overall p-value for each host/strain pair. An alternative would have been to subsample the host/strain with more fly emergence to force a balance between host/strains. However, since the results of the Fisher meta-analysis were both highly significant (see Results), we did not find this procedure to be necessary.

Prevalence analyses were performed using the program Quantitative Parasitology 3.0 (Reiczigel & Rózsa 2005); parasite abundance and host-associated phenology analysis were done using the STATISTICA 9.0 package (StatSoft Inc. 2009). GLMMs and MCMC calculations were performed using the R package (version 2.13.0, R development core team 2011, with libraries "lme4" and "languageR"). The code for the null models was also written in R.

Results

Effects of the experiments on prevalence and parasite abundance

Prevalence of *Carnus* was similar among the three host species (Fisher's exact test for comparing prevalence, P=0.058). No differences in prevalence were detected either between localities of origin (Fisher's exact test, P=0.5), between control and translocated samples grouped by assigned localities (2 groups: Tabernas Control + Castro Verde translocated vs. Tabernas Translocated + Castro Verde Control; Fisher's exact test, P=1.0) and among subsamples grouped by the combination of location x treatment (4 groups, Fisher's exact test, P=0.8). (Table 1)

	Common Garden Experiment (2006)			Translocation Experiment (2007)	
Host	Prevalence	Mean nº flies (range)	**Locality/ treatment**	Prevalence	Mean nº flies (range)
Hoopoe	72.7% (11)	15.2 (5-47)	Castro Verde (Cv)	88.2% (17)	267.1 (1-919)
Little Owl	54.5% (11)	66.6 (10-183)	Tabernas (Tb)	100.0% (17)	140.2 (16-357)
Rollers	100% (11)	118.6 (2-156)	Cv / Translocated	82.3% (17)	187.5 (6-768)
			Cv / Control	88.2% (17)	92.1 (1-395)
			Tb / Translocated	94.1% (17)	72.68 (1-200)
			Tb / Control	94.1% (17)	76.25 (7-157)

Table 1. Prevalence (number of nests in brackets) and abundance of *Carnus hemapterus* flies for different host species and for the two localities, and for the combination locality x treatment in the translocation experiment.

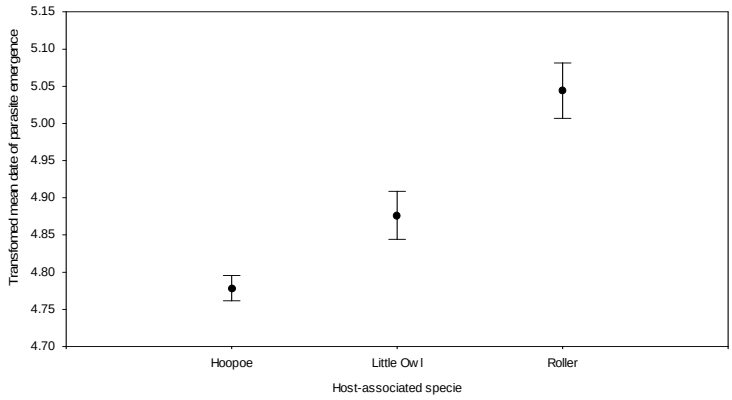
We did not find differences in *Carnus* abundance among samples coming from different host species (One-way ANOVA, $F_{2,22} = 2.5607$, P = 0.093) in 2006.

Treatment (translocated vs. control subsamples) did not influence the number of emerged flies per nest in either locality (Repeated Measures ANOVA, Castro Verde samples: $F_{1,13}$=0.8947, P = 0.36, Greenhouse-Geisser Adjusted P = 0.36; Tabernas samples: $F_{1,14}$=2.8945, P = 0.11, Greenhouse-Geisser Adjusted P = 0.11).

Host associated differences in parasite phenology

The common garden experiment showed that the mean dates of *Carnus* emergence significantly differed among host species. Parasites from Hoopoes emerged on average earlier, followed by flies from Little Owl nests, being the parasites from Rollers nests the ones emerging later (Repeated Measures ANOVA, $F_{2,10}$ = 53,64, P < 0.0001, Greenhouse-Geisser Adjusted P < 0.0001; Figure 2a). Comparisons between host species were all significant (Univariate test of significance for Planned Comparisons: Hoopoe vs. Little Owl $F_{1,5}$ = 14.1, P = 0.01; Hoopoe vs. Roller $F_{1,5}$ = 73.4, P = 0.0003; Little Owl vs. Roller $F_{1,5}$ = 74.0616, P = 0.0003). Although variability in the date of parasite emergence among replicates was remarkable for the three host species, allochronic emergence among samples from the different hosts was consistent (figure 2b).

2a.



2b.

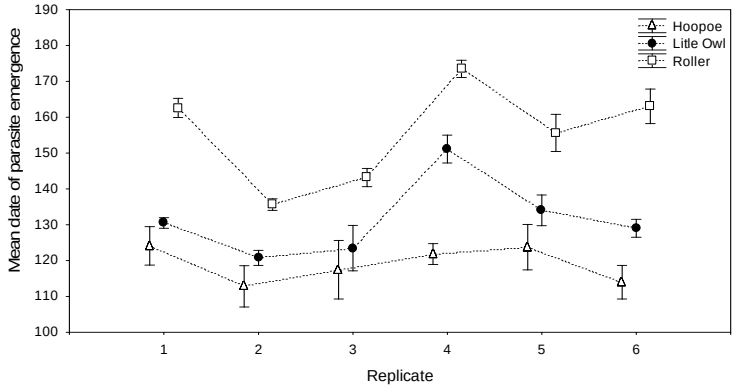


Figure 2. Mean *Carnus hemapterus* emergence date ± SE. (a) logtransformed mean host-associated parasite emergence date averaged for the six replicates, (b) mean host-associated parasite emergence date (in julian days) for every replica where emergence was recorded for the three host species.

Environmental associated differences in parasite phenology

The translocation experiment revealed a significant effect of locality, treatment and the interaction locality x treatment on the mean date of parasite emergence (Table 2). Spanish flies emerged later than Portuguese ones. The effect of

translocation varied with the origin of the samples since translocated Spanish flies emerged earlier than control Spanish flies whereas translocated Portuguese flies emerged later than control Portuguese flies (Figure 3).

Variable	*Fixed effects* Estimate	MCMCmean	HPD95lower	HPD95upper	pMCMC
(Intercept)	**154.534**	**154.564**	**151.097**	**158.190**	**0.0001**
Locality	**-25.453**	**-25.400**	**-30.573**	**-20.170**	**0.0001**
Treatment	**-7.328**	**-7.358**	**-8.564**	**-6.180**	**0.0001**
Locality x Treatment	**14.371**	**14.395**	**12.920**	**15.970**	**0.0001**

	Random effects Std.Dev.	MCMCmedian	MCMCmean	HPD95lower	HPD95upper
Nest identity (Intercept)	**8.1071**	**7.0408**	**7.1374**	**5.6547**	**8.7665**
Residual	**13.9867**	**13.9968**	**13.9973**	**13.7647**	**14.2464**

Table 2. Results of the Bayesian GLMM test for differences in date of parasite emergence between localities (Castro Verde and Almería) and treatments (translocated versus non translocated). Parameter estimation was done by means of Markov chain Monte Carlo (MCMC). Nest identity (individual nest origin) was entered as a random factor. P-values and confidence intervals calculated by means of MCMC sampling on posterior probability distributions of the parameters included in the model are showed. Significant p-values for fixed effects and effective MCMC means falling within HDP 95% intervals for random effects are bolded.

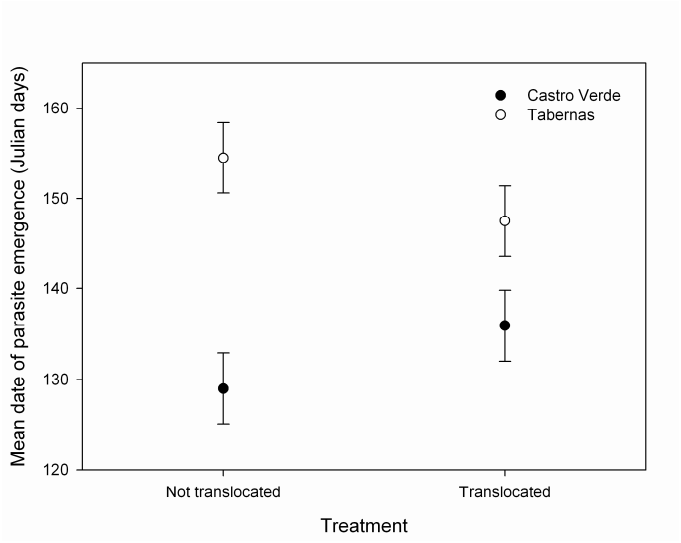


Figure 3. MCMC mean emergence date (Julian days) of *Carnus* flies in control and translocated samples from Tabernas and Castro Verde, after controlling for individual nest identity. 95% of confidence intervals were calculated by means of MCMC sampling parameters estimation.

Host-parasite synchronization and phenological co-occurrence

We explored host-parasite synchronization in the earlier and the latest hosts (Hoopoe and Rollers respectively) by means of null model simulation. The phenology of emergence of each of the two fly "strains" tended to match the breeding phenology of their original hosts. We found that host-parasite co-occurrence in time differed significantly from what it would be expected at random in 5 out 8 replicates for parasites from Hoopoe nests (with two non significant values being marginally significant, P = 0.052 and 0.057 respectively) and in 5 out of 8 replicates for parasites coming from Rollers nests (Table 3). Results of Fisher's combined probability test for meta-analysis showed that the combination of the p-values obtained in each replica yielded highly significant results in both Hoopoe and Roller - Carnus phenological co-occurrence (Fisher's χ^2 = 74.449 and 32.743 respectively; df=16 and P = 0.000 in both cases).

Host breeding phenology		**Replicate**	**Parasite emergence phenology**		**(Obs≠Exp.) p value**	
Upupa	*Roller*		From *Upupa*	From *Roller*	*Upupa*	*Roller*
128.8; (3.45) [136]	161.1; (2.69) [225]	**1**	124.1; (5.93) [14]	162.6; (5.70) [56]	**0.001**	**0.001**
		2	112.8; (8.62) [12]	135.7; (13.21) [156]	**0.001**	1.000
		3	117.5; (6.77) [6]	143.2; (12.58) [63]	**0.001**	1.000
		4	121.8; (9.36) [47]	173.5; (5.12) [68]	**0.001**	**0.001**
		5	123.8; (3.79) [10]	155.7; (15.86) [15]	**0.023**	**0.001**
		6	114.0; (1.45) [18]	163.0; (5.89)[17]	0.057	**0.001**
		7	116.0; (0.00)[5]	150.4; (12.89) [7]	0.052	0.317
		8	102.8; (4.02) [10]	148.5; (4.95) [2]	1.000	**0.019**

Table 3. Results of null models simulations of host-parasite co-occurrence, for each replica and host (Hoopoe and Roller). Observed phenology of both host (mean hatching date of the host population and standard deviation (SD)), and parasites (mean *Carnus* emergence date per nest and SD) is shown (in julian days). SD is show in brackets; number of host nests and flies per nest analysed in each simulation are shown in square brackets, and the significant p-values for differences between observed distributions of host-parasite phenologies versus expected host-parasite distribution by chance (after 999 permutations), are bolded.

Discussion

Our study is unique in that it tries to disentangle host and environmental related factors influencing the phenology of emergence of a haematophagous fly parasitising three different, usual host species. We found experimental evidence of: i) consistent allochronic emergence of carnid flies in three different host

species after controlling for environmental factors, ii) non-random temporal co-occurrence of carnid flies and two different, sympatric host species as a result of the differential emergence pattern, iii) environmentally-induced plasticity in parasite phenology after controlling for host-associated effects.

Host-parasite synchrony between *Carnus* and some of their hosts has been documented (Liker et al. 2001, Valera et al. 2003). However, only unimodal phenological distributions have been detected so far, probably because previous studies have focused on a single host species. Our multi-species study shows that *Carnus* pupae coming from different hosts with marked differences in breeding phenology emerge allochronically, and this pattern is consistent regardless the microenvironmental conditions experienced during the last phases of pupae dormancy. Furthermore, results of null models simulation show that phenological differences in host-associated parasites subgroups is highly synchronized with the average local breeding phenologies of the earlier and latest studied host species (Hoopoe and Roller respectively). Even though some breeding pairs of the different host species can overlap temporally, in general, Hoopoes start breeding first, followed by Little Owls and Rollers being the latest breeders (Martín-Vivaldi et al. 1999, Václav et al. 2011, Calero-Torralbo and Valera, personal observation). These results suggest that the temporal divergence in the phenology of *Carnus* "strains" is largely influenced by the breeding phenology of their hosts.

From an evolutionary point of view, allochronic phenology is considered a remarkable isolation mechanism than can increase divergence and/or diversification between populations or lineages. Models show that increasing phenological divergence can even lead to sympatric speciation (Devaux & Lande 2008) and many works have identified allochronic isolation as a common mechanism potentially causing incipient diversification in organisms with a previous life-cycle phenological variation, either as a by-product of any kind of adaptation (i.e. non-random mating, host fidelity, selection against hybridization between incompatible lineages; Coyne & Orr 2004), or acting directly as an allopatric barrier, by means of the reproductive isolation imposed by the temporal barrier (Yamamoto & Sota 2009, Santos et al. 2011, Kiss et al. 2011).

One important question when phenological isolation is detected in a group of organisms is to what extent the observed phenotypic pattern could be temporally or geographically fixed to allow for evolutionary diversification between host-associated lineages by means of long and stable allochronic and reproductive barrier (West-Eberhard 2003). If the phenological response of a genotype completely depends on the spatiotemporal variability of both host and environmental conditions, then temporal isolation of an individual (cohort or lineage) can fluctuate widely and, thus, should be considered as phenotypic plastic response (West-Eberhard 2003). In this case, an eventual process of diversification or lineage formation between host-associated parasites is unlikely, since genetic isolation between host-associated parasites is not generated (Coyne & Orr 2004). Our translocation experiment indicates that changes in local conditions experienced by *Carnus* in the last stages of diapause can modify the pattern of emergence. After correcting for the effect of the date of emergence of particular samples (that can bias the mean date of emergence towards nest with more emerged flies), we found that translocated samples from Tabernas emerged on average seven days earlier than control samples from Tabernas and, in turn, translocated samples from Castro Verde emerged seven days later than control samples from Castro Verde. We therefore found a very similar plastic response to changes in local conditions in both local populations. In other words, *Carnus* "strains" from the same host but from different localities and with different phenology of emergence (more than 25 days of difference in the mean date of emergence) showed a similar sensitiveness to changes in environmental conditions. Yet, the emergence of translocated samples did not overlap either because environmental differences were not large enough of because of physiological limits of plasticity.

Diapause is known to be a highly plastic trait (Tauber et al. 1986, West-Eberhard 2003) with a high potential for rapid evolutionary responses to selective pressures such as host-switching or changes in environmental conditions (Tsukada 1999, West-Eberhard 2003, Hairston et al. 2005, Dambroski & Feder 2007). Both previous work (Valera et al. 2006, Calero-Torralbo & Valera 2008) and this study confirm that this is also the case for *Carnus* diapause. Thus, this species has

potential to experience rapid rates of changes in diapause variability. The question then is whether variation in phenological traits (e.g. diapause length) associated to habitats or hosts could be genetically fixed. In our study system some factors could support fixation of diapause polyphenisms:

(1) Fixation of phenotypic alternatives will be produced if the ecological conditions (in our system, the different host breeding phenologies) that produce the different phenotypes are temporal and/or spatially recurrent (West-Eberhard 2003). Our results show that, at least, breeding phenology of the earliest and the latest host in our area are quite constant, namely because the Hopooe is a resident species and the Roller a transaharian migrant species. Although there is some interannual variability and phenological overlap (Martín-Vivaldi et al. 1999, Václav et al 2008, Chapter V), using data from several years did not change the phenological pattern between host species. Furthermore, simulations of host-parasite co-occurrence also suggest the existence of a divergent selective pressure towards synchronization at interspecific scale. Given that the infective phase of *Carnus* is short-lived (adult flies can survive up to 2 days in starvation, Calero-Torralbo unpublish data), temporal adaptation to different host's phenologies can be particularly useful for this ectoparasite. It could therefore allow for an increase in parasitation rate (Therom & Combes 1995), diminish energetic and reproductive costs derived from active dispersal searching for suitable resources (Strathman et al. 1981, Togashi & Hoshino 2003, Baker & Rao 2004), and favour reproduction (Filchak et al. 2000, Nyman 2002).

(2) Despite *Carnus* phenology seems to be highly dependent on environmental conditions, our results suggest that seasonal and microenvironmental changes (common garden experiment) do not eliminate host-associated differences among parasite lineages. Therefore temporal isolation among host-associated subgroups can be maintained.

(3) Even though we found phenological differences in hosts' phenologies, we cannot overlook that overlap among breeding individuals of the various hosts species occur, thus allowing for genetic flow between host-associated strains (e.g. *Carnus* colonization of various hosts during the overlapping period). Yet, genetic divergence and diapasue length fixation could occur between temporally distant

"strains", analogous to what parapatric speciation suggests (Balkau & Feldman 1973, Barton & Hewitt 1981, 1985, Felsenstein 1981). For instance, despite the plasticity in diapause length of this ectoparasite, flies parasitising late-breeding rollers could remain temporally isolated from flies parasitising early-breeding Hoopoes, thus allowing for a diversification processes by means of an ecological speciation or by allelic fixation by genetic drift (Schluter 2001, Coyne & Orr 2004). Some evidences in this regard have been obtained (see chapter VI). Genetic analysis of mitochondrial markers performed in *Carnus* individuals from a sympatric population in Tabernas coming from three different hosts (Roller, Little owl, Bee-eater), showed more genetic differences between flies emerging early and late in the season regardless the host species parasitized, than among flies obtained from different host/nests (see chapter VI).

Although a deeper assessment of the potential isolation effect of the observed host-associated phenology and of the limits of diapause plasticity in *Carnus* are needed, models and conditions of geographical allo-parapatric divergence processes (clinal changes of traits under divergence, hybrid zones between adjacent habitats and speciation occurring in distant areas even with low assortative mating within lineages, see Scriber 2002, Coyne & Orr 2004, Breisford & Irwin 2009) suggest that temporal clinal changes in *Carnus* emergence and co-occurrence with early and late host breeding phenologies could be a promising first step preceding allochronic diversification in this "apparently" generalist ectoparasite.

Mechanisms and consequences of temporal isolation in animal parasites have been poorly studied (Therom & Combes 1995, McCoy et al. 2001, 2005), despite the importance of allochrony in parasites speciation processes (McCoy 2003). Here we presented a promising system that could be useful in future research about ecological specialization and/or processes of diversification and temporal isolation in generalist parasites.

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CAPÍTULO V

Intraspecific variability in life-cycles synchronization between an ectoparasitic fly and its avian host

Abstract: Synchronization of host and parasites life-cycles and the role of environmental and host-associated variation over the final outcome of host-parasite relationships has been an important theme in evolutionary ecology for decades. However, studies at an intraspecific level are very scarce. Here we explore how host-associated traits, such as breeding phenology and habitat use, can influence parasite phenology and co-occurrence at different spatial levels (population and nest). During several years we studied the system formed by the generalist ectoparasitic fly *Carnus hemapterus* and one of their avian hosts, the European Roller *Coracias garrulus*. Interannual variation in phenology was larger for parasites than for hosts. Host predictability (in terms of both occurrence and phenological regularity) was moderate, suggesting that this resource can be difficult to be tracked by the parasite. A high number of flies consistently emerged before the appearance of suitable resources (nestlings), at both nest and population level. These results account for a low and highly variable interannual host-parasite synchronization rate. Nevertheless, we found that parasites from delayed host breeding pairs are less synchronized than parasites from early pairs within our roller population. Results on host suitability and parasite emergence distribution at population scale suggest that other mechanism (e.g. dispersal, exploitation of other host species) could be important for ensuring access to resources and counteract asynchrony at nest and intraspecific scale.

Keywords: host-parasite co-occurrence, environmental fluctuation, emergence, stochastic regulation, diapause, generalist, asynchrony.

Miguel A. Calero-Torralbo, Radovan Václav, Francisco Valera

Introduction

Understanding the dynamics of host-parasite interactions requires deep knowledge of the effects of environmental and host-related factors on the parasite's populations (Bush et al. 2001, Poulin 2007). Particularly, factors eliciting variation in host and parasite phenology can cause fluctuations of parasite densities and modify the abundance and capacity of a parasite infection over host populations (Godfray et al. 1994) and host-parasite co-occurrence (Münster-Swendson & Nachman 1978).

For many parasitic species, particularly nest-dwelling ectoparasites, phenological synchronization of their infective phases with the occurrence of sufficient resources is of vital importance to warrant the parasite's survival (van Asch & Visser 2007). One main question is whether parasites can reliably detect or predict both the beginning and the end of the period during which resources are available. In fact, many parasites have evolved developmental and dispersal strategies (e.g. diapause) based on both host related and environmental external stimuli to find a suitable host and synchronize their infective phase with the period when suitable hosts are available (Leather 1993, Poulin 2007, Jones 2001, Kostal 2006, Krasnov 2008).

The degree of phenological synchronization of interacting host–parasite species fluctuates widely due to multiple reasons such as differential sensitivity of each species to climate variation (heterotherms are more sensitive to climate than endotherms) or intraspecific variability in the response to environmental factors. In herbivorous insects and parasitoids, there is a wide intraspecific temporal and spatial variability in host-parasite co-occurrence within and among years due to variation in key environmental and host-related factors such as temperature (Visser & Holleman 2001), photoperiod (Denlinger 2002, Hegazi et al. 1988), drought (Smith and Bronstein 1996), snowmelt (Høye & Forchhammer 2008), budburst or fruiting phenology (Tikkanen & Julkunen - Tiito 2003, Feder & Filchak 1999, Van Dongen et al. 1997), or anthropogenic effects (Teixeira & Polavarapu 2003). Moreover, the strength of host-parasite synchronization observed at a smaller scale can be modified by ecological forces, constraining host or parasite population dynamics at larger scales (Van Nouhuys & Lei 2004).

Therefore, it is important to consider spatial and temporal variation in phenological synchrony (Tikkanen & Julkunen-Tiitto 2003, Van Nouhuys & Lei 2004). Similarly, a deeper understanding of the effect of key ecological factors (e.g. photoperiod, temperature) and life-history traits of the host (e.g. host predictability, distribution or density) at different spatiotemporal levels is important, since fluctuations of these parameters can determine the optimal degree of host-parasite synchronization and the reproductive tactics of the parasite maximizing its fitness (Powell & Logan 2005, Krasnov 2008, Barret et al. 2008). Despite the importance of synchronization of host-parasite life-cycles for the life-history strategies of "true" parasites (i.e. parasites that usually do not kill their hosts) of animals (Hakalahti et al. 2004), variation and heterogeneity in host-parasite synchronization have been largely neglected and well-documented cases of host-parasite synchronization are scarce (but see Foster 1969, Belozerov 1982, Drew & Samuel 1985, Larimore 1987, Roe 1988, Oliver 1989, Rolf 2000, Randolph 2004).

The system involving the blood sucking ectoparasitic fly *Carnus hemapterus* and one of its avian hosts, the Roller *Coracias garrulus*, provides an excellent system to study host-parasite synchronization. First, *Carnus hemapterus* parasitizes ephemeral resources, namely, unfeathered nestlings of various bird species. Thus, adaptations to synchronize the parasite's life cycle with the breeding cycle of the host are expected. Indeed, the emergence of the parasite's infecting phases has been reported to be partly synchronized with the occurrence of their hosts (i.e. avian nestlings) (Liker et al. 2001, Valera et al. 2003, Calero-Torralbo & Valera 2008). Second, since *Carnus hemapterus* can persist in the nest for several years (Valera et al. 2006), with no need to disperse actively, the nest itself can be seen as a discrete and isolated host entity, upon which fine level studies of host-parasite synchronization can be carried out. Moreover, information obtained at this level (e.g. host and parasite phenology) can be readily compared with those collected at larger scales (i.e. local patch or population levels). Third, temporal dispersal (by means of diapause) seems to be a major strategy for *Carnus* to synchronize its life cycle with the host (Valera et al. 2006). Diapause is characterized by a high degree of plasticity and variability (Tauber et al. 1986,

Danks 1987), so that diapausing parasites are ideal systems to study spatiotemporal variability of host-parasite co-occurrence. Fourth, breeding site fidelity has been reported for Rollers (Václav et al. 2011). This can increase resource predictability for *Carnus* and, therefore, enhance host-parasite synchronization at both species-specific and local patch host levels. Fifth, in our study area Rollers breed in three different nest types (natural holes in sandstone cliffs, holes in human constructions, and nest-boxes) involving different microclimatic conditions and host-related reproductive characteristics (Václav et al. 2011, Valera et al. unpublished data). Thus, macro and microclimatic variation throughout different spatiotemporal gradients are likely influencing both host and parasite phenologies. Finally, Roller is the last species breeding in our study area, setting the limit of seasonal parasite emergence, since no infective opportunities are available after nestlings Roller fledge.

Here we study host-parasite synchronization and their temporal and niche variability by monitoring host and parasite phenology during three years. We also analyse the influence of host predictability and parasite phenological constancy on the co-occurrence of both species. Specifically, we address the following hypotheses:

1. Environmental fluctuations can hinder host-parasite co-occurrence if their effects on both parasite and host phenologies are asymmetric (Van Nouhuys & Lei 2004). We predict that, in order to keep the high degree of host-parasite synchronization, inter-annual changes in environmental conditions should similarly affect the phenology of both hosts and parasites.

2. High host predictability can favour high parasite predictability (Barret et al. 2008). We predict that higher host predictability (in terms of nest reuse) at a fine spatial scale should be reflected by higher parasite predictability (i.e. interannual phenological regularity).

3. An accurate assessment of the period during which resources are available is vital for parasites diapause regulation. Such assessment is usually based on external factors, but also host-related life-story traits controlling the

parasite's life-cycle regulation over parental or subsequent generations (Tauber at al. 1986). We, therefore predict that host breeding phenology in year *t* should be an important host trait used by the parasite to time its emergence in year *t*+1.

Materials and Methods

Study area and species

The study area is located at the Desert of Tabernas (Almería, South-east Spain, 37º 05' N, 2º 21' W). The landscape mostly consists of badlands and wadis with olive and almond groves interspersed among numerous dry riverbeds. The climate is semiarid with long hot summers and high annual and seasonal variability in rainfall (Lázaro et al. 2004) and significant both intra and interannual fluctuations of temperature (mean monthly oscillation temperature between 12.8 to 15.6 ºC, summer and winter coefficient of variation 8-11 ºC and 22-23 ºC respectively; Lázaro et al. 2001)

Carnus hemapterus (hereafter *Carnus*) is a 2 mm long blood-sucking fly that parasitizes unfeathered nestlings of a wide variety of species (Grimaldi 1997). Its life-cycle comprises an adult stage, 3 larval phases encompassing around 21 days at 22ºC and 95% relative humidity and a pupal stage (Guiguen et al. 1983). The puparia are brownish-black and very cryptic, simulating nest remains of chitinous parts of arthropods consumed by the hosts. Usually, after a several months long winter diapause adult flies emerge in the spring when nestling hosts are available and emergence persists continuously throughout the whole nestling period of the host (Valera et al. 2003). Adult flies are initially winged, but typically lose their wings once they locate a suitable host (Roulin 1998). Carnid flies do not need a host for transmission and can colonise new host nests actively during the winged phase of their life cycle or can perpetuate by themselves in the nest for several years (Valera et al. 2006). Once adult *Carnus* has emerged, it cannot survive for a longer time without feeding (around 2-3 days; M.A. Calero-Torralbo, personal observation), and, thus, its dispersal period is short.

The European Roller *Coracias garrulus* (hereafter Roller) is a common avian host of *Carnus*. In our study area it breeds in burrows excavated by other birds in sandstone cliffs as well as in cavities in bridges. From the first year of our study in 2005 we provided Rollers with nest-boxes installed on trees and sandstone cliffs located near sandstone burrows and bridge cavities used by Rollers. We have progressively increased the number of nest-boxes along the study area during the last 6 years (see Václav et al. 2011). Microclimatic conditions vary widely among the various nest types. Generally, within-day cavity temperature variation in bridge and sandstone cavities is small compared to that in nest-boxes. Bridges show usually higher humidity levels compared to sandstone burrows and nest-boxes (unpublished data). Nest cavities are usually reused by Rollers but can also be used by Kestrels *Falco tinnunculus*, Jackdaws *Corvus monedula* or Little Owls *Athene noctua*. Rollers rear a single brood per year. Egg hatching is distinctly asynchronous with remarkable annual differences in hatching date as well as in clutch and brood size (Václav et. al 2008, 2011). Nestlings are naked at hatching, but, by the age of 13 days, their body is almost completely covered with closed feather sheaths. The sheaths open from around 15-17 days (Cramp 1985, R. Václav, pers. observ.). Nestling Rollers fledge approximately 20-22 days after hatching (R. Václav, pers. observ.).

Data collection

(a) Host monitoring

Fieldwork was carried out from 2005 to 2009. From the first observations of Rollers in early April, a population holding approximately 40 pairs was visited at least five times per week. After sighting first copulations, potential nest cavities were inspected every other day until the day of hatching of the last chick of the study population. During regular nest inspections, we monitored and recorded egg laying, clutch size, hatching dates and nestling survival. In order to control the effect of host nest reutilization on parasite population, we monitored host nest reoccupancy during the period 2005-2009. Hatching dates (in Julian days) of the first nestling of each brood was used as a measure of host phenology.

(b) Parasites phenology and abundance

Studying the phenology of emergence of Carnid flies in infected nests is not possible due to logistic (Rollers routinely expel alien material from their nests, including any devices for trapping emerging flies) and ethical reasons (continuous emergence monitoring would result in undesirable disturbance to Roller nestlings). Therefore, we studied the emergence of *Carnus* by monitoring nest subsamples stored under natural conditions (i.e. in natural cavities or nest boxes in the study area). This method may overlook the effect of the incubating host on diapausing pupae. However, Calero-Torralbo & Valera (2008) found that incubation by common avian host species does not result in significant changes in parasite phenology. *Carnus* phenology of emergence was studied in 2006, 2007 and 2009. Soil samples from the nest chamber of Roller nests used the previous season (likely containing *Carnus* pupae from parental generation of the previous year and probably some low percentage coming from individuals that have overwintered in the nest for 2 years or more (i.e. prolonged diapause, see Valera et al. 2006) were collected during the beginning of March 2006, 2007 and mid November 2008 (34, 19 and 21 nests respectively). After collection, samples were kept in transparent plastic bags and stored in cavities not used by overwintering or breeding birds, having been spread all over the study area. During 2006, samples collected from nests in sandy cliffs and bridges were randomly assigned to the same or different type of cavities, whereas samples collected in nest boxes were assigned to the other two nest types because all nest boxes were occupied by Rollers. In contrast, during 2007 and 2008-2009 each sample was assigned to the same type of cavity from where it was collected, except for the samples collected in nest boxes during 2007, which were assigned to holes in sandy cliffs. To account for any effect of the cavity type on Carnus phenology, we compared the emergence pattern recorded in samples stored in a cavity type different from the one where it was collected during 2006 and 2007 with those obtained in 2009 from samples of a particular nest type stored in a similar cavity the whole breeding season). We explore differences at the beginning of the emergence (first quartile, 25% of emergence) when differences in the variability of the emergence are higher (see Calero-Torralbo et al. 2008). In 2006 and 2007 25% of flies from

samples stored in a different cavity type emerged by the second and third week of May respectively, which was similar for samples kept in the same cavity type in 2009 when 25% of emergence was reached in the third week of May. Moreover, the emergence rate was similar regardless the type of nest where the samples was stored (GLMM, treatment fixed effect: $F_{1,93.16}$=0.03, p=0.86; nest identity random effect: Z= 36.42, p<0.001; see statistics section for the analysis procedure).

Samples were periodically monitored (each 2-3 days) for emerged *Carnus* from 1 March 2006, 2007 and 2009 until 20 July in 2006, 10 July in 2007 and 5 July in 2009 (in all cases more than 10 days after the last fly was recorded). Parasite emergence was detected in 19 out 34 nests in 2006, 17 out 19 nests in 2007, and 20 out 21 nests in 2009 (total nests: 56; parasite prevalence = 55.9%, 89.5%, and 95.2%, respectively). Flies emerging from each sample and during each inspection date were separately preserved in 99% ethanol and subsequently counted and identified with the aid of a binocular microscope.

Parasite emergence was defined by three variables: the weekly percentage of emerged flies, the length of emergence period (in days) per nest, and the date when 50% of *Carnus* flies emerged (in Julian days). We considered date when 50% of emergence is reached as a reliable estimator of mean date of emergence since most flies emergence was recorded around this date in previous works involving *Carnus hemapterus* emergence (Valera et al. 2006, Calero-Torralbo et al. 2008).

We also registered the total abundance of flies, which was defined as the total number of flies emerging in the nest sample, because the number of emerged flies was not related to the amount of nest material collected (Spearman rank correlations for each year: P>0.1, see also Valera et al. 2006, Calero-Torralbo et al. 2008).

(c) Host predictability and regularity of parasite emergence

We used two measures of host predictability and one of parasite emergence regularity:

a. Host occurrence: We calculated the rates of Roller nest reuse between consecutive years as well as the lag between intermittent uses of specific cavities by Rollers.

b. Phenology of host occurrence: Since host breeding phenology may vary between years and with the type of nest (Václav et al. 2011), host predictability was estimated on the basis of interannual differences in the hatching date of the first nestling in the same nest.

c. The regularity of parasite emergence was estimated by calculating the repeatability of the date when 50% of flies emerged in the same nest in different years.

(d) Host-parasite synchronization

In host-parasite interactions synchrony occurs when the life cycle of the parasite is timed so that it can exploit the host optimally. The availability of suitable hosts for *Carnus* was calculated at the nest and population level considering the requirements and life cycle of the parasite. For Rollers, the higher rates of *Carnus* infestation occur when the nestlings are unfeathered, quickly decreasing when feathers appear (about 14 days after hatching, Václav et al. 2008). Since *Carnus* can survive starving up to three days after its emergence, we extend the host availability period by three days before the first egg hatched in the respective nest. Consequently, the host availability period lasts 18 days (from day -3 to day +14, day 0 being the date when the first egg hatched). This period of host availability can increase depending on the number of surviving nestlings in the nest, since hatching is asynchronous in Rollers. We therefore extended the range of host availability according to the number of nestlings in the brood, assuming a mean hatching interval between two consecutive chicks to be 2 days (Václav et al. 2008). For our study system, the period of host availability ranged from 18 days (one nestling broods) to 28 days (six nestlings broods).

We calculated an index of host-parasite synchronization at the nest level as follows: we measured the percentage of flies emerging during the period of 18 days when the most suitable hosts are available (see above). This index is quite conservative because only the first hatched chick per nest was considered. The synchronization index requires that data of host hatching dates and parasites emergence dates are available for the same nest during the same year. Since parasite samples were collected before the Rollers breeding season and Rollers do

not always occupy the same nest in consecutive years (or breeding may fail), it was not possible to calculate the synchronization index for all nests. As a result, the sample size for the synchronization index (N=40 nests: 14 in 2006, 11 in 2007, and 15 in 2009) was lower compared with the number of nests with emerged parasites (N=56) and successful host breeding attempts (N=195).

Statistical analyses

To test the effect on emergence rate of translocation of nest box samples to other cavity types (see section (b) of material and methods), we used a generalized linear mixed model with binomial distribution error (the number of emerged flies during one week interval/the number of all emerged flies was used as a response variable). To account for the repeated measures effect (11 emergence observations for each nest sample), we used a mixed model analysis with the repeated measures design and the first order autoregressive, AR(1), covariance structure (Littell et al. 2006). Hereby, the sampling period was considered as a time measure (with one week intervals) and the sample's nest identity was used as a subject unit.

We evaluated niche variation in host and parasite phenology by analysing intra-annual differences among nest types and inter-annual differences in host hatching date, the length of parasite emergence and the date when 50% of Carnus flies emerged. We analysed inter-annual and cavity type differences also for parasite abundance. After checking the assumptions of parameterization, we performed a General Linear Mixed Model with normal distribution, except for parasite abundance, for which we used a Poisson distribution and Generalized Linear Mixed Model. Year and nest identity were analysed as repeated measures data including the AR(1) covariance structure (see above) ; year and cavity type were analysed as fixed effects. We performed post-hoc tests of significance when opportune. Three out of 56 nests were discarded for the analysis of parasite phenology due to the low (less than 5 individuals) and clumped emergence of flies.

We also analysed the effect of year and cavity type on the percentage of host-parasite synchronization by performing a GLMM with normal distribution. Year and nest identity were included as a repeated measures effect with the AR(1)

covariance structure, and year and cavity type as fixed effects. We conducted post-hoc tests of significance when opportune. Additionally, to test the effect of host breeding phenology on host-parasite synchronization in a given year (year *t*), we recorded previous (*t*-1) and current (*t*) host-hatching dates from the nest where parasite emergence was recorded during year *t*. A GLMM test with normal distribution was performed, including year and nest identity as a repeated measures effect with the AR(1) covariance structure, and year and the difference in host hatching date between year *t* and *t*-1 [$\Delta(t\text{-}(t\text{-}1))$] as fixed variables.

Results

Host phenology, predictability and nest reuse

Host hatching period at the population level (i.e. the period between the date of hatching of the first nestling in the earliest nest until the date of hatching of the first nestling in the latest nest) spanned from the fourth week of May until the fourth week of June for 2005-2008 and from mid May until mid July for 2009 (Figure 1). The mean length of the suitable host period during the five years study was 53.4 days (see Table 1a,b for additional information).

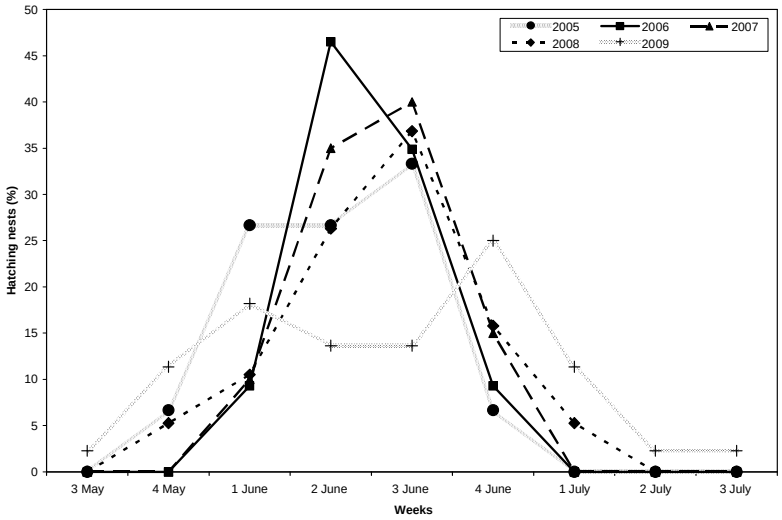


Figure 1. Percentage of hatching dates of the first Roller nestling in each nest along the breeding season for the years 2005 to 2009.

At the nest level, the mean range of suitable host days per nest was 22.63 days (SE = 0.14; *N* = 195). Host hatching dates did not differ among years during 2005-2009, but we observed significant differences among nest types (GLMM, normal distribution, repeated measures effect: year with nest ID as a subject, Z = 5.103, p < 0.001; fixed effects: year, $F_{4,105.2} = 1.93$, p = 0.111; cavity type, $F_{2,103.5} = 4.76$, p = 0.010; year × cavity type, $F_{8,109.6} = 0.86$, p = 0.555). Hosts hatched significantly earlier in nest-boxes than in bridges and tended to hatch earlier in nest-boxes than in sandstone burrows (Post hoc tests; b. vs. nb. $t_{107.9} = 3.07$, Sidak-adjusted p = 0.008; s. vs. nb. $t_{116.6} = 1.69$, Sidak-adj. p = 0.065; s. vs. b. $t_{92.24} = 5.97$, Sidak-adj. p = 0.69).

1a.

Year	Range	Minimum & Maximum	N	Mean	Median	Skewness
2005	27	145-171	30	158.3	158.5	-0.1145
2006	25	151-175	43	161.3	160.0	0.3415
2007	25	151-175	40	162.4	162.0	0.1890
2008	38	143-180	38	162.3	162.5	-0.2199
2009	60	134-193	44	165.0	165.0	-0.0767
05 to 09	60	134 to 193	195	161.7	162.0	0.1103

1b.

	Host availability			Parasite emergence		
Year	Earliest date	Latest date	Range	First fly	Last fly	Range
2005	142	189	48	-	-	-
2006	148	193	46	100	180	81
2007	148	193	46	109	184	76
2008	140	195	56	-	-	-
2009	131	201	71	97	176	80
05-09	131	201	71	-	-	-
06-07-09	-	-	-	97	184	86

Table 1. Descriptive data of (a) host hatching phenology (hatching date of the first nestling in the earliest and latest nest) and (b) dates of length of host availability and parasite emergence at population level, during the five - years study (05-09). Hatching dates and date of parasite emergence are measured in Julian calendar days.

Inter-annual host predictability was variable. Repeatability of host hatching dates in different years for the same nests during the whole study period was 0.26 (Table 2). However, repeatability varied widely depending on the time lags between different years, ranging from no repeatability for some years to a maximum of 0.70 for the 3-year period of 2007 to 2009 (Table 2).

HOST				PARASITE			
Years	Repeatability	P	N	Years	Repeatability	P	N
2005 to 2009 (5)	**0.26**	0,040	6	2006-07-09 (3)	0.38	0.080	5
2006 to 2009 (4)	**0.48**	0.006	6	2006-2007 (2)	**0.57**	0.029	10
2005 to 2008 (4)	0.16	0.177	6	2007-2009 (2)	**0.64**	0.030	7
2005 to 2007 (3)	-0.14	0.745	9				
2006 to 2008 (3)	0.46	0.167	8				
2007 to 2009 (3)	**0.70**	>0.001	13				
2005 and 2006 (2)	0.06	0.410	16				
2006 and 2007 (2)	0.18	0.234	18				
2007 and 2008 (2)	**0.60**	0.004	16				
2008 and 2009 (2)	**0.62**	>0.001	22				

Table 2. Host repeatability (interannual differences in hatching date of the first nestling in the same nest) in different periods. The number of years analysed are shown in brackets and the significant repeatability values are bolded. Values of signification of repeatability were calculated by means of ANOVA analysis.

The rate of nest reoccupancy in our area was not high. Monitoring nest cavities (all types) during the period 2005-2009 showed that approximately a half (53.3%; 56/105) of all cavities were used only once by the host, 24 (22.8%) were used 2 times, 15 (14.3%) were used three times, 4 cavities (3.8%) were used four times and 6 (5.7%) were used 5 times. Approximately a half of the nests used more than once were used intermittently: 50.0% (9/18) bridge cavities, 50.0 % (7/14) sandstone burrows, and 29.41% (5/17) nest-boxes. The lag between two nearest breeding attempts was one to three years (lags, 1 year: 16 ×; 2 years: 3 ×; 3 years: 2 ×).

Phenology of parasite emergence and interannual regularity

The parasite's emergence period at the population level (i.e. the period between the date of the emergence of the earliest fly until the date of the latest fly) spanned from the second week of April until the first week of July for 2006, from the third week of April until first week of July for 2007, and from the second week of April

until the fourth week of June in 2009 (see figure 2). The mean length of the period of 3 years was 79 days (see Table 1b for additional information). For 75% of samples containing flies, emergence started before the first week of June, when host hatching was widespread during the three years. Parasite emergence progressively increased along the season, with a sudden decrease after the peak was reached (Figure 2), as evidenced by the negative skew of the distribution of parasite emergence for all three years (skewness: *2006* = -0.556, *2007*= -0.730, *2009* = -0.568).

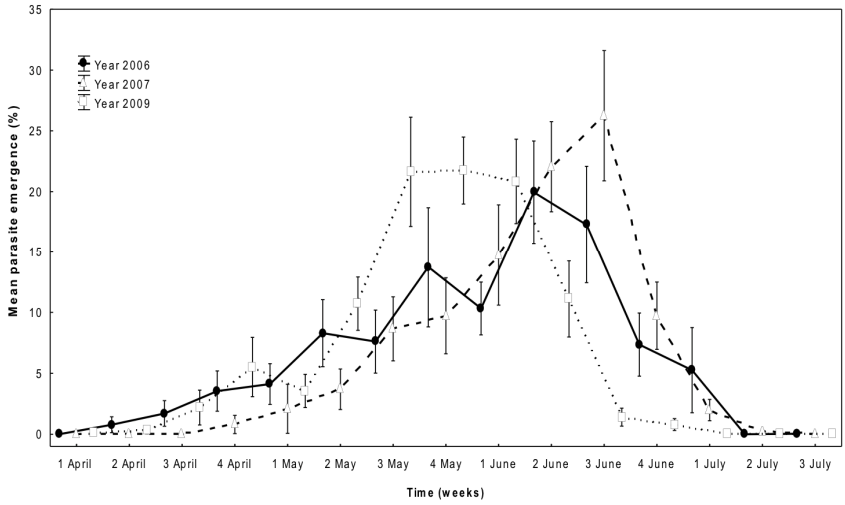


Figure 2. Mean percentage (±SE) of parasite emergence for years 2006, 2007 and 2009. (N = 31, 19 and 21 nests respectively)

In contrast, inside the nests the number of flies quickly increased, remaining relatively stable during the first four weeks of emergence and decreased progressively thereafter (Figure 3).

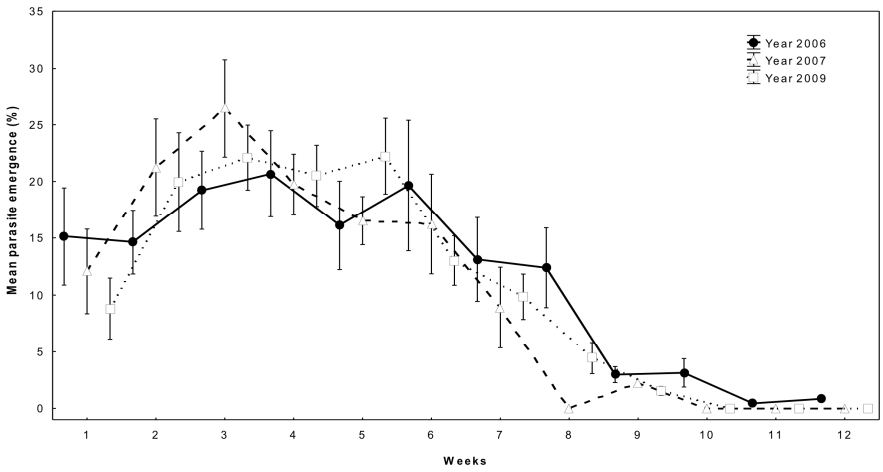


Figure 3. Percentage of the mean distribution (±SE), of parasite emergence within nests per week (N = 31, 19 and 21 nests respectively). Since the start of emergence varies among nests and years, nests of each year were batched taking week 1 as the start of the emergence in each nest, regardless of the actual parasite emergence date of each nest and year.

At the nest level, the length of the emergence period (the date of emergence of the first fly until the date of emergence of the last fly for the same nest), averaged 38.51 days during three study years; (*2006*: mean=41.31 days, SE=6.65, n= 16; *2007*: mean=37.18 days, SE=3.51, n=17; *2009*: mean=37.4 days, SE= 2.84, n=20) and differed between years and cavity types (GLMM test, normal distribution, repeated measures effect: year with nest ID as a subject, $Z = -1.99$, $p = 0.0466$; fixed effects: year $F_{2,40.91} = 5.58$, $p < 0.01$; cavity type $F_{2,26.59} = 13.64$, $p < 0.001$; year × cavity type $F_{4,42.06} = 5.18$, $p < 0.01$). However, as the interaction term year × cavity type suggests, these differences were only produced by the differences in the length of the parasite's emergence period in nest-boxes during 2006, but not during 2007 and 2009 (post hoc tests; b. vs. nb. $t_{36.02} = -5.07$, Sidak-adjusted $p < 0.001$; s. vs. nb., $t_{26.98} = 4.14$, Sidak-adj. $p = 0.009$; s. vs. b. $t_{26.79} = -1.09$, Sidak-adj. $p = 0.632$; 2006 vs. 2007, $t_{39.07} = 2.55$, Sidak-adjusted p =

0.043; 2006 vs. 2009, $t_{40.63}$ = 3.25, Sidak-adj. p = 0.007; 2007 vs. 2009, $t_{39.63}$ = 0.30, Sidak-adj. p = 0.987).

Parasite phenology (the date when 50% of parasites emerged) differed between years and cavity types (GLMM, normal distribution, repeated measures effect: year with nest ID as a subject, Z = 3.08, p < 0.01; fixed effects: year $F_{2,25.18}$ = 12.93, p < 0.001; cavity type $F_{2,34.78}$ = 22.94, p < 0.001. year × cavity type $F_{4,26.18}$ = 1.45, p = 0.247). Fifty per cent of emergence occurred earlier in nest boxes compared with sandstone burrows and bridge cavities (post hoc tests; b. vs. nb. $t_{36.02}$ = 6.39, Sidak-adjusted p < 0.001; s. vs. nb, $t_{36.24}$ = -5.62, Sidak-adj. p < 0.001; s. vs. b. $t_{32.49}$ = 5.97, Sidak-adj. p = 0.705) and occurred later in 2007 compared with both 2006 and 2009 (post hoc tests; 2006 vs. 2007: $t_{20.9}$ = -3.68, Sidak-adjusted p = 0.003; 2006 vs. 2009: $t_{37.54}$ = 0.46, Sidak-adjusted p = 0.958; 2007 vs. 2009 $t_{30.57}$ = 4.45, Sidak-adjusted p < 0.001). Parasite phenology within the same nests was repeatable between 2006 and 2007, 2007 and 2009, and tended to be repeatable among the three study years (2006, 2007 and 2009; see Table 2).

Determinants of parasite abundance

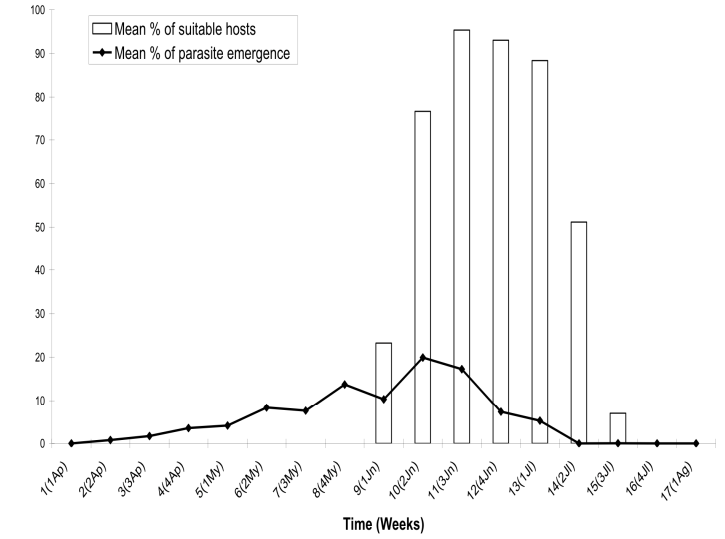
Carnus abundance (i.e. the number of flies emerged from nest samples) did not differ significantly between years (GLMM, Poisson distribution; repeated measures effect: year with nest ID as a subject, Z = 4.51, p < 0.001; fixed effect: year, $F_{2,42.91}$=1.20, p = 0.311). However, it did differ between cavity types (cavity type, $F_{2,42.97}$=6.36, p = 0.004; year × cavity type, $F_{2,43.07}$=0.62, p = 0.649) due to the higher *Carnus* abundance for nest-box samples compared to the samples from bridge cavities (nb. vs. b. $t_{42.7}$=-3.55, Sidak-adjusted p=0.028) and the marginally higher abundance for nest-box compared with sandstone samples (nb. vs. s. $t_{42.15}$=2.30, Sidak-adjusted p=0.062).

Host-parasite synchronization

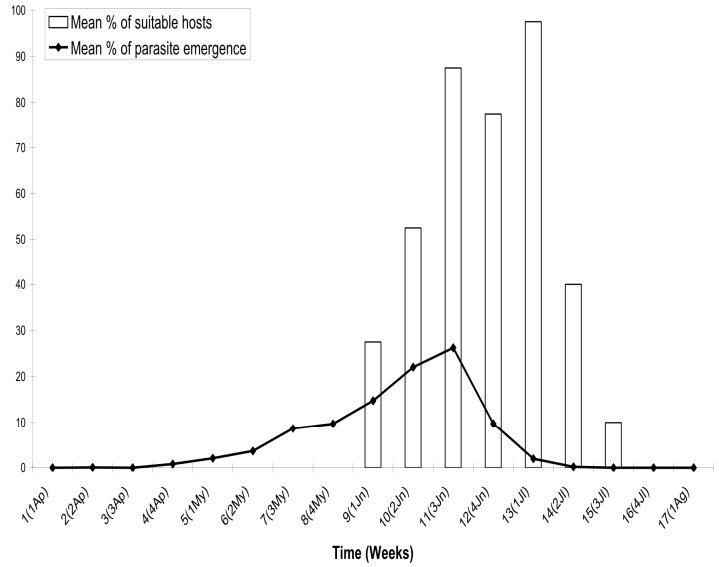
At the population level the mean period of fly emergence was 1.48 times longer than the mean period of host suitability (79 vs. 53.4 days). At the nest level the period of fly emergence was significantly longer (1.70 times longer, *t*-test for dependent samples t_{52} = -6.16, p < 0.001, data coming from nest where both fly

emergence and host suitability was recorded) than the period of suitable hosts availability (38.5 vs. 22.6 days). Consequently, in all years some flies emerged when no suitable Roller hosts were available (Figure 4a, b, c) and this mismatch affected the earliest flies so that, on average, one quarter of the studied population emerged before the occurrence of the earliest host (2006: 39.8%, 2007: 25.0% and 2009: 11.7%; see Figures 4).

4a.



4b.



4c.

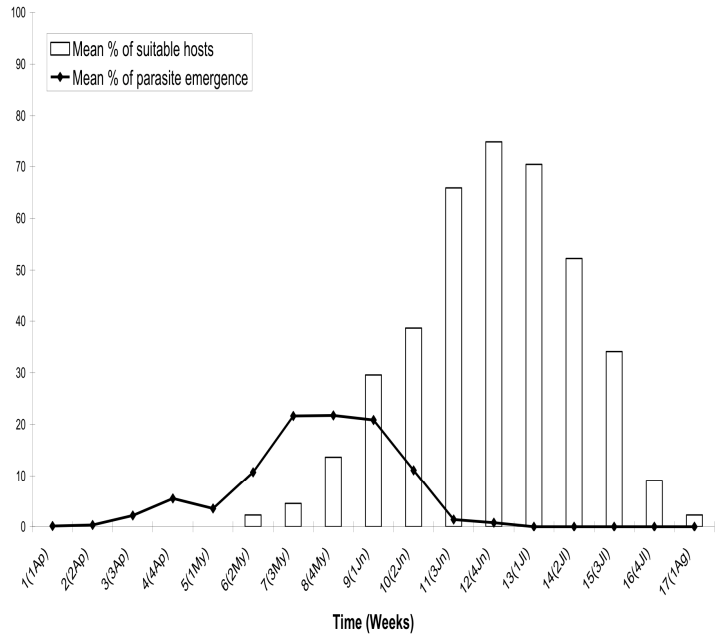


Figure 4. Host-parasite phenology of occurrence for (a) year 2006, (b) year 2007 and (c) year 2009. Bars show the mean weekly percentage of suitable nestlings over the Roller population. Lines show the mean weekly percentage of parasite emergence. Hosts sample size (nests): 2006 = 43; 2007 = 40; 2009 = 44. Parasite sample size (sampled nests): 2006 = 16; 2007 = 17; 2009 = 20.

Host-parasite synchronization at the nest level was highly variable (on average 27.3%, Table 3) and differed among years, but not among cavity types (GLMM, Poisson distribution, repeated measures effect: year with nest ID as a subject, Z = 0.10, p = 0.92; fixed effects: year, $F_{2,31}$ = 8.70, p = 0.001; cavity type, $F_{2,31}$ = 1.08, p = 0.35; year ×cavity type, $F_{4,27.49}$ = 2.52, p = 0.064). The year effect was related to the lower host-parasite synchrony in 2009 compared with 2006 and 2007 (post hoc tests; 2006 vs. 2007, $t_{.22.97}$ = -0.03, Sidak-adjusted p = 1.000; 2006 vs. 2009, t_{31} = 3.73, Sidak-adj. p = 0.0023; 2007 vs. 2009, t_{31} = 3.92, Sidak-adj. p = 0.0014; Table 3).

Year	Nest cavities			
	Sandstones	Bridges	Nest-boxes	Total
2006	49.55 (5)	55.26 (7)	4.21 (2)	*45.93 (14)*
2007	30.70 (4)	14.96 (2)	32.76 (5)	*28.78 (11)*
2009	1.25 (5)	15.87 (4)	10.31 (6)	*8.78 (15)*
Total	*26.92 (14)*	*36.95 (13)*	*18.01 (13)*	**27.28 (40)**

Tabla 3. Mean percentage of host-parasite synchrony (percent of flies emerging during the period when most suitable hosts are available, see methods for more details) per year and cavity-type . Sample size is indicated in brackets.

Host-parasite synchronization in a given year (*t*) was influenced by the breeding phenology of the host in the previous year (year *t*-1). An earlier breeding of the host in year *t* than in year *t*-1 resulted in a higher degree of host-parasite synchronization in *t*, whereas a later breeding of the host in year *t* than in *t*-1 produced poorer host-parasite synchronization in year *t* (GLMM, normal distribution, repeated measures effect: year with nest ID as a subject, $Z = 3.45$, $p < 0.001$; fixed effects: year $F_{2,36} = 16.40$, $p < 0.001$; Δ host hatching date between year *t* and *t*-1, $F_{1,36} = 8.94$, $p = 0.005$, Figure 5).

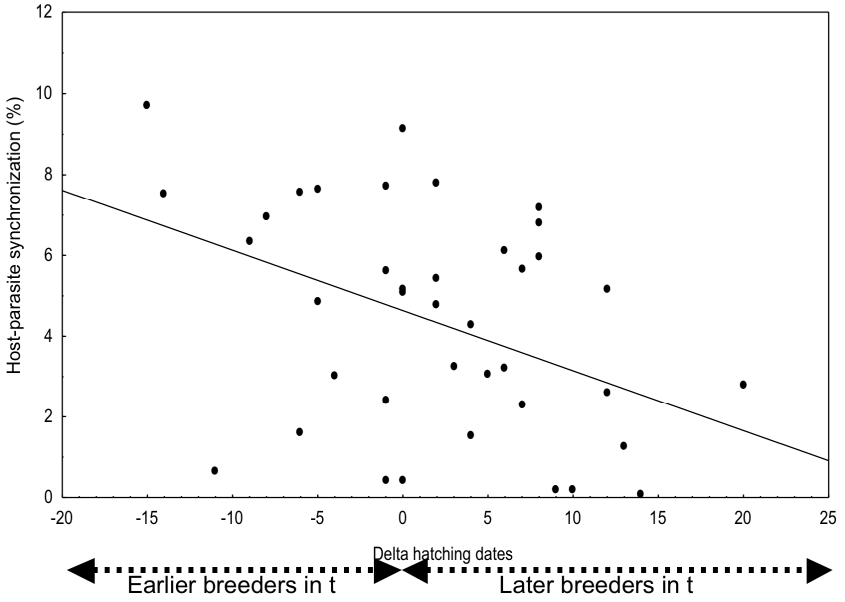


Figure 5. Effect of differences in breeding phenology between consecutive years (x axis) over the percentage of synchronization in year t (y-axis). Δ hatching dates were obtained subtracting date of hatching in year t (in julian days) by hatching date of the same nest in year t-1. Predicted values of host-parasite synchronization (after taking into account nest identity and year effects) are shown. Percentage of host-parasite synchronization was square root transformed to ensure normality.

Discussion

Comparison of host and parasite phenologies and parasite abundance

Our study revealed some similarities and differences in the phenology of the studied host-parasite system. At the population level, the length of the period when hosts are suitable for Carnus averaged 53.5 days. The shape of the distribution of host hatching dates varied among years, but the mean skew was positive for the five years of study. At this same level the mean *Carnus* emergence period (79 days) was 1.48 times longer than the period with suitable hosts, the former starting consistently several weeks before the majority of nestlings hatched. The distribution of *Carnus* emergence showed a strong, consistent negative skew, probably reflecting the disappearance of suitable hosts at the end of the breeding season. This result is in accordance with previous findings of decreasing infestations in other host species' nests at the end of the breeding season (Fargallo et al. 2001, Martín-Vivaldi et al. 2006, Calero-Torralbo et al. 2008).

The observed distribution of carnus emergence dates can be the result of the coexistence of various cohorts. The presence of flies cohorts from different parental genotypes may produce different Genotype x Environmental interactions (i.e. genotypes with different or limited thermal sensitiveness, Kemp & Bosch 2005). A population mixture of roller-associated univoltine flies with bivoltine generations coming from flies parasitizing earlier hosts, (such as regional *Leptidea* butterflies populations coming from different habitats, Friberg et al. 2008), or one-year life-cycle flies versus longer-than-one year life-cycle flies (prolonged diapause, Valera et al. 2006) would produce a wide emergence window. Nonetheless, regardless the functional origin of the observed distribution, from an evolutionary point of view, early emergence of a particular fraction of the parasite population might be an effective strategy for flies exploiting late breeders, since earlier flies emerging from late host species can also infect other host species of the local avian community. Alternatively, earlier phenotypes emerging in late host species might reduce the chance of complete or partial parasite reproductive failure in a given nest if host reproduction in that nest

fails (resource inconstancy, nest predation, reduced host brood) (see Wiklund & Friberg 2004 for stochastic processes of phenological and reproductive regulation). Interestingly, other works have found host-associated differences in length and shape of *Carnus* emergence (i.e. differences between single-brooded, late breeders such as Rollers or Bee-eaters compared with multi-brooded, early breeders such as Hoopoe; Valera et al. 2006, Calero-Torralbo et al. 2008, Calero-Torralbo et al in prep.), suggesting that specific host breeding phenologies may shape parasites life-cycles of their associated populations, as it has been showed for some plant-feeding insects and their host plant phenologies (i.e. Feder & Filchak 1999, Tabuchi & Amano 2010).

Our results show that at the nest level the emergence period of the parasite was also longer than the period of host occurrence. In fact, the difference in the length of the periods was slightly larger than at the population level (1.7× vs. 1.48×, respectively). Even though the occurrence of different cohorts could explain the long and variable emergence period, some synchronization between host availability and parasite emergence should occur, at least at the nest level. The most remarkable feature in this regard was the rapid increase in *Carnus* emergence at the beginning of the host breeding season, with more than 50% of flies emerging in the first four weeks. This high emergence rate at the beginning of the emergence period suggests that flies emerging from the Roller's nest substrate try to synchronize their occurrence with the early nestling period of Rollers when nestlings are unfeathered (see Václav et al. 2008). A positively skewed emergence is common in insects (see Danks 2006) since most individuals usually respond rapidly and similarly to relevant environmental cues. Knowledge about environmental factors used by *Carnus* to emerge is still incomplete, although ambient temperature combined with host-related cues has been suggested to play the main role in a rapid *Carnus* emergence and diapause arrest at the nest level (Calero-Torralbo & Valera 2008, see also Leather 1993, Tauber et al. 1986 for other systems).

Roller and *Carnus* phenologies were comparably influenced by some factors but not by others. Inter-annual variation in Roller hatching dates was minimal and slight inter-annual differences in skewness likely reflects certain

plasticity in hatching dates distribution around a stable population mean. In contrast, parasite phenology (50% emergence and the length of the emergence period) was more sensitive than host phenology to inter-annual changes. This is feasible because temperature is known to have a profound impact on heterothermic invertebrates (Tauber et al. 1986), whose growth and emergence patterns are frequently sharply adjusted by temperature. In contrast, for endotherm organisms, a direct effect of environmental temperature on breeding phenology is weaker and temperature is generally used as a cue to the occurrence of the most favourable period for reproduction (Visser et al. 2009, Forrest & Miller-Rushing 2010). Therefore, our study implies that the annual fluctuations of environmental variables can produce different effects on host and parasite phenologies and thus alter the degree of mismatch over host-parasite co-occurrence (see also Van Nouhuys & Lei 2004).

In contrast, nest type had a similar effect on the host and the parasite: both the Roller and carnid flies advanced their life cycle (hatching date and emergence, respectively) in nest-boxes. The advanced emergence of *Carnus* in nest boxes is probably due to different microclimatic conditions when compared with natural cavities. Microclimate can also account for earlier breeding of Rollers in nest boxes even though intra- or inter-specific host competition for nest sites can be other reasons (Václav et al. 2011). In addition to the increase in the degree of environmental heterogeneity, nest boxes also affected host-parasite relationships via parasite abundance. Nest boxes contained more *Carnus* flies than the other two nest types (see also Moller 1989; Fargallo et al. 2001, Wesolowski & Stanska 2001). Microclimate and nest substrate composition, affecting different stages of the parasite (Dawson et al. 2005, Krasnov 2008, Martinez – de la Puente et al. 2010), or difficulties in nest sanitation by the host due to the architecture of nest-boxes can account for a higher *Carnus* abundance in nest-boxes. Albeit the use of nest-boxes in the study of the ecology of hole-breeding birds is widespread, our results suggest that one should be cautious if general conclusions are extracted from phenological studies involving endothermic organisms and ectotermic species such as avian ectoparasites.

Host predictability and parasite phenological regularity

High host predictability across years is vital to promote parasite predictability and host-parasite synchronization (Barrett et al. 2009). Host predictability, both in terms of occurrence and phenology fluctuates in our study system: (i) Host occurrence was moderate, with some nests being used only once and others being used consistently for several years; (ii) inter-annual hatching date repeatability for the same nest was highly variable, ranging from no repeatability for some years to 0.70 for a 3-years period. In contrast, parasite phenological predictability for the same nest was less variable during the study period (Table 2).

Differences between host and parasite predictability are probably reflecting the different nature of the factors influencing phenology in each organism. *Carnus* emergence is highly dependent on diapause duration, which is rather rigidly regulated by ambient temperature (Calero-Torralbo & Valera 2008, Valera et al. in prep.). Unless a major environmental disturbance occurs, a given nest is likely to keep its thermal characteristics similar between years. In contrast, Roller phenology in a given nest can be influenced by a variety of biotic factors that are difficult to predict (changes in one of the members of the breeding pair, intra or inter-specific competition for nest sites…; but see Przybylo et al. 2000). Václav et al. (2011) showed for the same population that Roller nest reoccupancy in a given year was more likely if the nest had been previously used, although this effect was only detected for natural cavities and traditional nests in human constructions, but not for nest-boxes. Thus, although host predictability could be hardly forecasted by *Carnus* at the host population level, some opportunities for a high synchrony do exist in nests that are being occupied consistently and with a high phenological predictability by the same host. In these same nests, flies can also find a stable environment that could facilitate a high synchronization to local and regular host nest phenologies (see, for instance, van Dongen et al. 1997, Tikkanen et al. 2006).

Host – parasite synchrony

In host-parasite interactions synchrony occurs when the life cycle of the parasite is timed so that it can exploit the host optimally and that of the host is timed so that it is maximally exploited (Singer & Parmesan 2010). Our data show that, at the nest level, host-parasite synchronization was low (on average 27.3%; range = 1.25% to 55.26%; Table 3), mainly because *Carnus* consistently emerged before the occurrence of the host. This indicates that flies cannot precisely detect or predict the beginning of the period of host availability (see, for instance, Buse & Good 1996). The question appears to what extent *Carnus* can track the phenology of Rollers. Host-parasite synchronization in a given year (*t*) was influenced by the breeding phenology of the host in the previous year (*t*-1). A more advanced breeding of the host in year *t* compared to the previous year resulted in a higher degree of host-parasite synchronization in year *t*. In contrast, a later breeding of the host in year *t* compared to year *t*-1 produced a poorer degree of host-parasite synchronization in year *t*. This implies that birds progressively initiating their breeding attempts earlier in consecutive years should be more parasitised than birds progressively delaying their breeding attempts between years. Parasite synchronization with early breeders can be facilitated by the effect of host temperature (Calero-Torralbo et al. 2008). This effect may dilute as the season progresses and parasites reach the physiological time required for emergence.

The phenological host-parasite asynchrony showed in our study raises the question of whether perfect synchronization with their Roller hosts is indeed the optimal strategy for *Carnus hemapterus* to maximize its fitness or, on the contrary, a certain degree of stochasticity is desirable to face host unpredictability and/or take advantage of alternative resources. If individuals can precisely detect when resources are available, then a population of parasites could develop an ideal strategy of close co-occurrence and synchronization (Singer & Parmesan 2010). Nevertheless, in the absence of predictable cues and/or conditions, parasite generalists tend to have a broader phenology in an attempt to cover a broad phenological window at the community level (e.g. Tikkanen & Lyytikäinen-Saarenmaa 2002). The negative consequences of such a strategy can be partially offset by the acquisition of host searching skills during the dispersal period

(Combes 2001). In our system, the detrimental effect of low synchrony at the nest level can be balanced by means of spatial dispersion to close nests with suitable nestlings (e.g. host-parasite synchronization at nest level during 2009 was 8.78% whereas most flies in 2009 -88.3%- emerged during the whole period of suitable nestlings in 2009, see figure 4c). This suggests that there could be stronger selection for the parasite for synchronization at a larger scale (population or even community level, see Jepsen et. al 2009) than at a fine, nest, level. Additionally, records of massive and successful colonization of isolated nest-boxes by carnid flies (Calero-Torralbo pers. obs.) suggest that winged adults are equipped with some kind of searching skill to detect occupied nests.

Our results suggest that low host-parasite synchronization may be advantageous in parasitic system such as ours, where high rates of host and environmental unpredictability at both low geographical and short temporal scales are found, therefore supporting recent hypothesis over the functional and evolutionary significance of phenological asynchrony (Singer & Parmesan 2010).

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CAPÍTULO VI

Phylogeography and genetic structure of the generalist ectoparasite *Carnus hemapterus*: interplays between scale, geography and host-associated factors.

Abstract: Despite the ecological importance of generalist pathogens in terrestrial communities, our knowledge about agents of diversification and the way they shape the genetic structure of generalist and widespread parasites is poor. Here we explore the genetic structure of the avian ectoparasite *Carnus hemapterus* at different geographical scales (from continental to local scale), to elucidate the main barriers and sources of gene flow and their influence on genetic diversity and structure. At a large phylogenetic level (European and North America), *Carnus hemapterus* was strongly affected by historical events and physical barriers as well as by isolation by distance between remote localities. At a medium phylogenetic level (populations in Central Europe, Iberian peninsula, North of Italy and France), genetic diversification was dependent on particular historical and demographic events of each region, what may reduce the effect of other ecological factors over genetic divergence at lower scales. At a local scale (south east of Spain), genetic structure of sympatric lineages was partially shaped by extrinsic allochronic barriers due to temporal differences in parasite emergence, although host-associated genetic divergence was not detected. Parasite phenological allochrony could be a significant mechanism of genetic diversification in generalist parasites, even in those systems where host race formation or ecological speciation might not be detected. This work highlights the importance of studying widespread and generalist parasitic systems that show a multifarious range of factors shaping diversification and genetic structure, and stresses the need of considering several phylogenetic scales for a better understanding of host-parasite diversification processes.

Keywords: Ectoparasite, Diptera, long distance colonization, range expansion, temporal isolation, allopatry, speciation, phylogeny, Bering land bridge.

Miguel A. Calero-Torralbo, Lorenzo Zane, Federica Barbisan, Francisco Valera

Introduction

Understanding the evolutionary processes and the set of driving forces that are shaping population genetic structure of organisms is essential for improving our knowledge about the mechanisms that promote genetic diversification and speciation in nature. The observed structure of populations depends on the interaction between the rate of gene flow and increments of genetic variance between populations and can occur by changes along the spatial, temporal or ecological axes where the different populations are evolving (Fisher 1958). Therefore, for inference of population structure from genetic data, it is advisable to include the main driving factors (e.g. geographical and/or temporal features, historical events or ecological factors) that are interacting and influencing the amount of gene flow between populations at different spatiotemporal scales.

Parasites are ideal model organisms for the study of the forces modelling genetic structure at different scales. Ecological mechanisms of diversification can arise from the close and strong interactions between parasites and their hosts, the latter becoming ecological barriers that can induce changes in genetic structure and increase biodiversity of parasites (McCoy et al. 2003, Drès & Mallet 2002, Coyne & Orr 2004, Kemps et. al 2009). Geographical distance, physical barriers or historical events (past colonization or fragmentation, range expansion) can also promote diversification and regulate gene flow among populations in parasite taxa at different spatio-temporal scales (Simonato et al. 2007, Hoberg & Brooks 2008, Rich et al. 2008, Stefka et al. 2009, Vercken et al. 2010). Finally, the large diversity in life-history traits and adaptations of parasites interacting with the driving forces and barriers above mentioned, have led to a wide range of genetic structures and diversification within and among different groups of parasites (Poulin & Morand 2004, Barrett et al. 2008) which make them ideal systems to unravel the complex relationships among life-history characteristics and underlying mechanisms that induce or restrict gene flow and shape the genetic structure of populations.

In recent years, an increasing number of studies have focused on the genetic structure of polyphagous species with a broad host spectrum and distribution (e.g. McCoy et al. 2005, Bouzid et al. 2008, Archie & Ezenwa 2011). Generalist parasites with wide geographical distribution undergo highly variable selection pressures. Due to differences in local conditions and the nature, range and availability of the hosts exploited in each population, selection for several parasite traits could be important and active in some

locations but not in others (Lajeunesse & Forbes 2002, Thompson 2005). This can promote high variation in host-associated diversification mechanisms and in the effect of regional geographical barriers, isolation by distance or historical local events over the genetic structure of the populations distributed along latitudinal and longitudinal gradients.

The role of host-associated, geographical or historical factors over the genetic structure and diversification in animal parasites is still scarcely understood (Criscione et al. 2005, Poulin 2007). Although notable and enlightening efforts have been recently performed in some parasite taxa, particularly in nest-dwelling ectoparasites (McCoy et al. 2001, 2003, 2005, Dudaniec et al. 2008, Bruyndonckx et al. 2009, Kemps et al. 2009, Malenke et al. 2009), the remarkable variability within this group in key aspects (life-story strategies, complexity of life-cycle, method and rates of dispersion, or degree of intimacy with the host exploited; Marshall 1981) demands further attention. Recent studies with ectoparasites from different taxa have shown the importance of parasite life-history traits to explain genetic structure and diversification processes when parasites exploit the same species (Gómez-Diaz et al. 2007, Witheman et al. 2007, Toon & Hughes 2008). These results emphasize the need to perform studies in different parasitic taxa to properly understand the overall processes that shape genetic structure and diversification among ectoparasites. Here we present, to our knowledge, the first broad scale study of a generalist, widely distributed dipteran ectoparasite aiming at elucidating the genetic structure and detecting the main sources of genetic divergence at different geographical scales (from continent to local scale).

Carnus hemapterus (Nizscht 1818) is a 2 mm long, blood-sucking nest dwelling fly belonging to the Family Carnidae (Diptera: Acalyptratae). This species provides an excellent opportunity to study population genetic structure and mechanisms of diversification in animal parasites at different spatial scales since: i) it exploits a large host spectra (host range = 60 cited species, Brake 2011) along a wide area of distribution (Holartic ecozone, mainly recorded from Europe and North America); ii) it has been found parasitising a broad range of host species in small areas. For instance, up to ten different host species (with marked differences in morphology, behaviour and breeding phenology) have been recorded in the Desert of Tabernas (Almería, Spain, Calero-Torralbo and Valera, pers. obs.). Thus, high genetic variation in structure and diversification among and/or within populations due to changes in local conditions and nature of the available

hosts is expected. ii) *Carnus hemapterus* parasitizes ephemeral resources, namely unfeathered nestlings of various bird species with particular life-history traits and breeding phenologies. Furthermore, emergence of imagines has been reported to be partly synchronized with the occurrence of some of their hosts (i.e. hatching of nestlings) (Liker et al. 2001, Valera et al. 2003, Calero-Torralbo and Valera 2008, Chapter IV). This suggests that host-associated mechanisms of sympatric and/or allochronic divergence and isolation among parasite lineages could occur. Whereas allochronic divergence in phytophagous arthropods via host variation in phenology is well known (Craig et al. 1997, Filchak et al. 2000, Tikkanen & Julkunen - Tiitto 2003, Abbot & Withgott 2004, Feder et al. 2005, Santos et al. 2007, 2011, Ording et al. 2010), very few cases have been reported in animal parasites (see Therom & Combes 1995), despite the importance that this diversifying mechanism may have in parasites (Price 1980, Via 2001, McCoy 2003, Rundle & Nossil 2005).

Here we use mitochondrial molecular markers to study the genetic structure of *Carnus hemapterus* parasitizing a wide range of host species from south Europe to North America. And examine the demographic and historic events (past fragmentation, glacial refugia, range expansion…), that may have influenced the observed parasite structure. We also explore the main driving forces (namely host associated factors, geographical distances, physical barriers, historical events) that could generate genetic variation at different spatial scales: from local to continental geographical scales.

Materials and Methods

Study species, study area, sample collection and DNA isolation

The life cycle of Carnus hemapterus comprises an adult stage, 3 larval phases, and a pupal stage (Guiguen et al. 1983). Usually, after several months of winter diapause adult flies emerge in the following spring approximately when their nestling hosts hatch. Adult flies have a winged and wingless phase. After their emergence, adults are initially winged, but lose their wings as soon as they locate a suitable host (Roulin 1988). Since neither the adults nor the larvae have been found on adult birds, Carnid flies are assumed to actively colonize hosts' nests during the winged phase of their life cycle (Grimaldi 1997). Nonetheless, Carnus can persist by itself in the nest for several years since prolonged diapause has been recorded for this species (Valera et al. 2006a). Adult flies cannot survive

for long without feeding (around 2-3 days; Calero-Torralbo & Valera, unpublished data.), and, thus, its dispersal period may be short.

Overall 245 Carnus hemapterus flies were collected from different host species in various locations in Europe and North America (see Table 1 and Figure 1) from 2004 to 2007. Sampling was particularly intense in Tabernas (Almería) where 56 flies were obtained from three different hosts specie. Flies were obtained directly from parasitized nestlings or from pupae collected from nest detritus of infested nests. Flies were stored in 95% ethanol until assayed in the laboratory.

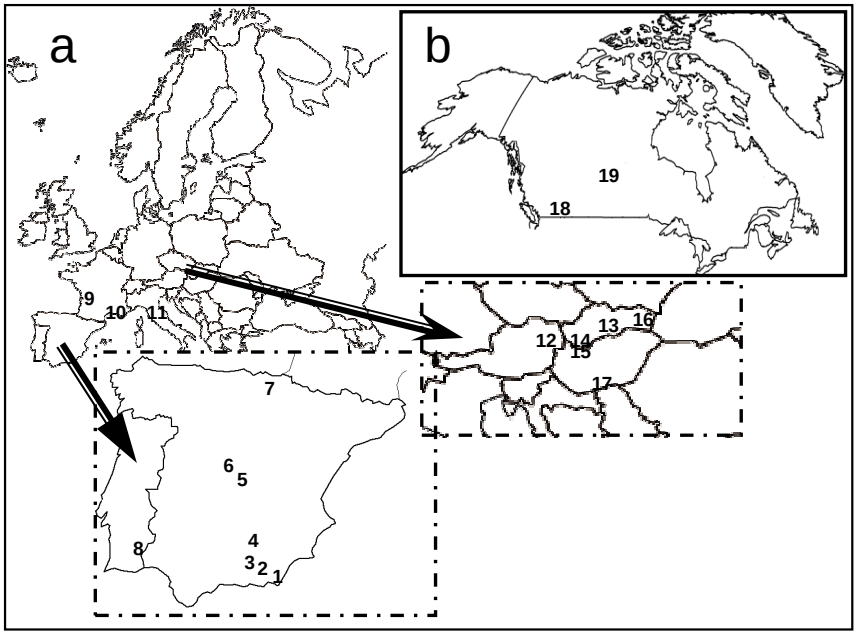


Figure 1. Sampling localities of *Carnus hemapterus* (1 to 19, see table 1); a. European localities; b. North American localities.

Total genomic DNA was extracted following Gloor & Engels (1992). The whole fly was minced in 30 µl of Extraction Buffer (10 mM Tris, 1 mM EDTA, 25 mM NaCl) solution and 2 µl of proteinase K per individual; subsequently it was incubated for 30 minutes at 56 ºC and for 5 minutes at 90 ºC to ensure the inactivation of proteinase. Extracted DNA was stored at -20 ºC.

DNA sequencing

Sequence information for 809 base pairs (bp) of a mitochondrial region situated within Cytochrome Oxidase subunits I and II genes (COI and COII), was obtained using two different amplification strategies. Initially, PCRs were performed using newly designed primers based on a COI and COII sequence obtained from a bigger fragment of around 1600 bp, amplified using universal primers C1J2183 (Sperling & Hickey 1994) and TKN3772 (Bogdanowicz et al. 1993). New primers were CF (5'-ACACGGTCCTCAACTTTCTT-3') and CR (5'-AGTTTATAGGAAGGACAACTCG-3') that allowed amplifying a single fragment of about 950 base pairs in 107 flies. In a second phase, since primers CF and CR failed in the amplification of individuals belonging to Slovak and North American populations, two additional internal primers were designed (CRN, 5'-ATATCTGTGTTCAGCAGGTG-3' and CFN, 5'-CTTCCCTCAACATTTTCTTGG-3') and two separate overlapping fragments were obtained from 138 individuals (CF-CRN, approx 460 bp and CR-CFN approx 700 bp), resulting in a final sequence of 809 bp.

Amplification of the first primer combination (CF-CR) was performed in a total volume of 25 µl containing 2.5 µl of Promega GoTaq® reaction buffer 10X, 1.5 µl of $MgCl_2$ [5 mM], 1 µl of dNTP's [2 mM], 1.5 µl of each primer ([10 mM]), 0.2 µl of GoTaq® Promega DNA Polymerasa [5 U/µl], 14.8 µl of H_20 and 2 µl of genomic DNA. Thirty five cycles were used on a DNA thermocycler with predenaturing at 94 ºC for 2 min, denaturing at 94 ºC for 30 s, annealing at 57 ºC for 40 s, primer extension at 72 ºC for 1 min and a final extension in 72 ºC for 5 min. Amplification for the CF-CRN and CR-CFN primer combinations was performed in a total volume of 25 µl containing 5 µl of Promega GoTaq® Flexi reaction buffer 5X, 2.5 µl of $MgCl_2$ [25 mM], 1 µl of dNTP's [2 mM], 1.5 of each primer ([10 mM]), 0.16 µl of GoTaq® Flexi Promega DNA Polymerasa [5 U/µl], 11.34 µl of H_20 and 2 µl of genomic DNA. Similar number of cycles and temperatures than in primer combination CF-CR were used, except for annealing period (56 ºC for 40 s).

Positive (DNA from flies previously amplified) and negative (PCRmix without genomic DNA) controls were used to check PCR performance and contamination in all the amplifications. Purification was performed incubating 5 µl of PCR product at 37 ºC for 15

min with 1 µl of ExoSAP-IT® (USB Corporation, Cleveland, USA) enzymatic reaction to remove excess of primers and a final 15 min period at 80 ºC to inactivate the ExoSAP activity. Sequencing was run on an ABI 3100 or an ABI 3700 Genetic Analyser. Nucleotide sequences were edited using the program Chromas v.1.45 (Conor McCarthy, Griffith University, Brisbane, Queensland, Australia), and combined into a unique sequence per individual. Different sequences were aligned with the program ClustalW (http://www.ebi.ac.uk/Tools/clustalw2; Chenna et al. 2003) and inspected by eye. No indels were found in any individual used in this study for this sequence. To exclude the possibility that a nuclear pseudogene was amplified, the nucleotidic sequence was translated by means of MEGA 3.1. and compared with known aminoacid sequences of other dipteran species using BlastX (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Our sequence covers the final part of COI (from position 1 to 356, codon start at third position, between 88-85 % match with other brachyceran flies) plus the region corresponding to tRNA-Leu gen (from positions 360 to 424), and the starting part of COII (from position to 425 to 809, codon start at first position, 88-85 % match with other brachyceran flies). The high percentage of similarity, the conservation of the position of stop codons and the lack of indels/stop codons in the coding region confirmed that we amplified a fragment of functional COI - COII copy.

Phylogenetic relationships

We used the software MODELTEST (Posada and Crandall, 1998), to select the best-fit models of nucleotide substitution for the Maximum Likelihood and Bayesian analyses. Hierarchical likelihood ratio tests and Bayesian information criteria (BIC) identified HKY + G as the most appropriate model, and Akaike information criteria (AIC) identified HKY + G + I as the most appropriate model. We choose HKY + G model following the Bayesian information criteria to avoid a possible overparametrization in model selection with our sequence data (Nylander et al. 2004).

We conducted ML analysis with PHYML (Guindon & Gascuel 2003), using a starting tree obtained from the program TREEFINDER (Jobb et al. 2004). Bootstrap analyses with 10000 replicates were performed to assess nodal support for the ML tree. Additionally, bayesian inference method analysis, as implemented in MRBAYES (v. 3.2; Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003), was performed. We

produced posterior probability distributions by allowing four MCMC to proceed for 10,000,000 generations, sampling every 100 generations; the initial 25% of trees (first 2,500,000 generations and 25,000 trees) were discarded as burn-in. We ran Bayesian analyses three times to ensure that they were not trapped on local optima. Since no similar sequences of mitochondrial DNA are available for other Carnidae species, we used Drosophila melanogaster (a close and relative nearby acalyptratae species) as outgroup for both Bayesian and Maximum likelihood analyses.

Tree-building methods tend to resolve intraspecific gene genealogies poorly when the different mitochondrial types are separated by few mutations and ancestral haplotypes may still be present in the populations (Crandall & Templeton 1993). Accordingly we used TCS 1.18 (Clement et al. 2000) to construct a minimum spanning network (Crandall & Templeton 1993) to improve the visualization of the relationships among the haplotypes of Carnus hemapterus. Haplotype network was estimated using the 95% reconnection limit between haplotypes. Ambiguities (loops) were resolved following Crandall & Templeton (1993) and based on expectations from coalescent theory (Posada & Crandall 2001).

Divergence time estimation

Age estimates of the most recent common ancestor of the main Carnus hemapterus clades were obtained using a Bayesian inference framework as implemented in the computer program BEAST v.1.4 (Drummond & Rambaut 2007). This approach allows evolutionary hypotheses to be tested taking into account phylogenetic uncertainty and provides 95% confidence intervals for the parameter estimate based on the posterior probability distribution over the parameter state space.

We constructed an input file with the program BEAUti, using birth-death prior distribution (given that we expect that some extinction has occurred during the Carnus hemapterus divergence time). We estimated relationships between lineages using the strict clock, the uncorrelated lognormal and the uncorrelated exponential relaxed clocks. To asses the preferred clock we checked the standard deviation of the uncorrelated lognormal relaxed clock. If the standard deviation is 0 there is no variation in rates among branches. If this parameter is greater than 1 the standard deviation in branch rates is greater that the mean rate and this means that there is among branch rate heterogeneity within the data and it is recommend the use of a relaxed molecular clock. We also calculated the coefficient of

variation (CV) of the root to tip distance (standard deviation divided by the mean of the root-tip tree distances), of our maximum likelihood tree without outgroup by means of the program Path-o-gen (Rambaut 2009). If CV is close to zero then the data are clock-like but if this is much greater than zero then they are not very clock-like. Additionally we calculated by means of the program TRACER (Rambaut & Drummond 2003) the harmonic mean of the likelihood values (as estimator of the marginal likelihood) from the posterior output of each of the clock models, and from these values, bayesian factors (BF) were calculated by taking the difference between the marginal likelihood values of one model topology T_1, and the alternative model topology T_0 (Newton & Raftery 1994; Suchard et al. 2001; Nylander et al. 2004); then the preferred clock model was selected following the guidelines provided in Kass & Raftery (1995). Two independents MCMC chains were run for 100,000,000 generations, sampling and logging parameters every 1000 steps. Model selected by MODELTEST was implemented and the different parameters values for the substitution model were used as priors.

The absence of fossil evidence or well-dated biogeographic events prevents the calibration of a molecular clock for Carnus hemapterus. We used a calibration rate of 2.3% pairwise sequence divergence per millions years estimated for mitochondrial markers in arthropods (Brower 1994). The widely used Brower's estimate is based mainly on dipteran species from different biogeographic and ecological origins. These flies are species recently diverged, like *Carnus hemapterus* populations; therefore both rates of substitution are supposedly in the initial rapid phase of mitochondrial divergence (Moritz et al. 1987). Furthermore, different parts of the mitochondrial genome were used for the calibration, including parts of the COI and COII gene. The robustness and reliability of this insect mitochondrial molecular clock has been further confirmed by linking an insect molecular phylogeny to paleontological and biogeographical data (Gaunt & Miles 2002). We therefore consider this calibration rate as the best estimate for our COI and COII mitochondrial data belonging to individuals recently diverged and close to the dipteran species used in Brower's study.

Population genetic structure

Since we do not have an a priori definition of populations, we used the program SAMOVA 1.0 (Dupanloup et al. 2002) to characterize the group structure of our European sampled

locations based on the genetic data. SAMOVA procedure permits to define geographically homogeneous populations that maximize the proportion of total genetic variance due to differences among population groups (F_{CT}). SAMOVA was run using 500 simulated annealing processes for k values from 2 to 20. Pairwise F_{ST} (Wright, 1951) estimates of genetic distances between (i) European sampled locations and (ii) homogeneous populations defined by SAMOVA analysis were calculated using ARLEQUIN 3.0 (Scheneider et al. 2000, Excoffier et al. 2005).

To test the hypothesis of isolation by distance and whether there was some kind of correlation between genetic and geographical distances at several geographic scales, we performed two - tailed Mantel tests using the software PASSaGE 2.0 (Rosemberg & Anderson 2011) for all European populations, and for the Iberian and the Central European populations. The test was carried out using pairwse F_{ST}'s and geographical distances in kilometres.

To highlight whether other factors apart from geographic distances between locations (e.g. host-associated factors) were influencing genetic variance among populations AMOVA analyses at different geographic scales (Europe, Iberian Peninsula and Tabernas) were performed with ARLEQUIN 3.0. We grouped the sequences by (a) sampling location, (b) flies emergence date, (c) host breeding phenology, (d) host aggregation tactic during breeding (solitary vs. colonial), and (e) host migration behaviour (resident vs. transharian migrants). For host breeding phenology we created three categories: early breeders (January, February and March), medium breeders (April, May) and late breeders (June, July). Assignment of the breeding phenology of each host species to such categories was done considering the period fitting most to the breeding data reported for each region sampled (see Cramp 1985). Breeding tactics and migration behaviour were obtained from Cramp (1985) and del Hoyo et al. (1994, 1999 and 2001).

Historical demography

In order to test the null hypothesis of no geographical association of haplotypes and to separate population structure from population history a nested clade network was constructed following Templeton et al. (1987), Templeton & Sing (1993) and Templeton et al. (1995). Then, a Nested Clade Phylogegraphical Analysis (NCPA) was performed in GEODIS, version 2.5 (Posada, 2000), with a number of permutations equal to 1000.

Significant results were interpreted using the inference key published (November 2005) and available at http://darwin.uvigo.es/. The haplotypes from the North American populations were excluded from the NCPA since they showed more than 40 substitutions with respect to European haplotypes, well above the 95% confidence criterion.

Recently, NCPA analysis has been severely criticized (Petit, 2008; Beaumont & Panchal 2008) due to the possibility of false positives, mainly for range expansion conclusions (Templeton 2004, Petit 2008). To support and better interpret our results, we performed additional analyses for each of the higher level clades at the total cladogram and for homogeneous grouped populations obtained by SAMOVA analysis. Mismatch distribution (Slatking & Hudson 1991, Rogers & Harpending, 1992) and statistical significance of the observed distribution were tested against a null distribution of recent population expansion using DnaSP 4.5 (Rozas & Rozas 1999). A unimodal mismatch distribution indicates a recent range expansion (Rogers & Harpending 1992; Excoffier et al. 1992), whereas multimodal mismatch distribution indicates diminishing population sizes, structured size or that the population is influenced by migration, is subdivided and/or has undergone historical contraction (Marjoram & Donelly 1994, Bertorelle & Slatkin 1995, Ray et al. 2003.). Fu's F_S and R_2 statistics (Fu & Li 1993, Ramos-Onsins & Rozas 2002) were also calculated with DnaSP 4.5, since they have more statistical power than SSD or Harpending's raggedness index when population sizes are small (Ramos-Onsins & Rozas 2002). We also calculated Tajima's neutrality test D (Tajima 1989). Significance values of all tests were determined by computer simulation (1000 replicates) using the coalescent algorithm implemented in DnaSP 4.5.

Results

Phylogenetic relationships

For the 809 bp mt sequence generated from 245 individuals, 119 variable nucleotide sites defined 64 haplotypes (haplotype diversity = 0,885) (see Table 1). Haplotypes Z, P and AN, found in 9, 5 and 5 out of 19 populations, were the most widespread ones. Specifically, haplotype P was found in remote localities from different countries (Tabernas in Spain, Parma in Italy, Somotor in Slovakia, Albertirsa in Hungary, Gosing in Austria), some of them more than 2000 km apart. Haplotype Z was found in all Iberian sampled

localities as well as in Paradou (Southern France). All populations, except one in southern Spain (Doña Maria), have unique haplotypes (haplotypes found only in that population).

Sampled location	Method	*N*	Host	Latitude	Longitude	Haplotypes	*n*	Hd
Spain								
1 Tabernas	N	56	CG, AN, MA	37.066 N	-2.354 E	P(3), U, W(2), **X**, **V**, Z(22), **AB**, **AC**, AD(2), **AF**, AJ, AN(3),BH(14), **BJ**, **BK**, **BL**	16	0.786
2 Doña Maria	N	6	UE	37.135 N	-2.711 E	W, Z, AE(3), BH	4	0.800
3 Guadix	N	17	UE	37.306 N	-3.135 E	Z(8), AJ(2), AN(3), **AO(4)**,	4	0.721
4 Andujar	N	13	MA	37.976 N	-4.122 E	U, Z(9), **AK(2)**, **AL**	4	0.526
5 Daganzo	N	15	MA	40.547 N	-3.457 E	U(2), Z(7), AE, **AI**, AJ, **AM**, AN, **BG**	8	0.790
6 El Espinar	N	16	FT	40.686 N	-4.350 E	W, Z(12), **AA**, **AH**, AN,	5	0.450
7 Balmaseda	H	3	FP	43.198 N	-3.181 E	J, Z, **BI**	3	1.000
Portugal								
8 Castroverde	N	35	CG,FN,FT	37.706 N	-8.071 E	U(5), Z(14), AE, **AG**, AJ(14),	5	0.677
France								
9 La Flotte	H	2	BB	46.189 N	-1.329 E	**BF**, J	2	1.000
10 Paradou	N	16	CG	43.690 N	4.787 E	**D(2)**, **F**, **I**, J(6), **K**, **L**, Z(2), AN, **BA**	9	0.858
Italy								
11 Parma	H	14	FT	44.884 N	10.173 E	**A(2)**, **B(2)**, **C**, **E**, **G(2)**, **H**, J, **M**, P, **AX**, **BB**	11	0.967
Austria								
12 Gosing	N	7	UE	48.469 N	15.812 E	P, **S**, **AZ**, **BC(3)**, **BM**	5	0.857
Slovakia								
13 Pavlova	H	11	MA	47.907 N	18.672 E	**Q (2)**, R(9)	2	0.327
14 Dunajska Streda	H	2	FC	47.994 N	17.617 E	**T**, **BE**	2	1.000
15 Piestany	H	4	FC	48.595 N	17.834 E	**N**, **BD(3)**	2	0.500
16 Somotor	H	7	MA	48.400 N	21.816 E	**O**, P(6),	2	0.286
Hungary								
17 Albertirsa	H	7	MA	47.252 N	19.608 E	P(5), R, **AY**	3	0.524
Canada								
18 Riskecreek	H	9	CA	52.066 N	-122.519 E	**AR(2)**, AS(3), **AT**, **AU**, **AV**, **AW**	6	0.889
19 Lake Bersnard	H	5	FS	55.436 N	-105.784 E	**AP**, **AQ**, AS(3)	3	0.700
TOTAL		**245**					**64**	**0.885**

Table 1. Sampled locations numbered as in figure 1, method of flies collection (H = directly from the host, N=From nest detritus containing *Carnus hemapterus* pupae), number of individuals sampled in each location, host species where flies were sampled (CG = *Coracias garrulus*; AN = *Athene noctua*; MA = *Merops apiaster*; UE = *Upupa epops*; FT = *Falco tinnunculus*; FP = *Falco peregrinus*; FN = *Falco naumanni*; BB = *Buteo buteo*; FC = *Falco cherrug*; CA = *Colaptes auratus*; FS = *Falco sparverius*), coordinates of the collection area, haplotype designation (in bold unique haplotypes not found anywhere else, with numbers in parentheses indicating the number of individuals sharing the same haplotype for each locality), number of haplotypes and haplotype diversity (Hd) for each location.

Maximum likelihood and Bayesian methods inferred a tree with strongly supported groups clustering individuals of the same geographic origin, indicating the existence of genetic differentiation among populations of *Carnus hemapterus* (Fig. 2).

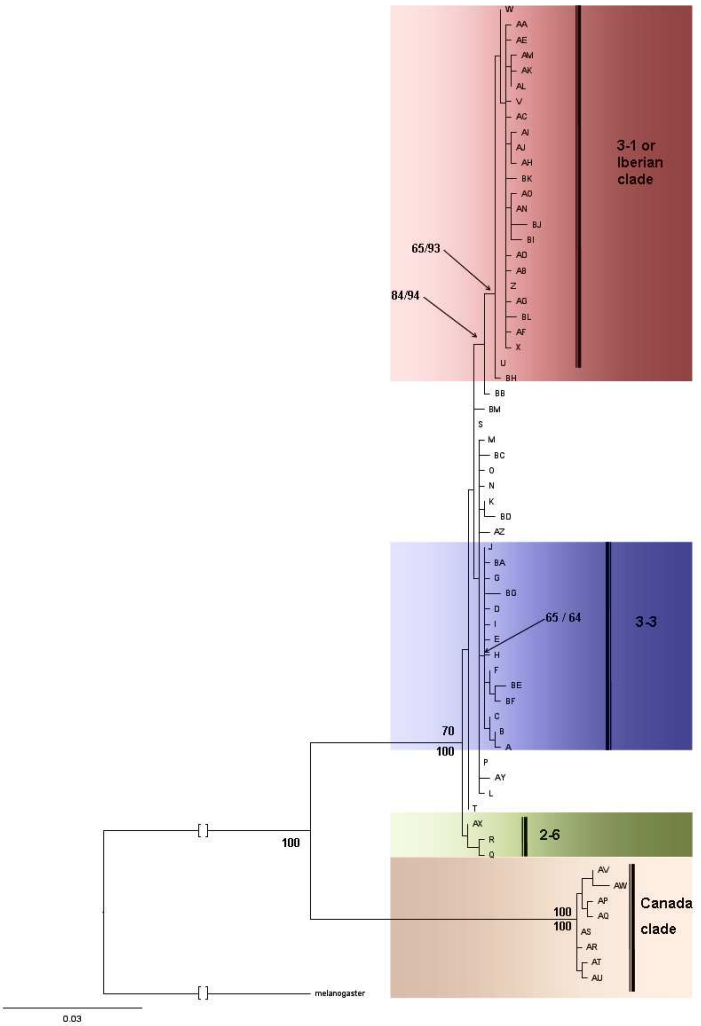


Figure 2. Maximum likelihood tree under the HKY + G model for 64 haplotypes of *Carnus hemapterus*. *Drosophila melanogaster* was used as outgroup taxa. Nodal support was assessed with nonparametric boostrap (for ML analyses; numbers above branches) and with Bayesian posterior probabilities (numbers below branches). Only explanatory and useful values for our objectives are indicated. Branches for basal node were cut to improve tree visualization. Haplotypes were also grouped following nested clade analysis results (see materials and method and result sections and Figure 3).

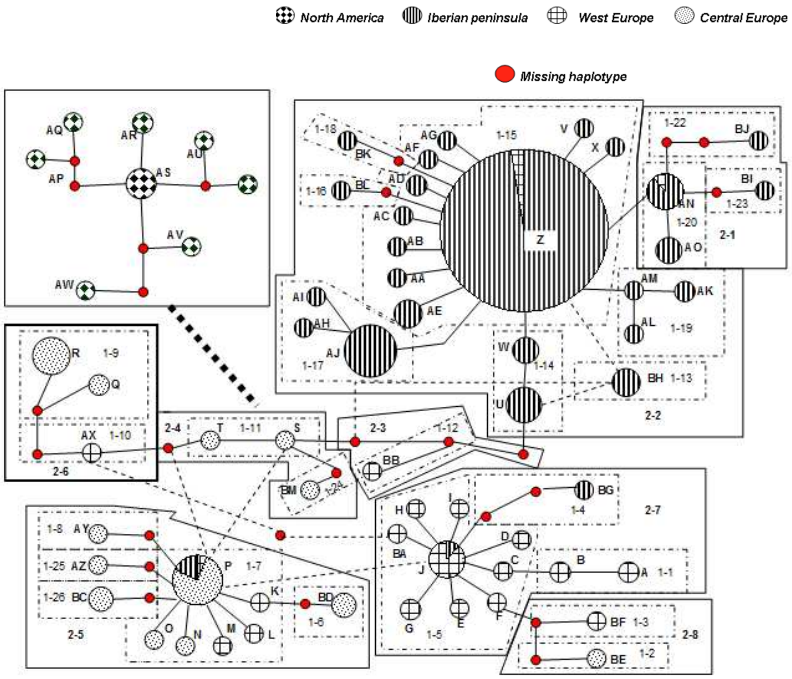
The separation between the European and the Canadian populations was well supported. All the haplotypes from the Iberian Peninsula, except for the BG haplotype, grouped together in a clade (hereafter clade 3-1). The "Iberian" clade included three weakly supported subclades.

Most of the Southern France and Italian unique haplotypes (12 out of 16 haplotypes), tended to form a weakly supported clade (hereafter clade 3-3, West European clade. An unclear structure was revealed for most Central European haplotypes.

The minimum spanning network and nested design support the clear distinction between the European and the Canadian populations (no haplotypes are shared between them), and the separation between the Iberian haplotypes (clade 3-1), and the great majority of French and North Italian haplotypes (West Europe haplotypes from clade 3-3). The Iberian and the West European populations only share 4 out of 43 haplotypes and the West European and Central European populations share just one out of 32 haplotypes (see Fig. 3). These results support the genetic structure suggested by the ML tree (Fig. 2). Furthermore, only 1 haplotype from a single individual sampled in a Central European population belongs to the 3-3 clade, (BE from Dunajska Streda, Slovakia), no haplotypes sampled in Central European populations belong to the 3-1 clade and only 6 haplotypes from 9 individuals belonging to West European and Iberian Peninsula populations are out of clades 3-1 and 3-3.

Concerning the rest of haplotypes in the network, haplotypes BD, K, P, AZ, BC, N, AY, O, M and L clustered together in a two level clade (hereafter 2-5) and haplotypes R, Q and AX clustered together in another two level clade (hereafter 2-6), weakly supported by the ML tree, suggesting that haplotypes from different origins are converging in the Central European sampled range, leading to a blurred genetic structure at this geographic scale.

3a.



3b.

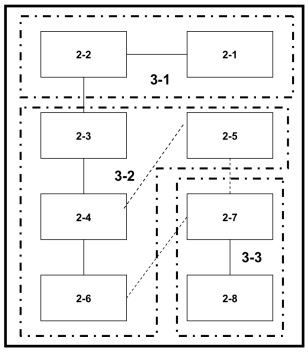


Figure 3. (a) TCS minimum spanning haplotype network based on 802 bp of concatenated COI and COII mtDNA fragments and nested design used in the NCPA levels 0-2. Haplotypes and network form North America populations were excluded from the NCPA since they showed more than 40 substitutions with respect to European haplotypes (see text). Locations were grouped in four main areas (Iberian Peninsula, Continental Central Europe, Continental West Europe and North America). Circles size is proportional to the total number of individuals sharing each haplotype and slices are proportional to the number of individuals per area. Dashed lines indicate alternative connections (loops). (b) European nested design level 3 and final nested cladogram.

Divergence time estimation

The Coefficient of Variation of our root to tip maximum likelihood tree distances was equal to 0,389 or 38'9%, which is within the range that is successfully modelled by a relaxed molecular clock model (Rambaut, pers. comm.). A comparison of the Bayesian factors of the trees obtained under the three clock model assumptions showed very strong (exponential clock model) and strong (lognormal clock model) preferences for relaxed clock models compared with strict clock model, and within relaxed models, relaxed model with uncorrelated exponential distribution was preferred compared with uncorrelated lognormal distribution model (Table 2a). Since preference against uncorrelated lognormal distribution was not very strong, and taking into account that exponential distribution relaxed model is not recommended unless the evidences favouring this model are clear (Drummond et al. 2007), we decided to use the uncorrelated lognormal distribution model for our relaxed clock. Bayesian age estimates for the most common recent ancestor of the main clades (All sampled haplotypes, North American and European haplotypes, clade 3-1, clade 3-3 and the rest of European haplotypes – clade 3-2 -), under the 2.3% pairwise sequence divergence substitution rates is summarized in table 2b. Clades 3-1 (Iberian haplotypes) and 3-3 (West European haplotypes) diverged approximately at the same time, what suggests that a common and similar historical or biological episode started the divergence.

2a.

Model clock		Evidence against T0	
T1	T0	2lnB10	Conclusion
Exponential	Lognormal	5,724	Positive
Exponential	Strict	13,886	Very Strong
Lognormal	Strict	8,162	Strong

2b.

Clade	Mean age estimates of Mrca
All sampled haplotypes	0.970 (1.766 – 0.786)
North American haplotypes	0.189 (0.319 – 0.070)
European haplotypes	0.370 (0.569 – 0.200)
3-1 clade (most of Iberian haplotypes)	0.205 (0.329 – 0.105)
3-2 clade (most of Central European haplotypes)	0.339 (0.544 – 0.178)
3-3 clade (most of West European haplotypes)	0.169 (0.301 – 0.070)

Table 2a. Summary of Bayesian factor analysis of alternative clock models.

Table 2b. Mean age estimates of the most recent common ancestor (Mrca, in millions years) of each main clade of *Carnus hemapterus* sampled populations obtained with Bayesian analysis. We used an uncorrelated lognormal relaxed clock model based on 2.3% pairwise sequence divergence per millions years estimated for mitochondrial markers in arthropods (Brower 1994). Values in parentheses correspond to the upper and lower 95% highest posterior density (HPD) intervals.

Genetic structure of European populations

SAMOVA analysis revealed the existence of genetically distinct groups broadly corresponding to sampling areas (all samples from each locality were grouped together except for Gosing locality that was split into two different groups). In fact, the F_{CT} values, the proportion of genetic variability explained by subdivision in groups, increased progressively with *k* (number of groups) but reached a plateau when *k*=7 to 9 (Table 3). Similarly, F_{SC} values, that indicate differences among populations within groups and thus, the genetic homogeneity within the created groups, decreased progressively with *k*, with a clear drop at *k*=7 (Table 3). Therefore we decided that the ideal and more conservative number of groups was *k* = 7.

kn-(kn+1)	F_{SC}	F_{ST}	F_{CT}
k2-k3	0.375	0.091	-0.050
k3-k4	0.027	0.000	-0.008
k4-k5	0.018	0.002	-0.003
k5-k6	0.011	0.000	-0.003
k6-k7	0.029	0.005	-0.003
k7-k8	0.009	0.001	-0.002
k8-k9	0.003	0.000	-0.001
k9-k10	0.003	0.000	0.000
k10-k11	0.000	0.000	0.000
k11-k12	0.007	0.001	-0.001
k12-k13	0.003	0.000	0.000
k13-k14	0.000	0.000	0.000
k14-k15	-0.001	0.000	0.000
k15-k16	-0.002	0.000	0.001
k16-k17	-0.003	0.001	0.002
k17-k18	-0.006	0.000	0.002
k18-k19	-0.003	0.000	0.002
k19-k20	-0.004	0.001	0.003

Table 3. F_{SC}, F_{ST} and F_{CT} differences between increasing adjacent values of k for different k-levels of grouping (from k=2 to k=20) used in SAMOVA analysis.

On the light of the SAMOVA result, populations were grouped as follows: G1 = All samples from the Iberian Peninsula; G2 = All Italian and French samples; G3 = Gosing 1; G4 = Gosing 2 + Somotor + Albertirsa samples; G5 = Piestany samples; G6 = Dunajska Streda samples; G7 = Pavlova samples. Hierachical AMOVA showed that 71.4 % of the variation was due to this partition (F_{CT}=0.714, $P < 0.001$, significance tests after 1023 permutations), while variation among populations within groups explained only 1.97 % of the total genetic variation (F_{SC}=0.069, P<0.001, significance tests after 1023 permutations).

Pairwise F_{ST} values between each location and between the groups defined by the SAMOVA, revealed significant genetic variation among almost all sampled populations, except for several locations within Iberian Peninsula (Table 4a) and among G6 versus G2, G3 and G5 SAMOVA groups (Table 4b). Additionally, several comparisons including localities like Balmaseda, Dunajska Streda or La Flotte, had high F_{ST}'s but did not reach

significance probably because of the low number of flies sampled in these localities (table 1 and 4a).

a.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Tabernas (1)	-	**0.091**	**0.159**	**0.056**	0.003	**0.045**	**0.187**	**0.109**	**0.728**	**0.603**	**0.691**	**0.688**	**0.846**	**0.701**	**0.772**	**0.725**	**0.708**
Doña Maria (2)	-	-	**0.299**	**0.15**	0.023	**0.214**	0.171	**0.195**	**0.77**	**0.574**	**0.649**	**0.689**	**0.915**	**0.709**	**0.806**	**0.87**	**0.745**
Guadix (3)	-	-	-	**0.22**	**0.123**	**0.18**	**0.293**	**0.263**	**0.833**	**0.657**	**0.73**	**0.757**	**0.907**	**0.804**	**0.853**	**0.863**	**0.799**
Andujar (4)	-	-	-	-	0.011	0.051	0.268	**0.148**	**0.807**	**0.619**	**0.696**	**0.738**	**0.91**	**0.77**	**0.833**	**0.852**	**0.776**
Daganzo (5)	-	-	-	-	-	0.007	0.054	0.038	**0.63**	**0.51**	**0.601**	**0.606**	**0.831**	**0.594**	**0.704**	**0.694**	**0.641**
El Espinar (6)	-	-	-	-	-	-	**0.353**	**0.103**	**0.875**	**0.658**	**0.734**	**0.791**	**0.934**	**0.845**	**0.884**	**0.902**	**0.828**
Balmaseda (7)	-	-	-	-	-	-	-	**0.382**	0.167	0.207	**0.347**	0.32	**0.799**	0.136	0.483	**0.571**	**0.4**
Castroverde (8)	-	-	-	-	-	-	-	-	**0.821**	**0.681**	**0.751**	**0.778**	**0.901**	**0.798**	**0.846**	**0.831**	**0.799**
La Flotte (9)	-	-	-	-	-	-	-	-	-	0.004	0.045	**0.282**	**0.877**	0.143	0.541	**0.698**	0.36
Paradou (10)	-	-	-	-	-	-	-	-	-	-	0.059	**0.209**	**0.704**	0.233	**0.403**	**0.183**	**0.165**
Parma (11)	-	-	-	-	-	-	-	-	-	-	-	**0.241**	**0.708**	0.262	**0.412**	**0.204**	**0.178**
Gosing (12)	-	-	-	-	-	-	-	-	-	-	-	-	**0.744**	0.259	**0.413**	0.179	0.081
Pavlova (13)	-	-	-	-	-	-	-	-	-	-	-	-	-	**0.843**	**0.867**	**0.9**	**0.742**
Dunajska Streda (14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.519	**0.672**	0.377
Piestany (15)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**0.651**	**0.429**
Somotor (16)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0
Albertirsa (17)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

b.

	G1	G2	G3	G4	G5	G6	G7
G1 (Iberian Peninsula)	-	**0.677**	**0.732**	**0.741**	**0.791**	**0.731**	**0.849**
G2 (France+Parma)	-	-	**0.263**	**0.185**	**0.387**	0.224	**0.658**
G3 (Gosing 1)	-	-	-	**0.320**	**0.482**	0.269	**0.780**
G4 (Gosing	-	-	-	-	**0.534**	**0.549**	**0.801**
II+Somotor+Albertirsa)	-	-	-	-	-	0.519	**0.867**
G5 (Piestany)	-	-	-	-	-	-	**0.843**
G6 (Dunajska Streda)	-	-	-	-	-	-	-
G7 (Pavlova)	-	-	-	-	-	-	-

Table 4. Pairwise F_{ST} values for (a) every European locality sampled and (b) groups defined by SAMOVA analysis. Significant values obtained by significance tests after 1023 permutations are shown in bold.

Mantel test did not reject the hypothesis of isolation by distance for all European populations (Observed Z = 207899.84; r = 0.6553; two tailed *P* = 0.001), supporting the

association between genetic distance and geography at this spatial scale. However, no significant correlation between genetic and geography distances was found at lower spatial scales like the Iberian populations (Mantel test: Z = 3440,40; r = 0.2901; two tailed *P* = 0.2614) or the Central European ones (Mantel test Z = 2403,49; r = -0,1475; two tailed *P* = 0.6873), suggesting that isolation by distance among sampled localities was not important to explain genetic structure at this geographical scale.

Significant variation among groups in AMOVA analysis at European scale were obtained when flies were grouped by sampled location and date of parasite emergence, whereas the remaining grouping variables (host species, host breeding phenology, migration behaviour and aggregation tactic during the breeding season) yielded no significant variation among groups (Table 5). The same, even though with a lower genetic variation for Iberian samples, was found for the Iberian Peninsula and central Europe, with an additional significant effect of host species for both regions and an effect of host breeding phenology for Central Europe samples. At a local scale (Tabernas) only date of parasite emergence showed significant genetic variation among groups (Table 5).

Scale	*Grouping variable*	*% of variation*			*Index of fixation*		
		Among groups	Among populations within groups	Within populations	FCT	FSC	FST
Europe	Sampled location	59.92	02.12	37.95	**0.5990; P<0.001**	**0.0530; P<0.001**	**0.6205; P<0.001**
	Emergence date	26.03	36.92	37.05	**0.2603; P<0.001**	**0.4991; P<0.001**	**0.6295; P<0.001**
	Host species	01.55	58.46	39.99	0.0155; P=0.236	**0.5937; P<0.001**	**0.6001; P<0.001**
	Host breeding phenology	-02.05	61.60	40.45	-0.0205; P=0.464	**0.6036; P<0.001**	**0.5954; P<0.001**
	Host aggregation tactic	-00.20	60.05	40.16	-0.0020; P=0.276	**0.5992; P<0.001**	**0.5984; P<0.001**
	Host migration behavior	02.12	58.20	39.68	0.0211; P=0.140	**0.5956; P<0.001**	**0.6032; P<0.001**
Central Europe	Sampled location	65.20	-0.70	35.50	**0.6520; P<0.001**	-0.0201; P=0.595	**0.6450; P<0.001**
	Emergence date	20.94	44.26	34.79	**0.2094; P<0.001**	**0.5599; P<0.001**	0.6521; P=0.071
	Host species	20.27	44.92	34.81	**0.2027; P<0.001**	**0.5634**; **P<0.001**	0.6519; P=0.063
	Host breeding phenology	20.27	44.92	34.81	**0.2027; P<0.001**	**0.5634**; **P<0.001**	0.6519; P=0.063
	Host aggregation tactic	18.04	47.23	34.73	0.1804; P<0.001	**0.5763; P<0.001**	0.6527; P=0.053
Iberian peninsula	Sampled location	09.43	02.68	87.99	**0.0934; P<0.001**	**0.0295; P=0.006**	**0.1201; P=0.001**
	Emergence date	03.32	07.95	88.73	**0.0332; P=0.005**	**0.0822; P=0.023**	**0.1122; P=0.002**
	Host species	03.87	07.35	88.78	**0.0387; P=0.030**	0.0764; P=0.076	**0.1127; P=0.001**
	Host breeding phenology	01.54	09.59	88.87	0.0153; P=0.082	**0.0974; P<0.001**	**0.1113; P<0.001**
	Host aggregation tactic	-00.97	11.06	89.91	-0.0097; P=0.617	**0.1100; P=0.001**	**0.1010; P=0.001**
	Host migration behavior	01.45	09.77	88.78	0.0144; P=0.060	**0.1000; P=0.003**	**0.1120; P=0.001**
Tabernas	Sampled nest location	-02.81	03.93	98.88	-0.0280; P=0.640	0.0380; P=0.300	0.0110; P=0.310
	Emergence date	15.27	-06.14	90.87	**0.1526; P=0.034**	-0.0724;P=0.400	0.0912; P=0.318
	Host species	-01.01	-01.88	102.89	-0.0100; P=0.660	-0.0186;P=0.600	-0.0920; P=0.750
	Host breeding phenology	-00.06	-02.60	102.66	-0.0010; P=0.360	-0.0260; P=0.760	-0.0260; P=0.780
	Host aggregation tactic	01.17	09.47	89.36	0.0117; P=0.390	**0.0950; P=0.008**	**0.1064 ;P=0.008**
	Host migration behavior	-00.06	-02.60	102.66	-0.0010; P=0.360	-0.0260; P=0.760	-0.0260; P=0.780

Table 5. AMOVA analysis at three different spatial scales. For all grouping variables the population units were individual host nests, except for *Carnus* emergence date. for which population units were individual host nest classified into months of parasite emergence. Hosts sampled in each geographic scale and data for each host included in the AMOVA analyses are as follows; **Europe**: *Buteo buteo* (Md, Tr, R), *Falco peregrinus* (Ea, Tr, R), *Falco cherrug* (Md, Tr, E), *Falco naumanni* (Md, Cl, E), *Falco tinnunculus* (Md, Tr, R), *Athene noctua* (Md, Tr, R), *Upupa epops* (Ea, Tr, R and E), *Merops apiaster* (Lt, Cl, E), *Coracias garrulus* (Lt, Tr, E). **Central Europe**: *F. cherrug, U. epops, M. apiaster*. **Iberian Peninsula**: *F. peregrinus, F. naumanni, F. tinnunculus, A. noctua, U. epops, M. apiaster* and *C. garrulus*. **Tabernas**: *A. noctua*, *M. apiaster*, *C. garrulus*. (Ea = Early breeder, Md = Mid breeder; Lt = Late breeder; Tr = Territorial, Cl = Colonial; E = Transaharian migrant, R = Resident).

Historical demography

NCPA showed significant inferences in several clades of different levels. Geographical inferences are showed in Table 6 (clades referenced in Figure 3a, b). Restricted gene flow with isolation by distance was inferred for clades 1-15, 2-2, 2-5, being 1-15 a low level nested clade of 2-2. 2-2 involved Iberian and South French populations and 2-5 involved populations from Tabernas (Spain), South France, Parma (Italy) and from Slovakia (Somotor and Piestany), Austria and Hungary. Haplotypes Q and R nested together in the 1-9 clade, corresponding to G7 SAMOVA population (Pavlova, in Slovakia, see figure 3 and tables 1 and 4b).

Continuous range expansion was inferred for clades 1-17, 3-1, 3-2, 3-3 and the total cladogram, even though for clade 3-2 it was impossible to distinguish between range expansion, past fragmentation or long distance colonization.

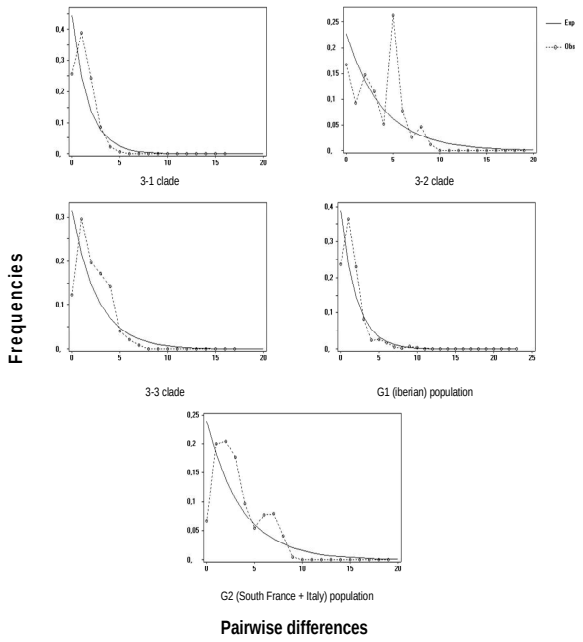


Figure 4. Mismatch distribution for observed frequencies of each of the three level clades obtained with NCPA and for the grouped populations G1 (Iberian Peninsula) and G2 (South France + Italy), compared with the expected frequencies for constant population sizes.

Nested clade	*P*	Interior clades	Dc	Dn	Steps	Pop involved	Inference
1-7	0.0810	Clade P (interior)	222.7819 S	268.0770	1-2-3-4-9-10-NO	G1,G2,G4,G5	INC
		I-T	210.4050 S	-242.2460			
1-15	0.4660	I-T	216.9778 L	11.4359	1-2-3-4-NO	G1,G2	RGF with ISB
		Clade AJ (interior)	211.9022 S	233.3442 S			
1-17	0.0340	I-T	211.9022 S	-71.6959 S	1-2-11-12-NO	G1	CRE
		Clade AN (interior)	323.0523 L	305.9172 L			
		Clade AO (Tip)	0.0000 S	157.4921 S			
1-20	0.1740	I-T	323.0523 L	148.4251 L	1-2-3-5-6 IGR 7-8 NO	G1,G2	INC
		1-13 (Tip)	15.4595 S	2164.8470			
		1-19 (Tip)	100.8812	109.7868 S			
2-2	0.0580	I-T	111.3568 L	-0.8881	1-2-3-4-NO	G1,G2	RGF with ISB
		Clade 1-7 (interior)	310.3883 L	318.9839 L			
		Clade 1-6 (tip)	0.000 S	92.2942 S			
		Clade 1-26 (tip)	0.000 S	179.0056			
2-5	0.0440	I-T	310.3883 L	177.1466 L	1-2-3-4-NO	G1,G2,G3,G4,G5	RGF with ISB
		Clade 1-1 (tip)	0.0000 S	509.0000			
		Clade 1-4 (tip)	0.0000	738.1465 L			
2-7	0.0130	I-T	379.3322 L	-182.3187 S	1-2-3-5-6 IGR 7-8 NO	G1,G2	INC
		Clade 2-2 (interior)	242.4295 S	246.1488 S			
		Clade 2-1 (tip)	327.5602 L	336.3215			
3-1	0.0020	I-T	-85.1306 S	-90.1727	1-2-11-12 NO	G1,G2	CRE
		Clade 2-4 (interior)	69.9535	78.8782 S			
		Clade 2-5 (interior)	249.9265	252.8521 L			
		Clade 2-6 (tip)	93.4048 S	119.7033 S			
3-2	0.0020	I-T	133.2910 L	129.8233 L	1-2-3-5-6-13-14 YES	G1,G2,G3,G4,G5, G6,G7	CRE or LDC or PF
		Clade 2-7 (interior)	400.0426 S	420.2663 S			
		Clade 2-8 (tip)	716.0784 L	755.2664			
3-3	0.00360	I-T	-316.036 S	-335.0001	1-2-11-12 NO	G1,G2,G6	CRE
		Clade 3-1 (tip)	257.4440 S	935.3074			
		Clade 3-2 (interior)	209.7731 S	1096.822 L			
		Clade 3-3 (tip)	521.7226 S	554.8209 S			
Total cladogram	0.0000	I-T	-85.0151	215.2795 L	1-2-11-12 NO	All populations	CRE

Table 6. Nested Clade Phylogeographic Analysis results obtained with the GEODIS key inference of November 2005. INC = Inconclusive results; RGF = Restricted gene flow; ISB = Isolation by distance; CRE = Contiguous range expansion; LDC = Long distance colonization; PF = Past fragmentation.; IGR = Insufficient geographic resolution.

The analysis of Mismatch distribution confirmed a range expansion for clades 3-1 and 3-3 (Figure 4 and Table 7) with a clear unimodal mismatch distribution for both clades. However, distribution of clade 3-2 appears slightly bimodal, it was not significantly ragged, and R_2 statistic was not significant (Table 7). Therefore sudden expansion model in this clade was not confirmed. Mismatch distribution of groups G1 and G2, that approximately coincide with clades 3-1 and 3-3, also confirmed range expansion in the distribution areas of these populations, although G2 population (South France + Italy) was slightly bimodal. Negative and significant values of Tajima's and Fu's tests for all NCPA clades (except 3-2 clade for Tajima's test) and G1 and G2 populations also suggest expansion population (Table 7).

Clade/Population	Test			
	Raggedness *rg*	R_2	Fu's F_S	Tajima's D
3-1	0.0684; *P*=0.152	**0.0236; *P*=0.010**	**-22.799; P<0.001**	**-2.1467; P<0.001**
3-2	0.0967; *P*=0.656	0.0653; *P*=0.064	**-5.614; P=0.017**	-1.2522; P=0.084
3-3	0.0518; *P*=0.151	**0.0598; *P*=0.001**	**-8.771; P<0.001**	**-1.8103; P=0.013**
Total cladogram	0.0209; *P*=0.072	**0.0319; *P*=0.026**	**-42.851; P<0.001**	**-1.7814; P=0.010**
G1	0.0596; *P*=0.167	**0.0234; *P*=0.002**	**-23.864; P<0.001**	**-2.6380; P<0.001**
G2	0.0300; *P*=0.075	**0.0630; *P*=0.013**	**-13.784; P<0.001**	**-1.4477; P=0.066**
G3	0.4100; *P*=0.701	0.2408; *P*=0.3785	1.0900; P=0.734	-0.5620; P=0.702
G4	0.2633; *P*=0.693	0.1317; *P*=0.233	-0.8650; P=0.242	**-2.1826; P<0.001**
G5	0.7500; *P*=0.887	0.4330; *P*=1.000	2.1970; P=0.922	-0.7801; P=0.167
G6	2.0000; *P*=1.000	0.5000; *P*=1.000	-	-
G7	0.6668; *P*=0.932	0.1636; *P*=0.1216	1.454; *P*=0.898	-0.1267; *P*=0.367

Table 7. Historical demography of *Carnus*: results and P values of the observed mismatch distribution against a sudden expansion distribution reported as the Harpending raggedness statistic test, the Ramos-Onsins and Rozas R_2 statistic test; Fu's F_s statistics and Tajimas's D for each of the three level clades and for the whole cladogram (corresponding to all European populations) obtained with NCPA and each grouped population obtained with SAMOVA analysis. Significant results (*P*>0.05) are shown in bold.

Discussion

Genetic structure and Carnus hemapterus biogeography

The analysis based on 245 *Carnus hemapterus* specimens belonging to 19 locations revealed high and variable levels of genetic divergence at different geographical scales.

<u>European and North America clades</u>

Canadian populations were genetically different from the European ones. Haplotype network showed these two distinct groups, also supported by maximum likelihood and

bayesian phylogenetic analysis. This is a typical pattern resulting from the geographical distance and physical barriers between both continents, whose populations shared no haplotypes (minimum number of substitutions between European and Canadian haplotypes > 40). This suggests that North American and European flies have evolved independently with no recent contacts between them. The definitive separation between North America and Europe occurred around 55-60 millions years ago (hereafter myr), nevertheless mean age estimates of most recent common ancestor indicate that the separation of both clades occurred around 1,8-0,8 myr, thus lineage divergence occurred after the separation of both continents. Many parasites use hosts as vehicle of transmission to cover long distances between remote sites and genetic structure of parasites derived from long distance movements of their hosts has been recorded for other ectoparasites (De La Cruz & Whiting 2003, Todd et al. 2004), This is unlikely for *Carnus*, since no records of flies parasitizing adult hosts has ever been observed, and parasite dispersal (active flight of the infective phase) only occurs during the host breeding period. Therefore, the divergence time between the European and Canadian clades is probably not the result of long distance parasite movements between continents using accidental dispersion of adult birds as a vehicle, but may reflect some kind of past contact, allowing gene flow between continents, that subsequently was interrupted by changes in physical barriers and/or changes in local conditions affecting both hosts and/or parasites. Present-day Alaska and eastern Siberia have been connected by a land bridge repeatedly during the Cenozoic era (Hopkins 1967); specifically, both continents were united during early to mid Pleistocene (2.0 to 1.2 myr approximately), coinciding with Riss glaciation (Ogasawara 1998). Although we must be careful with the estimation of *C. hemapetus* divergence time, since choice of molecular clock model and calibration rate of the molecular clock were inferred due to the lack of fossil records for this taxa (see material and methods), the opening of Bering straight (between 1.2 to 1.0 myr) coincides with the mean estimation divergence of European and Canadian lineages, supporting the idea that Bering land bridge could be an important corridor for Palaearctic and Neartic *Carnus* lineages. Palinological and fossil data suggest that climatic and vegetal conditions in Beringian region were warmer than adjacent regions in North America and Eurasia (Reppening 2001), tolerating the existence of habitats that allow the persistence and survival of significant number of birds species belonging to families typically parasitized by *Carnus* (see Saitoh et al. 2010) as well as other parasitic

taxa (Koehler et al. 2009). Furthermore, interchange and gene flow of different taxa (including vertebrate and invertebrate species) through Beringian land bridge was notable during this period (San Martin et al. 2001), and some host species like *Pica* genus used Beringian land to cross between continents during late Pliocene to early Pleistocene (Lee et al. 2003). Thus, persistence of host and associated parasite fauna in Beringian region during this glacial period (with the subsequent sea transgression and associated genetic isolation between Neartic and Paleartic *Carnus* clades and posterior colonization of new Paleartic and/or Neartic areas when ice sheets moved back to northerm latitudes) is the most valid explanation of the observed genetic structure of our data at Holartic geographical level.

European populations

Our results showed clear differences in phylogeographical and population structure of *Carnus* across Europe. Most Iberian haplotypes (89%) fell into a well-supported clade. A second, weakly supported clade was revealed by phylogenetic analysis and confirmed by the nested design of the haplotype network, with most sampled haplotypes in this clade belonging to localities in France and North Italy (78% of haplotypes exclusive from this region, compared with 7% of haplotypes exclusive from Central Europe and Iberian localities). Haplotypes from Central Europe did not show any clear phylogenetic relationships, but it is significative that only one sampled haplotype out of 13 fell into the two other clades.

The Iberian Peninsula has been recognized since long as a glacial refuge for many different organisms (Taberlet et al. 1998), including some parasites like mosquitoes (Aransay et al. 2003) or parasitic nematodes (Nieberding et al. 2004, 2005). Estimates of divergence of the Iberian clade coincide with glacial periods during middle Pleistocene and suggest the existence of glacial refuge of this ectoparasite in the Iberian Peninsula during Pleistocene. Posterior range expansion and genetic homogeneity of the Iberian population (Group 1 in SAMOVA analysis, Table 4b and 7, Figure 4) also confirm the idea of a glacial refuge and posterior constant range expansion along the Iberian Peninsula. Similar results on 3-3 clade divergence as well as evidences of range expansion for this clade also suggest the existence of some other glacial refuges for *Carnus hemapterus* along the Mediterranean area (probably the Italian peninsula or the French Mediterranean coast), as

it has been suggested for other organisms (Taberlet et al. 1998), although we ignore the origin and direction of the expansion of this clade.

The existence of a low number of individuals with shared haplotypes between the Iberian Peninsula and France-Italy locations suggests secondary contact and gene flow between those populations, nevertheless the Pyrenees may have been a major barrier of isolation during post-glacial *Carnus* expansion, shaping the genetic structure observed. Contrary to specialized parasites that are not capable to colonize new regions by themselves and, thus, with a high probability of co-speciation with their hosts (Gandon & Michalakis 2002 but see Gómez-Diaz et al. 2007), *Carnus hemapterus* expansion seemingly depends on own dispersal movements during free-living stages more than on the use of adults birds as vehicle of transmission, since: i) expansion has been produced gradually from their refugee, ii) no adult flies have been ever recorded in adults birds (see above), probably hindering long distance colonization movements. Nonetheless *Carnus hemapterus* could use coastal corridors to cross high altitude ranges and/or hostile climate area as it has been showed for other organisms with aggregated distribution (Vainio & Välnölä 2003, Teacher at al. 2009). This could explain the expansion of phylogroup 3-3, since shared haplotypes were found at both sides of the Alps, being even considered by SAMOVA analysis as a homogeneous population -population G2-. Thus, the generalist nature and self-dispersion ability of *Carnus* may be a successful strategy to colonize new habitats and avoid evolutive and/or geographical "points of not returns" that can characterize more specialized and strongly host-dependent parasites (Horberg & Brooks 2008).

Contrary to the Iberian and French-Italian populations, the distribution of haplotypes in Central Europe showed a very different genetic structure. The level of divergence among Central European locations was higher compared with the levels of divergence among Iberian populations (See table 4a). Some non-mutually exclusive hypotheses could explain such differences between both regions: Central Europe could be a contact zone of different parasite lineages from ancient origins prior to glacial isolation of the Iberian clade (as divergence time estimate showed for 3-2 nested clade phylogroup) or coming from other undetected glacial refuges. For example haplotypes AX, R and Q fell into a well supported and basal clade that may come from eastern territories or even from Central European river or forest habitats remaining during Pleistocene glacial periods

(Willis et al. 2000) that have been postulated as glacial refugia for other temperate species (Deffontaine et al. 2005). Current *Carnus hemapterus* distribution includes cold and northern habitats like Russia, Scandinavia or Canada (Matyukhin & Krivosheina 2008, Dawson & Bortolotti 1997). More samples from North Europe and other eastern Mediterranean locations such as Balcans or Turkey are necessary to clarify the observed structure in Central Europe. Additionally, the topology of the haplotype network together with the presence of the shared haplotype P, present in remote locations in Italy, Spain and Central Europe, also suggests that Spanish and French-Italian phylogroups appeared after a fragmentation of a larger European population during mid Pleistocene. After that, gene flow and contact between Iberian populations has been reduced by the presence of geographical barriers (Pyrenees) not present in Central European populations that may interchange individuals from other locations previously isolated during glacial periods. Although the presence of P haplotype in remote locations could be attributed to long distance colonization events, the lack of data of *Carnus flies* over adult hosts and the existence of winter diapause in this species that prevents post-breeding host-associated movements make this explanation very unlikely.

At a local scale, and in contrast to what happen with Iberian populations, the genetic structure of the populations in Central Europe is probably shaped by varying selection pressures or local genetic drift. AMOVA analyses grouped by sampled location in Central Europe showed a maximum percentage of genetic variation among location and minimum value for genetic variation within locations (See Table 5). Furthermore, Mantel Test showed that the genetic divergence between populations was not produced by a pattern of isolation by distance, and not considerable geographical barriers divide the different central Europe sampled locations. This scenario could have been produced by fluctuations in population size that could create genetic bottleneck episodes at local level. Such episodes could strongly influence the genetic composition by founder effects derived from emigrants colonizing new areas and genetic drift eroding diversity of remaining low size populations (Conord et al. 2006).

A different situation seems to occur in Iberian locations. Although the values of within localities genetic variation (Table 5) could also indicate an effect of genetic drift, low values of genetic variation among localities, F_{ST} values between localities and nested clade and mismatch analyses (Tables 4 to 7), support the idea that genetic structure in this

region has been the result of a population expansion following by a mixing and interchange of haplotypes between locations.

Host richness or density may influence parasite spread and genetic structure (Tsai & Manos 2010). If host density is large enough, the number of available resources will increase and consequently the number of host breeding sites no longer represents a population limiting factor. Parasite populations can remain dense in high host density habitats, promoting high levels of genetic mixing between populations, allowing the contact of previously isolated population and increasing gene flow between them (Chapuis et al. 2008). Therefore, differences in host density between the Iberian peninsula and Central Europe could be an important factor explaining genetic differences in *Carnus* diversification and should be taken into account in future works of this and other parasites.

Host-associated factors as genetic diversifying mechanism

Detection of host-associated factors influencing genetic diversification revealed different results depending on the spatial scale. AMOVA analysis including allopatric (Europe, Iberian Peninsula and Central Europe locations) samples, showed that a substantial part of genetic divergence was explained by emergence date (Table 5). This divergence probably reflects latitudinal differences in parasite emergence dates and host breeding phenologies across localities. This is also supported by the fact that the percentage of variation among locations within emergence dates cluster is greater than the percentage of variation among emergence date groups in AMOVA analysis. Although some of this divergence in emergence date or other host-associated traits may be explained by similar host breeding phenologies at similar climatic or latitudinal conditions, it is difficult to distinguish general host-associated traits that may influence genetic divergence in allopatric populations from parasite diversification associated with geographical divergence. Nevertheless, sympatric lineages can remove the noise produced by geographical differences in host-associated traits. At a local spatial level, sympatric samples from Tabernas achieved the maximum level of divergence when individuals were grouped by parasite emergence date (Table 5). This means that temporal clustering was a better predictor of *Carnus* genetic diversification at local level than spatial clustering (Table 5), suggesting some kind of allochronic isolation between ectoparasite groups. Differences in host phenology are a key trait in allochronic divergence in animal or phytophagous parasites (Tauber et al. 1986,

Theron & Combes 1995). Therefore, temporal differences in hosts' phenologies are required as a first step to produce temporal isolation that eventually can finish in diversification between parasite lineages. Our results show that allochronic isolation in *Carnus hemapterus* is not associated with host species, or other host-associated traits and suggest that temporal divergence is produced by the lower probability of encounters and mating opportunities between *Carnus* emerging in different periods during the breeding season and not as a result of parasite genotype adaptation to a particular host phenology. Thus, barrier to gene flow and allochronic divergence among sympatric *Carnus hemapterus* lineages is not part of selective process of speciation but more probably resemble a diversification in allopatry (Giraud 2006) where temporal barriers and mating probabilities are extrinsic to parasite genotype adaptations (Kondrashov & Mina 1996). Genetic differentiation by means of host race formation is the main explanation of sympatric diversification processes in other generalist parasites (Vía et al. 2000, McCoy et al. 2001, Founier & Giraud 2008, Blair et al. 2005). Future sampling and experimental efforts together with the use of other genetic markers that may can detect finer host-associated parasite genetic divergence should highlight whether differences in *Carnus* emergence dates have an adaptive value, as it is the case for other insects systems (see introduction).

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DISCUSIÓN GENERAL INTEGRADORA

Uno de los elementos fundamentales que predicen el éxito y la eficacia biológica de cualquier organismo es la habilidad para completar su ciclo de vida. Todas las especies deben pasar por una serie de fases que incluyen la selección de los hábitats y recursos idóneos para completar su desarrollo y reproducción, así como diversas fases de generación y dispersión de propágulos reproductivos que aseguren la transferencia de la información genética de cada individuo a las siguientes generaciones. En la mayoría de los organismos de vida libre, el desarrollo y terminación de estas fases se producen sin la necesidad de la asociación con otro organismo. Para los parásitos, sin embargo, la presencia de un hospedador en al menos una de las fases de su ciclo de vida es condición necesaria para completar satisfactoriamente su reproducción y desarrollo. Por tanto, no es de extrañar, que la mayoría de las adaptaciones y estrategias que han desarrollado los parásitos tengan la función de detectar, explotar y transmitirse de forma efectiva entre los hospedadores a los que parasita. Los parásitos por tanto, necesitan superar dos tipos de barreras para poder infectar exitosamente a sus hospedadores, son los denominados filtros de encuentro (habilidad para encontrar al hospedador adecuado y poder coincidir con él en el espacio y el tiempo) y los filtros de compatibilidad (habilidad para superar las defensas del hospedador frente a la explotación y transmisión, así como la capacidad de explotar los mejores recursos desde un punto de vista de optimización nutricional y metabólica para el parásito) (Combes 2001). La distribución y abundancia de los hospedadores, así como la predecibilidad y persistencia de éstos en el espacio y en el tiempo son parámetros ecológicos fundamentales que influirán sobre el éxito de superación de las barreras de encuentro, y en gran medida de las barreras de compatibilidad (la posibilidad de elección por parte del parásito de un tipo de hospedadores con unas características de salud y/o condición nutricional concretas aumentan las probabilidades de completar con éxito el ciclo de vida del parásito), (Combes 1997, 2001, Vázquez et al. 2005). El grado de impredecibilidad de los hospedadores es, por tanto, un factor clave que puede modelar las características ecológicas y estrategias evolutivas de la mayoría de las especies parasíticas. Frente a esta impredecibilidad surgen dos estrategias principales (Bush et al. 2001): (1) estrategias de detección y transmisión pasiva, en las que los parásitos utilizan una serie de

factores y pistas para situarse en los hábitats o periodos donde la densidad y presencia de los hospedadores adecuados será más probable (por ejemplo sincronización estacional, quimio o geotactismos de diversa naturaleza, procesos de quiescencia, dormancia o hibernación, etc.); y (2) aquellas estrategias denominadas activas, en el que los parásitos desarrollan una serie de habilidades de búsqueda, selección de hospedador, dispersión, transmisión y colonización de nuevos hospedadores de forma dinámica. El estudio de los dos tipos de estrategias, así como el grado de predecibilidad de los hospedadores y su entorno se hacen indispensables si se quieren conocer con exactitud los requerimientos ecológicos de los parásitos para completar con éxito su ciclo de vida, a lo largo de los diversos ejes espaciotemporales en los que la interacción parásito-hospedador se desarrolla.

A lo largo de esta tesis, los argumentos que intentan explicar la función de la variabilidad en el ciclo de vida y la sincronización de *Carnus hemapterus* hacen frecuente referencia al término "impredecibilidad": impredecibilidad de la fenología de cría de los hospedadores, impredecibilidad de la supervivencia de los hospedadores a nivel de nido y población o impredecibilidad de la reocupación de los nidos por parte de los hospedadores. Determinados resultados obtenidos en la presente tesis y en trabajos anteriores sugieren que este sistema, y más concretamente, la presencia temporal y espacial de los hospedadores se caracteriza por cierto grado de incertidumbre e impredecibilidad: (1) el tamaño de puesta y nidada para las carracas varía significativamente entre años, incluso en distintas localidades (Avilés et al 1999, Capítulo III, R. Václav datos no publicados), además la mortalidad de los juveniles de carraca dentro de la nidada y entre nidadas puede ser alta y varió entre años (Capítulo III, R. Václav datos no publicados). Este patrón de variabilidad en los parámetros reproductivos y alta mortalidad de juveniles es característico de otros hospedadores típicos de nuestras latitudes con asincronía en la eclosión de la puesta como la Abubilla (Martín-Vivaldi et al. 1999), Cernícalo (Dijkstra et al 1982, Beukeboom et al. 1988, Avilés et al. 2001), o Grajilla (Soler 1988, 1989); (2) la tasa de reocupación de nidos entre años para la carraca en nuestra área de estudio es moderada y muy variable; (3) en cuanto a la fenología de cría, nuestros datos muestran que aunque la distribución fenológica de las fechas de eclosión del primer huevo en cada nido de la población de carraca estudiada no varió significativamente entre años, la repetibilidad de la fecha de eclosión del primer huevo en cada nido reutilizado fue mucho más variable,

dependiendo del periodo y del número de años incluidos para calcularla, con valores de repetibilidad significativos de entre 0.26 y 0.70 (Capítulo IV); (4) el hecho de que la carraca sea el hospedador habitual más tardío en criar en nuestras latitudes podría añadir un componente mayor de impredecibilidad espacial, debido a la baja abundancia de hospedadores disponibles en la fase final de la estación reproductora de la comunidad aviar hospedadora de este parásito. Como ya hemos indicado anteriormente, todos los datos arriba mostrados sugieren que *Carnus hemapterus* se enfrenta frecuentemente a diversos grados de incertidumbre e impredecibilidad temporal y espacial de sus hospedadores, aunque ha desarrollado diversas estrategias ecológicas y comportamentales a múltiples niveles para poder contrarrestar la imposibilidad de detectar con precisión la presencia de sus hospedadores.

En general, nuestros resultados confirman que *Carnus hemapterus* es un parásito con un ciclo de vida y estrategias vitales muy variables, con una gran capacidad de responder plásticamente a cambios ambientales o aquellos relacionados con sus hospedadores. Existen gran cantidad de rasgos en esta especie que muy probablemente hayan surgido como respuesta frente a la impredecibilidad y/o persistencia en el tiempo de sus recursos.

La preferencia de *Carnus hemapterus* por pollos con una mayor respuesta inmune dentro de las nidadas de carraca, en detrimento de otros rasgos como el tamaño del pollo o su condición corporal, estuvo condicionada por parámetros tales como la condición física de la nidada o la densidad de moscas dentro del nido. Cuando la condición de la nidada era alta (mayor probabilidad de supervivencia) o la cantidad de moscas en el nido era baja (menor competencia intraespecífica por el recurso), esa predilección por pollos con mayor respuesta inmune (más fuertes y sanos), desaparecía. Esto sugiere que la selección de hospedador en *Carnus hemapterus* va dirigida hacia aquellos recursos que tienen una mayor probabilidad de persistir en el tiempo, más allá de la abundancia o calidad nutricional relativa de éstos. Este parásito, por tanto, parece seleccionar preponderantemente aquellos individuos que puedan garantizarle el tiempo suficiente de acceso a la sangre y/o secreciones necesarias para completar su ciclo de vida y optimizar su eficacia biológica. Muy probablemente, otros rasgos de la selección de hospedador en *Carnus hemapterus*, como su preferencia por pollos no emplumados o el hecho de que no suela parasitar a especies cuyos pollos tienen un ciclo de desarrollo corto (como por

ejemplo, muchas especies de paseriformes, Brake 2011), pueden ser parcialmente explicados por la necesidad de establecerse un periodo de tiempo mínimo en el hospedador para completar satisfactoriamente su reproducción y ciclo de vida.

Otros rasgos ecológicos que se han estudiado en este parásito parecen seguir ese "camino común" de variabilidad y respuesta plástica en la lucha contra la impredecibilidad de su entorno. La existencia de diapausa prolongada, y el hecho de que ésta sea considerada como una alternativa a la dispersión espacial para evitar periodos en los cuales los recursos son escasos o de baja calidad (Danks 1992, Hopper 1999), sugiere que esta modificación del ciclo de vida de *Carnus hemapterus* ha podido surgir como una estrategia de escape frente a posibles fracasos en la reproducción de los hospedadores o a la presencia irregular e imprevisible de estos últimos en un periodo y/o estación reproductora determinada. En el caso de que hubiera algún tipo de catástrofe a nivel poblacional (por ejemplo una mortalidad de pollos elevada en un año, cambios climáticos que modifiquen drásticamente la fenología de cría local de la comunidad de hospedadores) o a nivel de nido (por ejemplo, nidos que permanecen vacíos durante una o más estaciones reproductoras, puestas o pollos depredados, nidos donde cría otra especie de fenología más temprana o tardía), la diapausa prolongada aparece como una solución plausible frente a fluctuaciones estocásticas temporales del medio ambiente y sus hospedadores.

Carnus hemapterus es capaz de responder a otros niveles de impredecibilidad. Las variaciones y cambios de temperatura que se producen dentro del nido durante el periodo de diapausa, ya sea a través de cambios ambientales o bióticos, son los principales responsables de los cambios en la fenología de emergencia de *Carnus hemapterus*, tal y como muestran las simulaciones de cambio de hospedador y los resultados del experimento de translocación de parásitos realizados entre Tabernas (Almería) y Castro Verde (Portugal). Aunque no podemos descartar que otros factores (como el fotoperiodo, humedad, densidad poblacional o disponibilidad de alimento, ver Tauber et al. 1986), no sean decisivos en la inducción o desarrollo iniciales de la diapausa en *Carnus hemapterus*, nuestros resultados indican que la temperatura es un factor determinante que controla el desarrollo y la terminación de la diapausa, así como la posterior emergencia de los imagos de esta especie. Muy posiblemente el efecto de la temperatura sea directo, afectando a la aceleración del desarrollo metabólico durante la última fase de la diapausa (Kostal et al. 2006).

Continuando con el ciclo de vida, nuestros resultados muestran que el periodo de emergencia de este parásito puede variar lo largo de diversos ejes espaciotemporales. *Carnus hemapterus* presenta una fenología de emergencia que varía entre los años de estudio y también entre los tipos de nidos usados en el presente trabajo (las cajas nido presentaban una emergencia más temprana que el resto). En contraposición, la fenología de cría de la carraca no varió entre años, aunque hubo diferencias asociadas al tipo de nido. ¿Cómo afecta esta variabilidad a la sincronización entre *Carnus hemapterus* y uno de sus hospedadores habituales (carraca)? A los resultados bajos de sincronización que encontramos, se une el hecho de que los valores fluctúan enormemente entre tipos de nido y años. La variación interanual en sincronización se debe a la variación de la fenología de los parásitos. Las diferencias interanuales entre las fenologías de *Carnus* y carraca probablemente se producen porque los cambios ambientales afectan intensamente a la duración y terminación de la diapausa en especies heterotermas como *Carnus* cuyo metabolismo y desarrollo está asociado a la variación ambiental (principalmente temperatura; Kostal et al. 2006). Los valores bajos de sincronización parásito-hospedador encontrados indican que los ciclos de vida de estas dos especies no están tan finamente sincronizados como cabría esperar si *C. hemapterus* estuviera adaptada al ciclo de vida de este hospedador en concreto, tal y como se ha encontrado en sistemas altamente especializados (Dres & Mallet 2002, Tilmon 2008), lo que redunda en la caracterización de *Carnus* como especie generalista. Resultados adicionales muestran que el periodo de emergencia del parásito es mayor que el periodo de disponibilidad de pollos de carraca, con lo que existe una gran cantidad de moscas que emergen antes de que haya pollos disponibles de carraca en el nido o en la población estudiada. Todo lo anterior sugiere que la asincronía encontrada podría ser una estrategia efectiva en esta especie para parasitar otros hospedadores más tempranos y/o evitar procesos estocásticos ambientales que pudieran comprometer la reproducción de una especie de hospedador concreto y condenar la supervivencia y eficacia biológica de las poblaciones parásitas asociadas a un hospedador (e.g. una fenología reproductora) determinado. Diversos autores, usando insectos fitófagos como modelo, han conjeturado que cierto porcentaje de asincronía fenológica con la aparición de los recursos podría ser una estrategia efectiva frente a la impredecibilidad de la aparición de los recursos (Wiklund & Friberg 2004, Singer & Parmesan 2010). Esta visión de la asincronía en sistemas insecto-planta podría ser

fácilmente extrapolada a sistema análogos de explotación de recursos con cierto grado de imprevisibilidad, como muchos parásitos de animales y, particularmente, al sistema objeto de estudio de esta tesis.

El efecto de la variación ambiental sobre la fenología de emergencia y sincronización de *Carnus hemapterus* que muestra nuestros resultados sugiere que la contribución de los hospedadores sobre la variación de la fenología de emergencia de este parásito podría poco significativa. Sin embargo, otros resultados obtenidos en el presente trabajo indican que sí existe cierta influencia y que no debe ser obviada si se quieren conocer con precisión los factores que intervienen en el control de la sincronización parásito-hospedador en esta especie. El efecto de la temperatura de incubación por parte de hospedadores más tempranos que el hospedador de origen de las muestras produjo un adelanto en la fecha de emergencia media de los parásitos sometidos a los distintos tratamientos experimentales de temperatura. Por otro lado, la fenología de los parásitos dentro de cada nido sigue una distribución con asimetría positiva, en la que la mayoría de los individuos emergen en las primeras 3-4 semanas, coincidiendo con el periodo medio de presencia dentro del nido de pollos no emplumados de carraca. Además, la variación en el periodo de reproducción de los hospedadores en un año afectó al resultado final de la sincronización en el año siguiente, siendo asimétrico el resultado de este efecto (las parejas de carraca que adelantaron su reproducción con respecto al año anterior experimentaron una sincronización mayor con sus parásitos que las parejas que retrasaron su reproducción con respecto al año anterior).

La variabilidad y plasticidad del ciclo de vida de *Carnus hemapterus*, así como la baja predecibilidad de su entorno son factores fundamentales a la hora de abordar el potencial de diversificación y/o especiación en esta especie parásita generalista. Para que un organismo generalista experimente un proceso selectivo de diversificación es necesario que se cumplan los siguientes pasos: (1) la expresión polifénica de alguno de sus rasgos; (2) que alguno o todos los distintos fenotipos existentes acaben seleccionando diferencialmente los hábitats o recursos que les permiten una eficacia biológica máxima, y (3) que esos hábitats o recursos se mantengan estables y predecibles a lo largo del tiempo (West-Eberhard 2003). ¿Qué partes de este proceso se cumplen en nuestro sistema? Nuestros resultados indican que la fenología de emergencia en este parásito se comporta como un rasgo polifénico; no solo porque aquella es capaz de variar dentro de individuos

parasitando a la misma especie de hospedador, sino porque además, este trabajo ha demostrado que *Carnus hemapterus* puede responder diferencialmente a diversos hospedadores, sincronizando su ciclo de vida con la fenología reproductora de varias especies. *Carnus hemapterus* sincroniza su fenología de emergencia con al menos dos hospedadores con fenología reproductora marcadamente diferente (Abubilla, de fenología más temprana y extensa; Carraca, con fenología mas tardía y corta; Martín Vivaldi et al. 1999; presente tesis). Además, otros trabajos han mostrado sincronización *Carnus*-hospedador en otras especies como Abejaruco (Valera et al. 2003) y Estornino (Liker et al. 2001), por lo que esta respuesta y sincronización con múltiples especies podría estar bastante extendida. Por tanto, a la vista de los resultados obtenidos, los dos primeros puntos antes mencionados (existencia de polifenismo y adaptación de los fenotipos a diversos hábitats/recursos) se cumplen en nuestro sistema. El tercer paso (la predecibilidad y estabilidad de los distintos recursos -hospedadores- que este especie explota), parece ser una característica del sistema mucho más improbable; sabemos por nuestros resultados que la fenología de cría y la presencia y/o supervivencia de los hospedadores es un rasgo con un alto grado de estocasticidad, añadiéndose además el hecho de que las distintas fenologías de cría de los distintos hospedadores no están aisladas unas de otras y que cierto solapamiento es esperable entre especies. Por ejemplo, en nuestra área de estudio principal no es raro encontrar en determinados momentos de la estación de cría, varias especies (por ejemplo Carraca, Abejaruco, Mochuelo y Abubilla) criando a la vez, aunque bien es cierto que la fenología media de emergencia de muchas de estas especies difiere significativamente entre y dentro de localidades. A la vista de este panorama es inevitable pensar en las posibilidades reales de que pueda desencadenarse un proceso de diversificación ecológica y/o especialización en *Carnus hemapterus* asociado a las distintas fenologías de cría de sus hospedadores, puesto que el aislamiento y la barrera temporal que impediría el libre flujo genético entre los distintos linajes asociados difícilmente se producirá. Parece pues más lógico pensar que esta especie se mantendrá como un estratega generalista capaz de hacer frente a la variabilidad e irregularidad que ofrecen sus hospedadores a través de diversos mecanismos de respuesta, como por ejemplo aquellos asociados a su ciclo de vida o comportamiento de selección de hospedador.

Con el fin de buscar alguna evidencia genética que corroborara las predicciones arriba desarrolladas a partir de las observaciones y experimentos en el campo, se realizó un

intenso esfuerzo de muestreo para realizar un análisis molecular de ejemplares de varias localidades de Europa y Canadá. Los resultados obtenidos a partir del análisis del ADN mitocondrial de los distintos especímenes muestran que las poblaciones estudiadas de *Carnus hemapterus* presentan cierto grado de diferenciación genética en las distintas escalas geográficas estudiadas. Diversos eventos históricos como las glaciaciones europeas, así como barreras geográficas como los Pirineos o el estrecho de Bering han podido influir sobre la diferenciación de estos parásitos en alopatría. La distancia geográfica que separa las distintas localidades estudiadas también parece haber influido en la diferenciación y el aislamiento de esta especie, aunque esta correlación entre distancia geográfica y genética se diluye cuando analizamos escalas geográficas menores (por ejemplo poblaciones de la Península Ibérica o Europa Central), muy posiblemente debido que a esta especie posee una gran capacidad para dispersarse y colonizar nuevos hábitats, pero sin la ayuda de transporte pasivo para recorrer grandes distancias en un periodo corto de tiempo, como se sabe que realizan otros parásitos más íntimamente ligados al ciclo de vida de su hospedador (Marshall 1981). Nuestro estudio indica que si existe algún tipo de efecto selectivo de los hospedadores a escala local en esta especie, éste se ve diluido por la mayor importancia de las barreras geográficas o el aislamiento por la distancia. Los resultados de la presente tesis son insuficientes para saber si la divergencia genética encontrada entre poblaciones aisladas es un producto de la selección natural (por ejemplo adaptaciones locales) o si otros procesos no selectivos de diferenciación genética (deriva genética, efectos fundadores o cuellos de botella) han sido los responsable de provocar las diferencias obtenidas. Sin embargo, el estudio realizado en Tabernas (la única localidad con un número suficiente de moscas provenientes de distintos nidos y hospedadores para hacer un análisis molecular de la divergencia genética en simpatría de esta especie), arroja interesantes conclusiones. *Carnus hemapterus* muestra la máxima divergencia genética (aunque con valores de diferenciación moderados) cuando las moscas provenientes de distintos nidos y hospedadores son agrupadas por la fecha de emergencia, lo que sugiere que existe una barrera temporal que impide el libre flujo genético y contacto entre los individuos muestreados. Esto implica que la emergencia alocrónica de *Carnus hemapterus* puede tener algún efecto sobre la diversificación de esta especie a nivel local. Curiosamente, está asociación desaparece cuando los individuos son agrupados por la especie hospedadora, posiblemente debido a la alta variabilidad y al solapamiento parcial

de la fenología de cría de las especies hospedadoras estudiadas en nuestra área de estudio. Por tanto, con la metodología utilizada en la presente tesis no encontramos evidencias de un proceso de diversificación selectiva ecológica (Rundle & Nossil 2005) asociada a los distintos hospedadores. Sin embargo, el hecho de encontrar cierta diferenciación genética asociada a la fenología de emergencia de *Carnus hemapterus* implica que la emergencia alocrónica y sincronización parásito-hospedador encontrada no se debe únicamente a la plasticidad fenotípica total propia de un generalista extremo, en el que un mismo individuo puede responder fácilmente a cambios entre o dentro de años y ajustar su ciclo de vida y fenología de emergencia al hospedador que parasita en cada momento. Los resultados moleculares de este trabajo sugieren, por tanto, que dentro de una misma localidad podrían existir ciertas cepas en las que se ha fijado una longitud del periodo de diapausa o sensibilidad térmica determinadas para emerger en distintos periodos de la estación de cría de su comunidad aviar de hospedadores. Esta fijación de determinados rasgos de la diapausa en distintos hábitats es conocida sobre todo en especies adaptadas a cambios ambientales asociados a clinas latitudinales o altitudinales (ver ejemplos en Tauber et al. 1986, West-Eberhard 2003). También se conocen algunos casos de variación del ciclo de vida y longitud de la diapausa en simpatría (Coyne & Orr 2004); aunque estos últimos son mucho menos abundantes en la literatura. A la vista de los resultados y del conocimiento de este sistema, sugerimos que la diferenciación encontrada en simpatría podría responder a un modelo de diversificación análogo a un proceso de especiación parápatrica (Coyne & Orr 2004) en el que pueda existir divergencia genética siguiendo un eje clinal, dentro de un rango continuo de especies, poblaciones o linajes, pero principalmente entre los extremos de la distribución más que entre las poblaciones adyacentes (Barton & Hewitt 1981, 1985). En nuestro caso el eje clinal de cambio no sería espacial, sino temporal, generado por la distribución continua y solapada de las fenologías de cría de los distintos hospedadores. Esto implica que las moscas de emergencia tardía y emergencia temprana se aparearán y contactarán entre sí con una probabilidad mucho menor que aquellas moscas emergiendo en momentos adyacentes en el tiempo. En este caso, la barrera temporal sería análoga a la barrera física de los procesos alopátricos, una barrera extrínseca al parásito, diferente de las barreras intrínsecas (barreras selectivas provocadas por el control del genotipo sobre la probabilidad de apareamiento y contacto entre dos individuos) que establecen los modelos de especiación simpátrica (Mina & Kondrashov 1986). En resumen, con nuestro trabajo

hemos podido demostrar que diferentes barreras geográficas y eventos históricos han modelado la diversificación y la estructura genética de *Carnus hemapterus*. No hemos podido detectar una diversificación selectiva asociada a los diversos hospedadores de este parásito generalista, debido muy probablemente a: (1) la impredecibilidad, variabilidad y solapamiento de las fenologías de cría de los distintos hospedadores a los que parasita; y (2) a las diversas adaptaciones y estrategias plásticas de *Carnus hemapterus* para solventar esta variabilidad en la disponibilidad de sus hospedadores, que incrementan el flujo genético y mezcla entre los grupos asociados a cada hospedador. Sin embargo de forma indirecta, a través de la barrera externa temporal impuesta entre los extremos de la fenología de cría de la comunidad aviar en nuestra área de estudio, detectamos cierta diferenciación genética.

La presente tesis ha considerado el estudio de rasgos biológicos básicos de *Carnus hemapterus* como paso previo para conocer la potencialidad de diversificación y diferenciación genética en este sistema generalista. El estudio de los procesos ecológicos y evolutivos que rigen a los organismos pasa inevitablemente por tener un profundo conocimiento de la historia natural de las especies implicadas en cada investigación. El extenso conocimiento previo de los organismos modelo que se usan tanto en campo como en laboratorio es fundamental para desarrollar hipótesis fundadas sobre los procesos y factores que influyen sobre una especie determinada. Con esta idea en mente, entre otras, ha sido concebida y desarrollada esta tesis: la investigación sobre cuestiones básicas acerca de la biología de *Carnus hemapterus* puede ayudarnos al avance del conocimiento ecológico y evolutivo de los procesos de diversificación y especiación en parásitos así como a desentrañar el cajón de sastre en el que hoy en día se mantienen las denominadas estrategias generalistas. Como ya nos alerta West-Eberhard en su fantástico y enriquecedor trabajo "Developmental plasticity & Evolution": "*Although this is not always recognized, the generalist category is actually composed of a continuum between two importantly different patterns –true generalists and polyspecialists*".

Creemos que nuestro trabajo presenta muchas limitaciones debido al desconocimiento de rasgos básicos sobre el ciclo de vida y estrategias vitales de este parásito, pero supone un avance que alienta a seguir hacia delante en el desarrollo del conocimiento de este sistema. Aunque el uso de este sistema generalista como modelo de investigación en interacciones ecológicas antagonistas sea bastante prometedor, serán

necesarios trabajos adicionales focalizados en otros aspectos básicos de este sistema (por ejemplo, conocimiento profundo de su ciclo de vida y reproducción, capacidad de dispersión espacial a escala local, comportamiento de detección y búsqueda de hospedadores, factores que afectan a su eficacia biológica, etc.…). También será indispensable ampliar alguno de los resultados de esta tesis (uso de marcadores moleculares adicionales como microsatélites, AFLP's o marcadores nucleares, incremento del esfuerzo de muestreo en poblaciones simpátricas y alopátricas de este parásito, experimentos de selección de hospedadores a escala interespecífica, series temporales más largas de sincronía parásito-hospedador en diversas poblaciones y cómo se relacionan éstas con diversos parámetros climatológicos y con la dinámica de las poblaciones de las especies hospedadores, etc.). Una vez hecho esto estaremos en disposición de seguir utilizando este sistema para avanzar en el conocimiento de la ecología evolutiva de parásitos, y podremos determinar con precisión el aporte y utilidad de este fascinante sistema en el avance del conocimiento de la ecología evolutiva de parásitos.

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Conclusiones

1. *Carnus hemapterus* selecciona preferentemente pollos con una mayor respuesta inmune y no emplumados, en detrimento de criterios de selección como el tamaño o la condición corporal. No obstante, la preferencia por pollos más sanos (mayor respuesta inmune) sólo se mantiene cuando la densidad de parásitos dentro del nido es alta (mayor competencia intraespecífica) y la condición física de la nidada es baja (mayor probabilidad de mortalidad dentro del nido). Esto sugiere que esta especie posee una plasticidad en los criterios de selección de los hospedadores más adecuados y que para completar su ciclo de vida con garantías prefiere hospedadores persistentes en el tiempo más que la calidad nutricional o el tamaño corporal de éstos.

2. Nuestros datos confirman la existencia de diapausa prolongada en *Carnus hemapterus.* Esta forma de dispersión temporal puede haber surgido como respuesta a la fluctuación e impredecibilidad de sus hospedadores y/o hábitat, alzándose como una estrategia alternativa a la dispersión espacial en esta especie.

3. El estudio de marcadores mitocondriales revela procesos demográficos de expansión continua en las poblaciones de la Península Ibérica y Francia y Norte de Italia, así como falta clara de evidencias de colonización a larga distancia. Esto sugiere una alta capacidad de dispersión hacia nuevos hábitats por parte de esta especie, aunque esta capacidad se basaría en sus propios medios y no en el uso de transporte pasivo mediante hospedadores, vectores o vehículos foréticos.

4. El efecto de un cambio de hospedador de fenología de cría más temprana puede adelantar parcialmente la fenología de emergencia en *Carnus hemapterus* a través de la temperatura de incubación del nuevo hospedador.

5. El estudio de la fenología de emergencia de *Carnus hemapterus* procedentes de distintas especies de aves revela una emergencia diferencial asociada a la fenología de cría de cada especie de hospedador estudiada. Esta emergencia diferencial resulta en un patrón alocrónico que podría ser un importante mecanismo de aislamiento temporal, potencialmente desencadenante de un proceso de diversificación en esta especie generalista.

6. El estudio de la sincronización parásito-hospedador a nivel intraespecífico en el sistema *Carnus*-carraca revela unos niveles de sincronía bajos y fluctuantes a lo largo de 3 años de estudio. Esta baja sincronización se produce fundamentalmente por la emergencia de un gran número de parásitos antes de la aparición de los pollos de carraca y una mayor variación interanual en la fenología de cría del parásito que en la del hospedador.

7. La baja predecibilidad (tasa de reutilización del nido y repetibilidad de fenología de cría) del hospedador puede explicar la baja sincronía parásito-hospedador a nivel intraespecífico. No obstante, el tiempo de reproducción de los hospedadores influyó sobre la tasa de sincronización parásito-hospedador, lo que indica que la variación de la fenología de cría del hospedador tiene cierta influencia sobre la fenología de emergencia del parásito. Otros mecanismos no estudiados distintos de la sincronización parásito-hospedador (dispersión espacial, explotación de otras especies) podrían ser importantes para asegurar el éxito infectivo y reproductor en este ectoparásito dentro del marco temporal estudiado.

8. Los resultados de los experimentos de cambio de hospedador y de translocación de parásitos, así como la variabilidad de la fenología de emergencia observada en los trabajos de sincronización parásito-hospedador de la presente tesis, confirman que el desarrollo y terminación de la diapausa en este parásito son rasgos que pueden ser fácilmente modificados como respuesta a cambios ambientales y/o bióticos

(relacionados con la temperatura del hospedador) de su entorno más cercano.

9. El análisis mitocondrial muestra una acusada estructura filogeográfica de las poblaciones de *Carnus hemapterus* a nivel continental. Diversos eventos geológicos durante el Pleistoceno, como las glaciaciones en Europa o la apertura del estrecho de Bering entre Eurasia y Norteamérica, pueden haber tenido un papel relevante en la conformación de la estructura y diferenciación genética de este parásito a lo largo del área de distribución estudiada. Estos resultados, junto con la correlación positiva entre distancia geográfica y genética encontrada, indican que esta especie es bastante sensible a las barreras físicas y al aislamiento de sus poblaciones por la distancia, al menos cuando estudiamos escalas geográficas amplias.

10. *Carnus hemapterus* presenta una cierta diferenciación genética en simpatría asociada al periodo de emergencia del imago, pero independiente del hospedador parasitado, lo que sugiere la existencia de una barrera temporal que impide el libre flujo genético en esta especie.


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