Mecanisme d'acció de l'hormona juvenil en la metamorfosi dels insectes

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Tesi doctoral realitzada per

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Mecanisme d'acció de l'hormona juvenil en la metamorfosi dels insectes

Memòria presentada per Jesús Lozano Fernàndez per optar al grau de doctor per la Universitat de Barcelona

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Sobre la portada:

Com es transforma una eruga en papallona? Aquesta pregunta, que resumeix la meravella i el misteri de la metamorfosi, ha constituït un enigma intemporal, que ha fascinat l'home des dels temps més remots. Actualment coneixem amb més profunditat quins són els mecanismes que la desencadenen tot i que encara queden algunes preguntes per resoldre. Quin és el factor que determina el moment òptim d'iniciar la metamorfosi? La resposta la trobem en el factor de transcripció Krüppel-homolog 1, el qual està implicat en la via de senyalització de l'hormona juvenil, hormona que impedeix la metamorfosi. Krüppel-homolog 1 s'expressa durant les fases juvenils de l'insecte i deixa d'expressar-se en l'última fase juvenil, quan desapareix l'hormona juvenil i es desencadena la metamorfosi. Si eliminem prematurament l'expressió d'aquest gen a la panerola *Blattella germanica* provoquem una metamorfosi precoç, i l'insecte es salta una fase juvenil. L'adult precoç (imatge de la dreta) sempre és més petit que l'adult normal (a l'esquerra).

Agraïments

Durant els anys de realització d'aquesta tesi doctoral he tingut l'oportunitat i plaer de conèixer a grans companys de professió i millors persones. Vull fer un esment i agrair el seu suport durant aquests darrers anys. En primer terme vull agrair especialment al meu supervisor de tesi, Xavier Bellés, l'oportunitat de treballar amb ell i endinsar-me en el coneixement de la història natural. Gràcies per la confiança dipositada en mi des d'un inici i per ser tan bon mestre, espero haver-te correspost com a deixeble. La segona persona a la qual vull agrair tot el seu suport és a M. Dolors Piulachs; has sigut la meva mentora i consellera des del primer dia de treball. Tens en compte les necessitats de cada persona, per això no només ets una investigadora de primer nivell sinó també una gran companya, gràcies. No em vull oblidar d'agraïr a M. Àngel Arnedo la possibilitat que em va oferir d'endinsar-me en el món científic i l'ajuda que m'ha ofert durant aquests anys.

A les persones que han fet de la meva estança al laboratori una segona llar: Mercedes Rubio i Alba Herraiz, les estudiants de doctorat de la meva generació amb les que he compartit tant els bons moments com els no tan bons, deguts a moments de treball molt intens. A les veteranes de lab P64, Erica Donato Tanaka, Paula Irles i Eva Gómez per ser tan acollidores i ser unes grans amigues. A Jia-Hsin Huang, un magnífic científic i un amic Taiwanès per a tota la vida. Al sector masculí del laboratori: Raúl Montañez, Aníbal de Horna, Carlos Vásquez, Guillem Ylla, Moysés E. Neto i Jelle Caers, me n'alegro d'haver-vos conegut i haver gaudit de tants bons moments. I no vull oblidar-me de les darreres incorporacions al laboratori, Carolina Gonçalves, Ana Fernández, Nashwa Elshaer i Elena Navas, m'heu fet costat i m'heu donat moments d'alegria en els moments d'escriptura del present treball. A Cristina Olivella, per la seva comprensió i amabilitat.

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Contingut

Glossari i Abreviatures

20E:	20-Hidroxiecdisona. Forma activa dels ecdisteroides com a hormona de muda més comuna en insectes, produïda a partir d'Ecdisona en diferents teixits perifèrics.
Al.latectomia:	Extracció quirúrgica dels *corpora allata*.
Ametàbol:	Tipus de desenvolupament directe, on l'individu juvenil té característiques gairebé idèntiques a l'adult, a excepció dels òrgans sexuals.
Antimetamòrfic:	Que inhibeix la metamorfosi.
Apterigots:	Sense ales.
bHLH-PAS:	Família de proteïnes que contenen un domini *basic* i *helix-loop-helix*, seguit de dos dominis descrits inicialment en les proteïnes Period, ARNT i Single-minded.
BR-C:	Broad-Complex. Grup de factors de transcripció dependents de 20E i d'HJ.
Corpora allata:	Glàndules endocrines dels insectes que sintetitzen i secreten l'HJ.
dsRNA:	RNA de doble cadena.
DNA:	Àcid desoxiribonucleic.
E:	Ecdisona. Producte sintetitzat per les glàndules protoràciques, precursor de la 20E. També, terme usat genèricament per a les hormones esteroïdees.
E75:	Ecdysone induced protein 75. Receptor nuclear.
E93:	Factor de transcripció que determina la metamorfosi.
EcR:	Receptor d'Ecdisona, de la superfamília dels receptors nuclears.
Estadi:	Període entre dues mudes de les fases juvenils postembrionàries.
Glàndula protoràcica:	Glàndula que produeix l'ecdisona.
Hemimetabolia:	Tipus de desenvolupament postembrionàri en el qual les nimfes són morfològicament similars als adults, excepte en alguns caràcters exclusivament imaginals, com les ales o la genitalia. També es pot descriure com metamorfosi gradual.

HJ:	Hormona Juvenil. Terme genèric per descriure les HJ actives, sintetitzades pels *corpora allata*.
Holometabolia:	Tipus de desenvolupament postembrionàri en el qual les larves són morfològicament clarament diferents respecte als adults, la qual cosa fa necessària la fase de pupa, intermèdia entre la larva i l'adult. També es pot descriure com metamorfosi sobtada.
ILP-1:	Insulin-Like-Peptide 1.
Imago:	Estadi adult de l'insecte.
Indel:	Inserció/deleció de bases en una secuència.
Isoforma:	Variants de producte gènic, usualment produïdes per "splicing" diferencial.
kJHRE:	Element de resposta a HJ.
Kr-h1:	Krüppel-homolog 1. Factor de transcripció.
Larva:	Fase juvenil dels insectes holometàbols.MEKRE93: Methoprene tolerant - Krüppel homolog 1 - E93. Cascada de senyalització de l'HJ per al control de la metamorfosi, universal en insectes..
Met:	Methoprene-tolerant.
miRNA:	MicroRNA. RNA petit no codificant que regula nivells de mRNA a escala post-transcripcional.
mRNA:	RNA missatger.
Muda:	Síntesi periòdica de cutícula i reemplaçament de l'antiga per permetre el creixement de l'insecte.
Muda imaginal:	Muda que conduirà a la formació de l'insecte adult.
Nimfa:	Fase juvenil dels insectes hemimetàbols.
ORF:	*Open Reading Frame*.
Pterigots:	Clade monofilètic dels insectes alats.
RISC:	*RNA-induced silencing complex*. Complex proteic encarregat de transportar RNA petits al mRNA diana.
RNA:	Àcid ribonucleic.
RNAi:	RNA d'interferència. Reducció de nivells d'un mRNA, mitjançant l'administració d'un RNA de doble-cadena amb una seqüència complementària a la de l'mRNA del gen que volem interferir.
siRNA:	RNAs petits d'interferència.

Tai/SRC/FISC:	Factor de transcripció bHLH-PAS anomenat amb aquestes nomenclatures sinònimes: Taiman (Tai), Steroid Receptor Coactivator (SRC) i βFtz-F1 Interacting Steroid receptor Coactivator (FISC)
Usp:	Ultraspiracle. Receptor nuclear.
UTR:	Regió de l'mRNA que no es tradueix.

1. Introducció general

1. Introducció general

La metamorfosi és un procés de canvi morfològic radical que succeeix en un període específic durant el desenvolupament postembrionari de diverses espècies animals, tals com amfibis o insectes. Els primers estudis sistemàtics sobre la metamorfosi dels insectes van ser duts a terme per entomòlegs del Renaixement, que van establir que els canvis postembrionaris són més espectaculars en insectes com les papallones, els escarabats i les mosques, els quals pateixen una transformació morfològica sobtada, de larva a pupa i a adult, en un fenomen conegut com holometabolia. Altres insectes, com els saltamartins, llagostes i paneroles, també fan una metamorfosi, però és progressiva, des de la darrera fase nimfal a l'adult, tot i que el canvi de forma no és tan radical donat que les nimfes s'assemblen als adults. No obstant, pateixen canvis metamòrfics qualitatius, com la formació d'ales i de la genitalia externa, en un fenomen conegut com hemimetabolia. La metamorfosi dels insectes sembla doncs un model ideal per portar a terme estudis on es vulgui esbrinar com es coordinen els senyals del desenvolupament cel·lular per dur a terme morfogènesis complexes a nivell genètic i de teixits. La comparació entre els mecanismes de regulació hormonal a escala molecular que succeeixen en les transicions no metamòrfiques i metamòrfiques poden donar resposta a les preguntes subjacents a aquest procés.

a) Origen de la metamorfosi

La metamorfosi ha sigut una innovació clau en l'evolució d'insectes com reflecteix que la major part de la biodiversitat existent a la Terra es composta d'insectes metamòrfics, amb aproximadament 1 milió d'espècies descrites, i 10-30 milions d'espècies encara per descobrir (Grimaldi and Engel 2005, Hammond 1992). La metamorfosi es un tret comú a tots les insectes alats recents (Pterigots). Durant la metamorfosi, la majoria d'òrgans passen per un canvi de forma o estructura marcat i abrupte, la mida de les ales s'incrementa dramàticament i es forma l'articulació de l'ala (Kukalová-Peck 1991, Sehnal *et al.* 1996). Amb l'excepció dels efemeròpters, la metamorfosi té lloc en la transició d'estadis juvenils, anomenats nimfals a les espècies hemimetàboles i larvaris a les espècies holometàboles, a adults. Les restes fòssils permeten saber que les formes més antigues d'insectes eren apterigotes i mostraven un desenvolupament directe (ametàbol) i que posteriorment van aparèixer els insectes metamòrfics, procés el qual els va propiciar una radiació ràpida (Carpenter 1992, Kukalova-Peck 1978). Els primers

indicis sobre l'origen de la metamorfosi els podem trobar al registre fòssil. Els fòssils suggereixen que la metamorfosi ha evolucionat com a conseqüència necessària producte de l'emergència de les ales (Kukalová-Peck 1978, Kukalová-Peck 1983, Kukalová-Peck 1991). Els primers insectes alats van aparèixer al Paleozoic. Els estrats del Carbonífer, fa uns 350 milions d'anys, aporten ja una diversitat notable d'espècies amb ales funcionals. En les espècies hemimetàboles actuals el desenvolupament de les ales en les nimfes és retardat, els esbossos alars són immòbils, firmament fusionats amb els terguits i mancats d'articulació. No obstant, els insectes del Paleozoic desenvolupaven gradualment les ales, les quals estaven articulades i probablement permetien el vol als estadis juvenils (Rasnitsyn 1981, Kukalová-Peck 1991). Possiblement aquestes ales d'insectes ancestrals fossin vulnerables i poc útils per al vol. Pels individus juvenils això suposaria un inconvenient i la pressió selectiva possiblement afavorí aquells individus que endarrerien l'emergència de l'ala fins a estadis més avançats del desenvolupament. Aquesta supressió esdevindria tan avançada que caldria un canvi sobtat metamòrfic per restaurar les ales a la seva condició funcional. Cap a finals del Carbonífer i inicis del Permià, fa uns 300 milions d'anys, pràcticament tots els pterigots presentaven un desenvolupament postembrionari dividit en fases nimfals, transicions metamòrfiques, i una fase adulta, és a dir, s'havia produït la hemimetabolia, o metamorfosi gradual. Els primers fòssils d'insectes que poden considerar holometàbols apareixen en estrats del Permià, fa uns 280 milions d'anys (Kristensen 1975, Kristensen 1999). El registre fòssil i la reconstrucció filogenètica ens suggereixen que la innovació de la holometabolia, o metamorfosi sobtada, només s'hauria produït una vegada.

b) Tipus de metamorfosi

En els insectes moderns, no només les ales sinó altres òrgans, com la genitalia i la cutícula, porten a terme la metamorfosi. Des de l'emergència dels primers insectes metamòrfics, han tingut lloc diversos tipus de desenvolupament post-embrionari i metamorfosi revisat a Sehnal *et al*. (1996) (Figura 1.1).

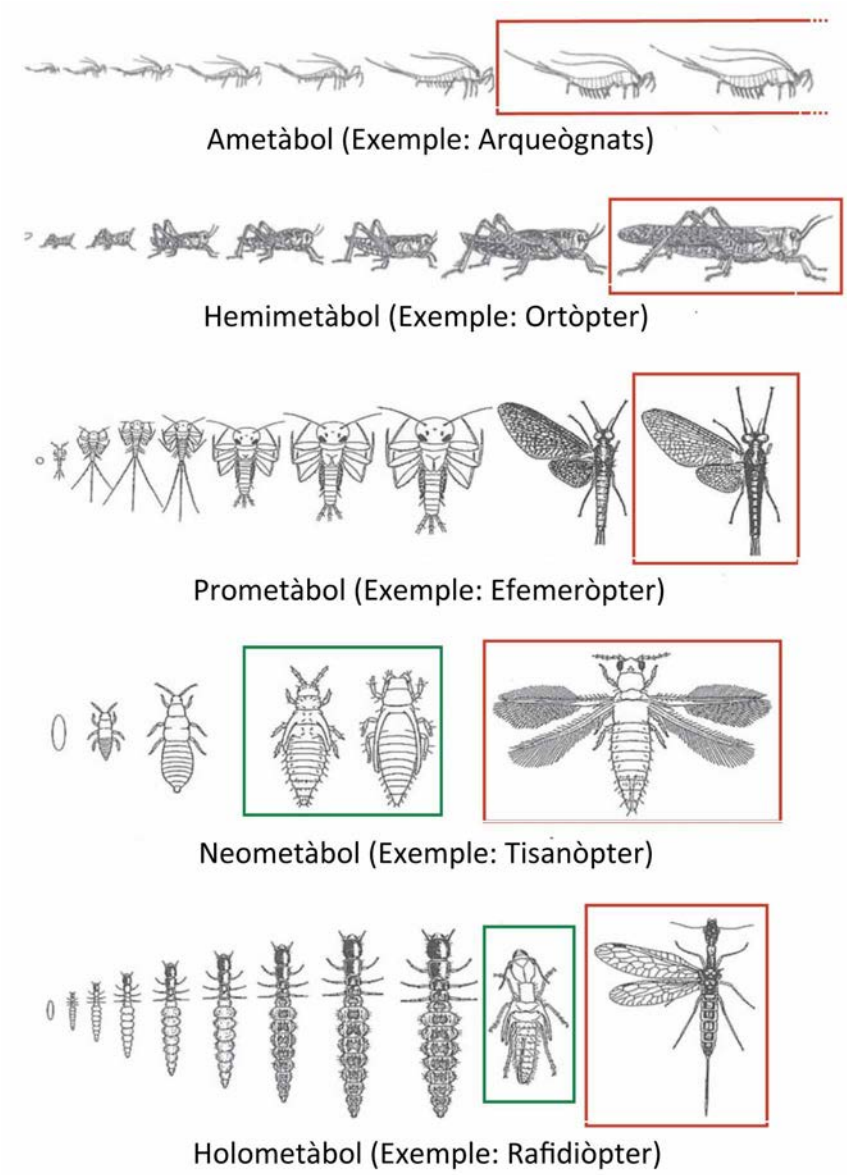

Figura 1.1: Principals tipus de metamorfosi en els insectes. Ametàbola (sense metamorfosi), hemimetàbola i holometàbola, i les categories internes hemimetàboles, prometàbola i neometàbola. Els estadis quiescents estan marcats amb un requadre verd. Els adults estan marcats amb un requadre vermell. En els insectes ametàbols, el requadre vermell està obert degut a que l'adult continua mudant. Modificat de Sehnal *et al*. 1996.

Les efímeres (Efemeròpters) són l'únic grup d'insectes vivents que conserven dues mudes post-metamòrfiques (alades): un subadult no-reproductiu i l'adult. En la resta d'insectes, la metamorfosi es seguida d'un sol estadi adult. En els insectes amb metamorfosi hemimetàbola, manifestat per exemple en els Hemípters, les paneroles (Blatoïdeus) i saltamartins (Ortòpters), els rudiments d'ales externes (esbossos alars) comencen a aparèixer des de certa fase nimfal o com a molt tard a la darrera fase nimfal. Les ales funcionals, genitalia i altres estructures imaginals es desenvolupen totalment durant la metamorfosi al final de la darrera fase nimfal. D'acord amb els estudis filogenètics més recents els hemimetàbols formarien un grup parafilètic heterogeni

(Letsch *et al* 2012). Un dels clades d'aquest grup, els paraneòpters, serien el grup germà dels endopterigots (=holometàbols) (Figura 1.2). Dins dels paraneòpters s'inclouen diversos grups amb casos de neometabolia, és a dir, un tipus de desenvolupament postembrionari en què s'intercalen entre 1 i 3 estadis quiescents que s'assemblen a l'estat de pupa dels holometàbols (Sehnal *et al*. 1996) (Figura 1.1). Amb la qual cosa, el més parsimoniós és pensar que els holometàbols s'haurien originat a partir d'ancestres hemimetàbols, i el cas de la neometabolia ens suggereix que la pupa podria no haver estat una innovació exclusiva dels holometàbols. L'estratègia de metamorfosi recent més estesa i millor estudiada es l'holometàbola. A diferència dels hemimetàbols, els insectes holometàbols(=endopterigots, = oligoneòpters) (Kristensen 1999) constitueixen una unitat monofilètica (Figura 1.2). Els insectes holometàbols presenten diferents estadis larvaris, seguits d'una estadi pupal, que és l'estadi juvenil final, quiescent, i de l'adult. També han aconseguit que els cicles de vida siguin molt curts. Les diferències considerables entre les formes larvàries i adultes permeten les etapes que exploten diferents hàbitats i fonts d'aliments, i també permeten l'adaptació extrema d'una etapa per a una funció particular, tal com la dispersió. Les larves difereixen morfològicament dels adults i no presenten ni primordis alars externs ni genitalia, els quals apareixen a l'estadi pupal. En molts casos, els primordis d'òrgans adults com les ales es troben a l'interior de la larva en forma d'estructures indiferenciades, o, en espècies més modificades, en forma dels anomenats discos imaginals, el desenvolupament complet i exteriorització dels quals es produeix bruscament en la transició metamòrfica (Nijhout 1998).

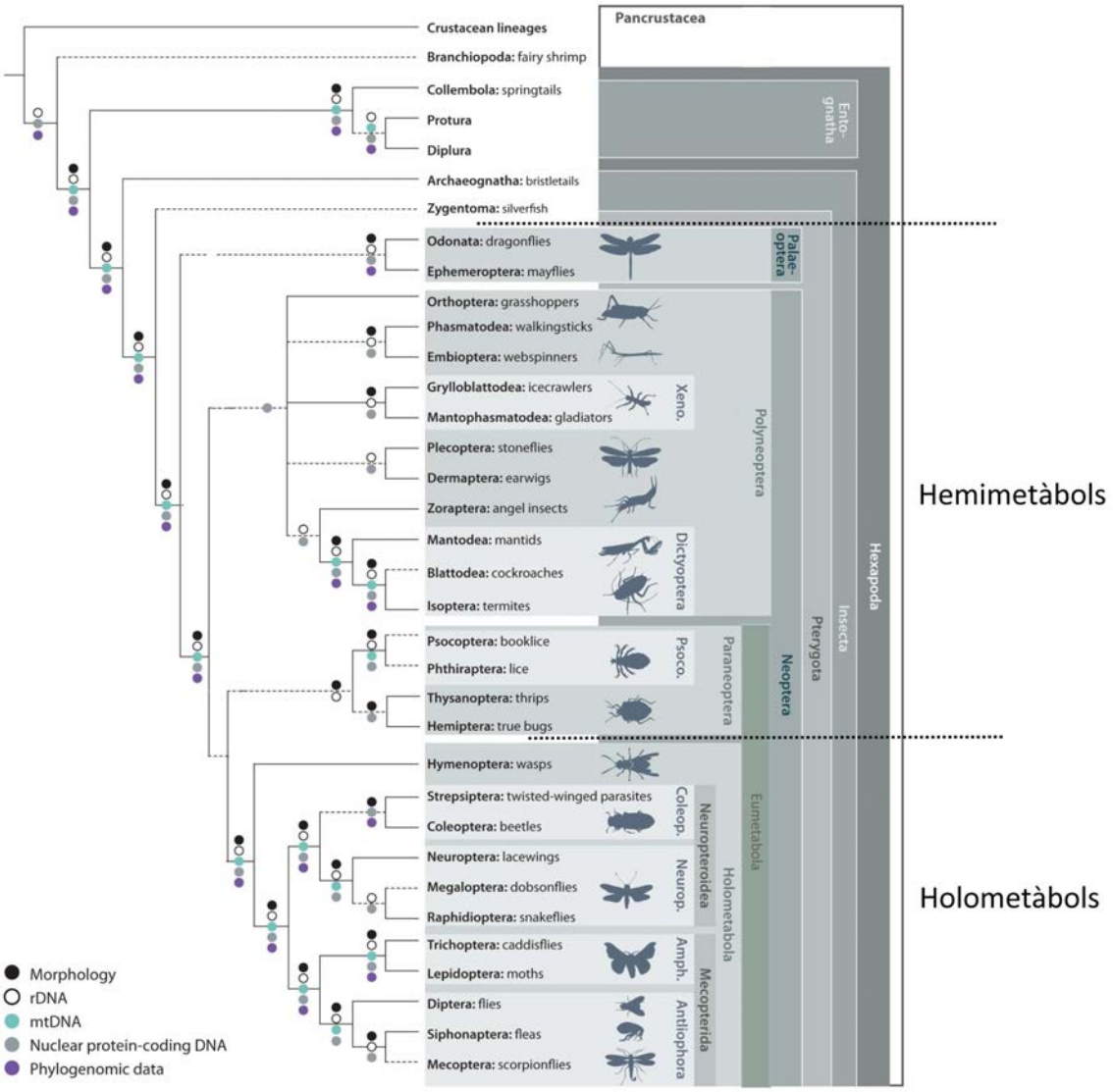

Figura 1.2: Arbre que representa les relacions filogenètiques dels hexàpodes. Als nodes s'indica amb cercles de colors el tipus dades que donen suport al clade corresponent (morfologia; rDNA: inferència filogenètica amb DNA ribosòmic; mtDNA: inferència filogenètica amb DNA mitocondrial; inferència filogenètica amb DNA nuclear codificant de proteïna; inferència filogenètica amb dades filogenòmiques). La reconstrucció filogenètica està basada en Trautwein *et al*. (2011) i Letsch *et al*. (2012).

c) Teories sobre l'origen i la evolució de la metamorfosi

La metamorfosi va evolucionar d'hemimetàbola a holometàbola, i aquesta última innovació va ser la que tingué més èxit pel fet que més del 80% dels insectes actuals són espècies holometàboles (inclosos els "quatre grans" ordres: lepidòpters, coleòpters, dípters i himenòpters) (Grimaldi and Engel 2005, Hammond 1992) (Figura 1.3). Per tant, explicar aquesta transició evolutiva pot aportar una nova mirada a explicar com es va originar aquesta increïble biodiversitat (Truman and Riddiford 2002). S'han proposat un nombre de teories per explicar la transició evolutiva de la metamorfosi

hemimetàbola a holometàbola (Belles 2011, Truman and Riddiford 1999). Una teoria clàssica originalment argumentada per Lubbock (1890), i posteriorment formalitzada per Berlese (1913), proposa que les larves de les espècies holometàboles sorgeixen per "desembrionització", de manera que la larva és essencialment una mena de embrió vermiforme que fa vida lliure i s'alimenta fora de l'ou. La teoria de la "desembrionització" va ser ressuscitada i reforçada amb dades endocrines modernes per Truman i Riddiford (1999), on proposaven que la larva holometàbola correspon a l'última etapa embrionària de les espècies hemimetàboles i la pupa holometàbola és homòloga a tot el conjunt de tots els estadis nimfals de les espècies hemimetàboles. La hipòtesi de Truman i Riddiford es basa principalment en dades morfològiques i endocrines (Truman and Riddiford 1999, Truman and Riddiford 2002, Riddiford 2008). Sota aquest enfocament, la seva hipòtesi original postulava que els hemimetàbols eclosionarien després de tres 'mudes' embrionàries, donant una nimfa semblant a la d'adults, mentre que els holometàbols eclosionarien després de dues 'mudes' embrionàries, donant lloc a una larva vermiforme, molt diferent de l'adult. La hipòtesi és atractiva tant per la seva senzillesa com pel seu poder explicatiu. No obstant això, els estudis ultraestructurals basats en un nombre d'espècies representants d'una diversitat d'ordres d'insectes (efemeròpters, odonats, plecòpters, neuròpters, coleòpters, lepidòpters, dípters i mecòpters) indiquen que l'embrió de totes les espècies produeix tres deposicions de cutícula (tres 'mudes); (Konopova and Zrzavý 2005) i eclosionen en un estadi homòleg. Els dípters ciclòrrafs (els dípters més modificats, com *D. melanogaster* en la qual s'han basat tradicionalment la majoria dels estudis experimentals) seria una excepció, atès que es mostren dues cutícules embrionàries, probablement pel fet que la tercera ha estat perduda secundàriament (Konopova and Zrzavý 2005). L'altra teoria contraposada postula que els insectes eclosionen tots en un estat equivalent, on el desenvolupament postembrionari seria equivalent a hemimetàbols i holometàbols i la fase de pupa dels holometàbols equivaldria a la darrera fase nimfal dels hemimetàbols (Hinton 1948, Sehnal *et al*. 1996).

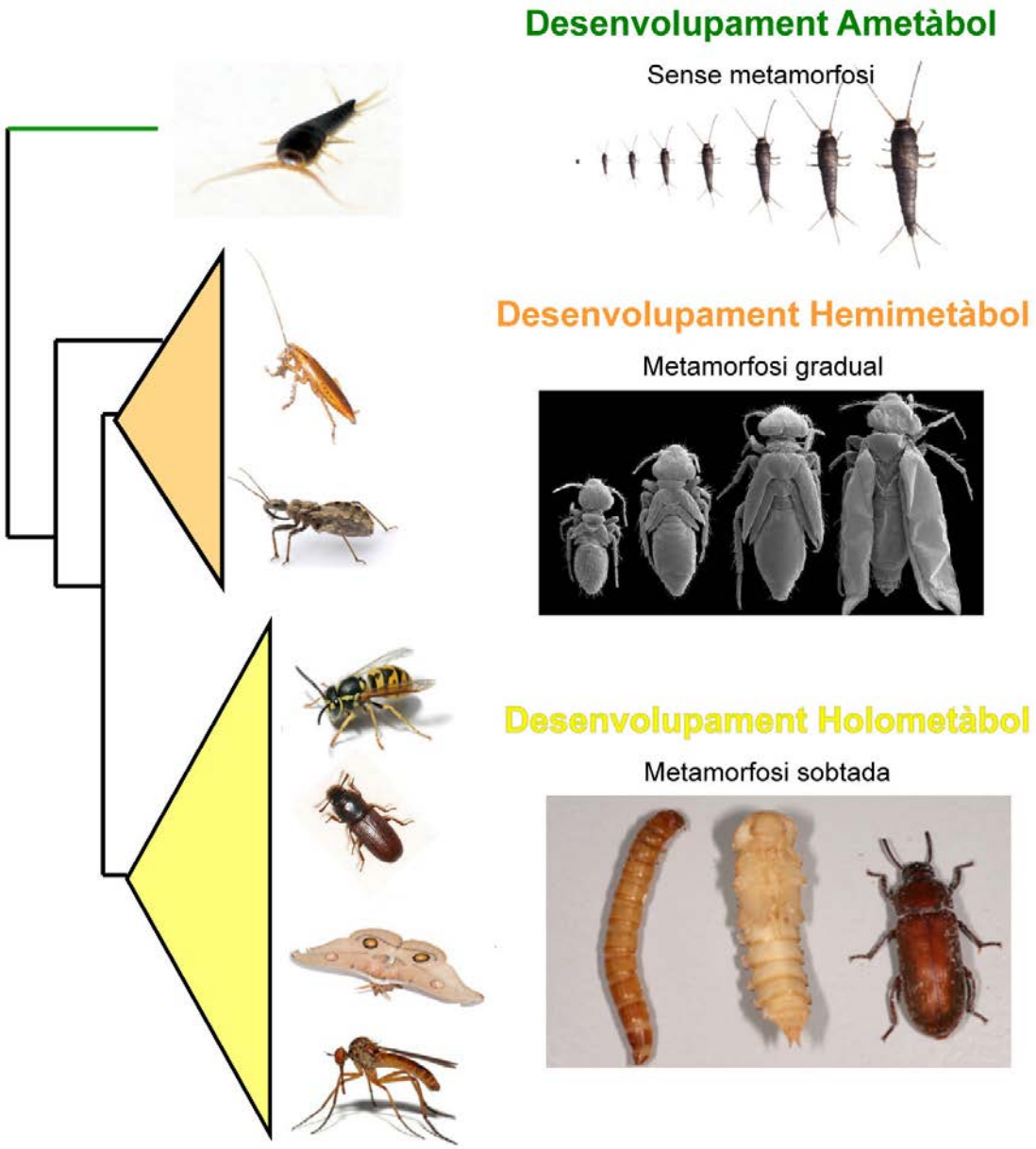

Figura 1.3: Tipus de desenvolupament postembrionari en insectes i cladograma representant les relacions de filogenètiques. La categoria més basal correspon als insectes Ametàbols (sense metamorfosi). Els insectes metamòrfics presenten desenvolupament hemimetàbol (metamorfosi gradual) i holometàbol (metamorfosi sobtada). A la dreta, imatge del cicle vital d'un insecte representant de cada categoria.

d) Control endocrí de la metamorfosi

La informació fonamental sobre la base endocrina de la metamorfosi va ser establerta per Vincent B. Wigglesworth a partir de la dècada de 1930 (Wigglesworth 1934, Wigglesworth 1954, Sehnal *et al*. 1996). Els insectes, en virtut de tenir un exosquelet rígid, han de mudar per créixer, i la muda és la base de la metamorfosi, ja que les transformacions es produeixen a través de les mudes. La metamorfosi dels insectes és

regulada per l'acció de dues hormones, l'esteroide 20-hidroxiecdisona (20E) o hormona de la muda i el sesquiterpenoid Hormona Juvenil (HJ) (Nijhout 1998). Mentre que la 20E indueix la metamorfosi, l'HJ reprimeix la metamorfosi. Els ecdisteroides larvaris són sintetitzats a les glàndules protoràciques (Gilbert 2004), i en resposta a senyals del cervell, aquestes glàndules secreten ecdisona, una prohormona relativament inactiva que es convertida a la activa 20E al cos gras i a les cèl·lules epidèrmiques (Nijhout 1998). A més d'actuar en la metamorfosi, la 20E és requerida per les mudes larvals periòdiques (secreció d'una cutícula nova i ècdisi de l'antiga) i per això els nivells de 20E augmenten varies vegades durant el desenvolupament larvari. La funció prometamòrfica de la 20E és bloquejada en estadis primerencs juvenils degut a la presència d'HJ, un producte de les glàndules retrocerebrals anomenades *corpora allata*. Després d'arribar a la massa de cos crítica, l'HJ declina i és llavors que la 20E indueeix canvis metamòrfics. La funció antimetamòrfica de l'HJ fou primerament demostrada en l'hemimetàbol hemípter *Rhodnius prolixus* (Wigglesworth 1934, Wigglesworth 1936). Quan les larves de la penúltima fase (no-metamòrfica) eren decapitades, per tant els *corpora allata* eren eliminats, alguns dels individus mostraven un desenvolupament parcial de caràcters adults com ales, genitalia, o estructures cuticulars; posteriorment, aquest efecte va ser obtingut també en fases juvenils prèvies (Wigglesworth 1985). El paper essencial de l'HJ d'impedir la metamorfosi precoç ha estat validat en una sèrie d'espècies d'insectes hemimetàbols i holometàbols (Wigglesworth 1954, Wigglesworth 1985, Stall 1986). Els mateixos resultats han estat recentment confirmats per estudis genètics. Un exemple el trobem en els cucs de la seda *Bombyx mori* transgènics que sobre-expressen l'esterasa de l'HJ, un enzim que degrada aquesta hormona, de manera que aquests insectes fan la metamorfosi a pupa i després a adult abans d'arribar a la darrera fase larvària, saltant-se el nombre normal de fases larvàries (Tan *et al*. 2005), a l'igual que els cucs que havien estat al.latectomitzats quirúrgicament (Bounhiol 1938, Fukuda 1944). També s'ha obtingut una pupa precoç a l'escarabat *Tribolium castaneum* després d'interferència d'RNA (RNAi) de l'*O*-metiltransferasa de l'àcid de l'HJ (JHAMT en anglès), un gen que codifica un enzim clau en la biosíntesis d'HJ (Minakuchi *et al*. 2008). L'aplicació d'HJ ectòpica i els seus anàlegs administrats a fases larvàries impedien la transformació adulta i causaven una muda a larva supernumerària. De manera similar, en les pupes de cuc de seda, que normalment presenten nivells baixos d'HJ endògena, l'aplicació ectòpica d'HJ impedia la metamorfosi adulta, i portava als cucs de seda tractats a realitzar una segona fase pupal

(Zhou and Riddiford 2002). La funció de l'HJ és, doncs, mantenir l'estadi juvenil de desenvolupament, l'hormona que manté l'*status quo* (Williams 1959, Williams 1961).

e) Estudis genètics en senyalització hormonal

En els darrers anys, s'ha fet un esforç continuat per entendre la senyalització hormonal que dirigeix el desenvolupament dels insectes a nivell genètic. Un estudi detallat dels puffs trobats en els cromosomes politènics de *Drosphila melanogaster* va permetre explicar com la 20E activava un conjunt de gens de manera seqüencial (Ashburner 1974). Aquest treball, i d'altres posteriors respecte a l'acció molecular de les hormones de la muda, va demostrar el senyal es transdueix a través d'una cascada de factors de transcripció, una vegada que la 20E s'uneix al receptor heterodimeric composat pel receptor d'ecdisona (EcR) i Ultraespiracle (Usp), factors de transcripció que pertanyen a la superfamília dels receptors nuclears. Amb modificacions, el model d'Ashburner és encara acceptat després de més de 30 anys des de la seva formulació. La caracterització molecular dels puffs primerencs van portar a l'identificació de factors de transcripció, com E74, E75 i Broad-Complex (BR-C) (revisat a Thummel 1996, Kozlova and Thummel 2000) o el mateix receptor de 20E, EcR (Koelle *et al*. 1991, Yao *et al*. 1992, Thomas *et al*. 1993). Malgrat el tipus de desenvolupament evolutivament derivat de *D. melanogaster*, aquest model genètic ha proporcionat pistes vitals gràcies al descobriment de gens funcionals durant la metamorfosi que són regulats per HJ, com l'abans esmentat BR-C, Methoprene-tolerant (Met), i Krüppel-homolog 1 (Kr-h1) (Berger and Dubrovsky 2005, Riddiford 2008). Amb els mètodes actuals de genètica funcional reversa i primordialment amb el sorgiment de l'RNA d'interferència (RNAi) (Fire *et al*. 1998), hom té ara la possibilitat d'estudiar la funció d'aquests factors en una gran varietat d'espècies. L'RNAi es basa en seleccionar un gen i estudiar després la seva funció mitjançant l'ús d'una doble cadena d'RNA (dsRNA) complementària al mRNA del gen que es pretén interferir. Aquest dsRNA serà fragmentat en fragments d'uns 21 nucleòtids (siRNAs) per l'enzim Dicer i seguidament deshibridarà a cadena simple. Posteriorment, les cadenes simples seran guiades pel complex RISC a l'mRNA diana, on s'uniran a aquest per complementarietat de bases nucleotídiques. El complex RISC amb el siRNA fragmenta l'mRNA diana molècula i es generen nous fragments de cadena simple que amplificaran l'efecte. Aquesta estratègia és diametralment oposada a la genètica funcional clàssica que es basa en seleccionar una funció i buscar el gen o gens responsables. Aquesta tècnica ha esdevingut un nou referent en l'estudi genètic, ja

que permet estudiar espècies no transformables genèticament, és a dir, nous estudis més enllà de l'espècie *D. melanogaster*, un holometàbol summament modificat. Ara és possible realitzar els estudis genètics sobre l'acció de l'HJ a altres insectes models apropiats.

f) *Blattella germanica*, un bon model experimental per l'estudi de la metamorfosi

Blattella germanica és un insecte exopterigot polineòpter que mostra una transformació morfològica gradual durant els sis estadis nimfals del seu cicle de vida, i és un bon representant dels hemimetàbols menys modificats. La Figura 1.2 mostra la posició basal de *B. germanica* dins del grup dels dictiòpters, en comparació amb altres espècies hemimetàboles, com els hemípters, que estan més emparentades amb les espècies holometàboles. La seva posició filogenètica, i el fet que és especialment sensible al silenciament gènic per RNAi (Belles 2010), han fet de *B. germanica* un model hemimetàbol favorit per estudiar la metamorfosi. D'altra banda, els nivells de 20E i JH s'han descrit a fons per a les etapes premetamòrfiques, metamòrfiques, i adulta (Cruz *et al*. 2003, Pascual *et al*. 1992, Romana *et al*. 1995, Treiblmayr *et al*. 2006) (Figura 1.4). A més, també s'ha estudiat en aquest insecte un nombre important de factors de transcripció encarregats de la transducció del senyal hormonal

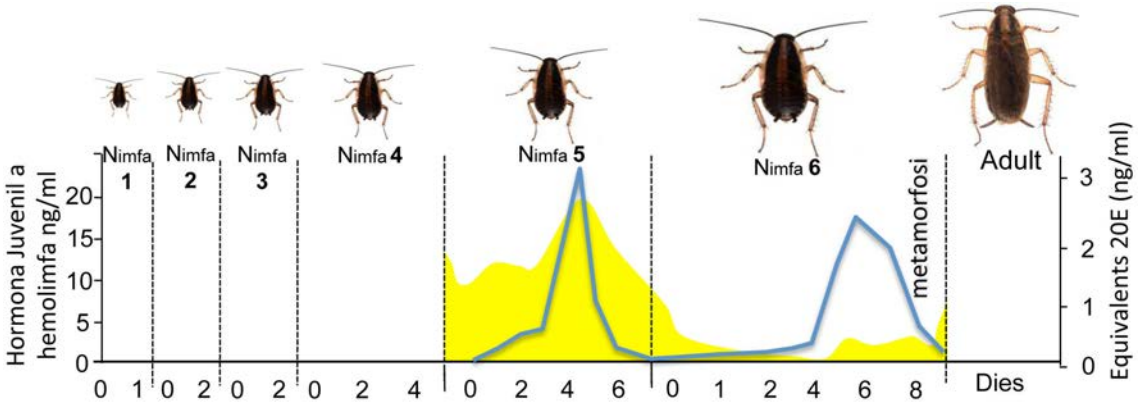

Figura 1.4: Nivells d'HJ i 20E durant les dues últimes fases del desenvolupament postembronari de *Blattella germanica*. Esquema representant les diferents fases nimfals del cicle de vida postembrionari de *B. germanica.* A cinquena i sisena fase nimfal s'indiquen els nivells d'HJ (ombrejat groc) i de 20E (línia blava) i HJ (ombra groga). Dades obtingudes de (Cruz *et al.* 2003 i Treiblmayr *et al*. 2006).

g) Receptor de l'hormona juvenil

Tot i que les proteïnes receptores de la hormona de la muda 20E van ser identificades fa més de 20 anys (Koelle *et al*. 1991, Yao *et al*. 1992, Thomas *et al*. 1993) aquest no ha estat el cas del receptor de l'HJ, el qual ha estat identificat recentment. Com l'ecdisona, l'HJ es una molècula petita i lipofíl·lica capaç de penetrar a la membrana cel·lular fins al nucli, on presumiblement regula la transcripció de gens. S'havia especulat prèviament sobre la possibilitat que el receptor de l'HJ fos el receptor nuclear Usp o bé el factor de transcripció Met (Gilbert *et al*. 2000, Truman and Riddiford 2002, Berger and Dubrovsky 2005, Goodman and Granger 2009, Riddiford 2008). Només en els darrers 5 anys s'ha confirmat que Met és la proteïna encarregada d'actuar com a receptor de l'HJ.

Methoprene-tolerant (Met)

Met és un factor de transcripció de la família basic helix–loop–helix/Per–Arnt–Sim (bHLH-PAS) (Ashok *et al*. 1998, Miura *et al*. 2005). Els membres d'aquesta família de proteïnes exerceixen la seva funció com a heterodímers i ho fan normalment amb altres proteïnes de la mateixa família. El gen Met fou descobert l'any 1986 com un factor que conferia resistència als efectes tòxics del metopré, un anàleg d'HJ, a algunes soques de *D. melanogaster* (Wilson and Fabian 1986, Ashok *et al*. 1998). Posteriorment, es va descobrir que Met presentava una afinitat molt alta amb l'HJ, donant ja una primera pista de la seva funció en la recepció de dita hormona (Miura *et al*. 2005). Met de *D. melanogaster* té un gen paràleg anomenat germ-cell expressed (GCE) (Godlewski *et al*. 2006, Wang *et al*. 2007). En altres genomes d'insectes, com els de dípters basals com el mosquit, només s'ha trobat un ortòleg de Met/GCE (Wang *et al*. 2007). Tot i que el significat funcional de cada un dels paràlegs de *D. melanogaster* en la senyalització d'HJ encara no s'ha aclarit del tot, els estudis sobre el seu únic ortòleg a l'escarabat *T. castaneum* van aportar les proves funcionals clau que suggerien que l'acció repressora de l'HJ en la metamorfosi es realitzava mitjançant Met (Konopova and Jindra 2007). L'RNAi de Met realitzat a estadis larvals primerencs d'aquest insecte holometàbol basal induïa una metamorfosi precoç. Es va comprovar també que Met de *T. castaneum* era necessari per a l'expressió de gens regulats per HJ, com són BR-C i Kr-h1. Aquests experiments encara no van aclarir definitivament si Met era el component essencial de recepció de l'HJ, i si aquest actuava sol o dimeritzant amb altres proteïnes. Com a membre de la família de proteïnes bHLH/PAS, es presumeix que Met pugui funcionar

interactuant amb altres proteïnes. La parella més probable podria ser una proteïna bHLH/PAS i/o un receptor nuclear. Assajos amb immuno-precipitació van demostrar que Met de *D. melanogaster* tenia la capacitat de formar homodímers o heterodímers amb el seu paràleg, GCE; tot i que aquestes interaccions eren desafavorides per la presència d'HJ (Godlewski *et al*. 2006). Hom comprovà que Met tampoc interaccionava amb Tango, un membre de la mateixa família bHLH/PAS (Godlewski *et al*. 2006). Assajos amb doble híbrid de llevat van indicar que Met de *D. melanogaster* podia formar complexos d'unió amb cada component del receptor de l'ecdisona, format pel dímer de EcR-Usp (Li *et al*. 2007), la qual cosa suggeria un mecanisme pel qual l'HJ podria modular la resposta d'ecdisteroides (Dubrovsky 2005, Guo *et al*. 2012). L'any 2011 es va publicar un treball que confirmava experimentalment la unió de Met amb HJ. En aquest article es va descriure que la unió es produïa a traves del domini PAS-B de Met, tot demostrant efectivament el paper de Met com a receptor de l'HJ (Charles *et al*. 2011).

Taiman

Els models actuals que expliquen els mecanismes que reprimeixen la metamorfosi dels insectes postulen el factor de transcripció de la família bHLH-PAS Tai com la parella heterodimèrica de Met en la recepció de l'HJ. Aquesta funció de Tai com a transductor de l'HJ s'ha comprovat funcionalment *in vitro* en proteïnes d'insectes i crustacis (Zhang *et al*. 2011, Kayukawa *et al*. 2012, Kayukawa *et al*. 2013, Charles *et al*. 2011, Miyakawa *et al*. 2013). La paradoxa és que cap evidencia *in vivo* demostra que Tai complexi aquest paper. Tots el intents previs per adreçar la participació de Tai en la metamorfosi mitjançant RNAi han sigut infructuosos degut al 100% de letalitat obtinguda després del tractament, tot havent-se provat en nimfes de *Pyrrhocoris apterus* i larves de *T. castaneum* (Bitra *et al*. 2009, Smykal *et al*. 2014). Aquests resultats divergeixen molt dels obtinguts després d'eliminar Met en estadis juvenils amb RNAi, els quals resulten en un avançament de la metamorfosi, i més aviat suggereixen que Tai exerceix funcions vitals en el desenvolupament de l'insecte. Els nostres estudis preliminars van mostrar que alguns insectes posseeixen varies isoformes de Tai, resultat que ens dona indicis de possible diversitat de funcions, entre les quals la repressió de la metamorfosi.

h) Altres factors de transcripció

Broad-Complex (BR-C)

Un altre factor de transcripció important que participa en la senyalització de l'HJ i també és dependent d'ecdisona és Broad-Complex (BR-C) (DiBello *et al*. 1991). BR-C codifica per un grup de factors de transcripció amb dits de zinc-BTB del tipus C_2H_2 produïts per *splicing* alternatiu a partir d'un únic gen. Les isoformes difereixen en el domini d'unió a DNA carboxi-terminal (DiBello *et al*. 1991, Bayer *et al*. 1996). La BTB comú a totes les isoformes permet a BR-C homo- i heterodimeritzar i reclutar altres corepressors (Bardwell and Treisman 1994, Perez-Torrado *et al*. 2006). A les espècies holometàboles, com el dípter *D. melanogaster*, els lepidòpters *Manduca sexta* i *Bombyx mori* o el coleòpter *T. castaneum*, els factors BR-C s'expressen al darrer estadi larvari i la seva expressió transitòria és essencial per una formació correcta de la pupa (Zhou and Riddiford 2002, Uhlirova *et al*. 2003, Konopova and Jindra 2008, Parthasarathy *et al*. 2008b, Suzuki *et al*. 2008). L'HJ administrada exògenament durant la fase pupal d'insectes holometàbols indueix la re-expressió de BR-C i això fa que l'insecte generi una segona muda pupal. A les fases larvàries, en canvi, sembla que l'HJ inhibeix l'expressió de BR-C degut a que els nivells d'expressió d'aquests només incrementen després d'un petit pic de 20E produït en absència d'HJ al final de la darrera fase larvaria (Konopova and Jindra 2008, Suzuki *et al*. 2008). Sota el control de l'HJ, els factors BR-C també intervenen en el desenvolupament hemimetàbol (Erezyilmaz *et al*. 2006). Experiments duts a terme als hemípters *Oncopeltus fasciatus* i *P. apterus*, que són espècies hemimetàboles filogenèticament distals, suggereixen que, en aquestes espècies, els factors de transcripció BR-C només regulen gradualment el creixement de l'ala. Les nimfes expressaven alts nivells de BR-C els quals queien durant la diferenciació a adult, de manera similar a la situació en els holometàbols. El silenciament de BR-C mitjançant RNAi a nimfes d'*O. fasciatus* impedia el creixement adequat de l'ala i provocava canvis en el patró de color de la cutícula (Erezyilmaz *et al*. 2006). En aquests hemimetàbols paraneòpters, l'expressió de BR-C va lligada a l'expressió de l'HJ durant les fases nimfals. Aquests resultats suggereixen que les seves funcions han divergit radicalment entre espècies hemimetàboles i holometàboles, on els hemimetàbols semblen posseïr un desenvolupament menys dependent de BR-C.

Krüppel-homolog 1 (Kr-h1)

Un altre element important que participa en la transducció de l'HJ en relació amb la metamorfosi és el factor de transcripció Krüppel-homolog 1 (Kr-h1). Kr-h1 és un factor de transcripció que conté dits de zinc del tipus C_2H_2. L'alt grau de conservació d'aquest domini en totes les espècies estudiades, suggereix que la funció de Kr-h1 podria haver estat generalment conservada al llarg de la classe Insecta. Els primers treballs amb Kr-h1, fets a *D. melanogaster*, van demostrar que aquest factor era regulat per la 20E i s'encarregava de modular processos metamòrfics dependents d'ecdisteroides. No obstant, els treballs més recents indiquen que l'expressió de Kr-h1 és induïda per l'HJ també a la mosca *D. melanogaster*, l'escarbat *T. castaneum* i als tisanòpters *Frankliniella occidentalis* i *Haplothrips brevitubus* (Minakuchi *et al*. 2008, Minakuchi *et al*. 2009, Minakuchi *et al*. 2011). En estudis funcionals realitzats a *D. melanogaster* i *T. castaneum*, Kr-h1 s'ha caracteritzat com a regulador crucial de la metamorfosis, ja que la seva supressió a estadis larvals mitjançant RNAi indueix una metamorfosi precoç (Minakuchi *et al*. 2008, Minakuchi *et al*. 2009). A *T. castaneum*, Kr-h1 davalla a l'estadi pupal, tot just abans de passar a adult (Minakuchi *et al*. 2009). Hom va considerar Kr-h1 com el transductor de l'HJ més distal en la jerarquia de transducció, l'acció antimetamòrfica la qual havia sigut conservada en els insectes holometàbols, d'escarabats a mosques. Treballs posteriors han demostrat que la regió promotora del gen Kr-h1 conté elements de resposta (*k*JHRE) als quals s'uneix al complex Met-Taiman en presència d'HJ, amb la qual cosa s'activa la seva transcripció (Kayukawa *et al*. 2012, Kayukawa *et al*. 2013).

i) Regulació dels transductors per microRNAs

Els microRNAs (miRNAs) són una classe d'RNAs no codificants petits (al voltant de 22 nucleòtids) que s'uneixen principalment a l'extrem 3' de la regió no traduïda dels RNAs missatgers i juguen un paper crític en molts processos biològics tot modulant l'expressió gènica a nivell post-transcripcional (Bartel 2004, Bentwich *et al*. 2005, Berezikov *et al*. 2005) (Figura 1.5).

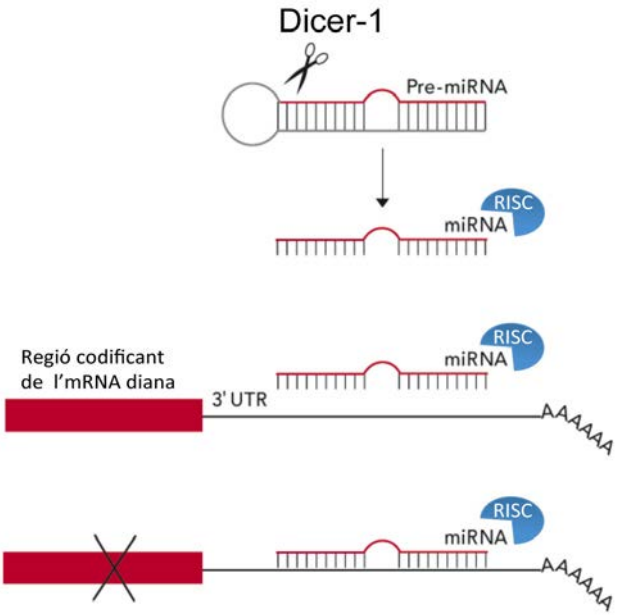

Figura 1.5: Representació esquemàtica del mode d'acció dels miRNAs. Els precursors de miRNAs (pre-miRNA) passen del nucli cel·lular al citoplasma on seran trossejats en dobles cadenes de miRNA per l'acció de l'enzim Dicer-1. D'aquestes dues dobles cadenes una és degradada, i l'altra, el miRNA madur, s'incorpora al complex RISC que el guiarà fins unir-se a l'mRNA diana. Una vegada produïda la unió, normalment a la regió 3'UTR, s'impedeix la possible traducció a proteïna i l'mRNA es degrat.

El nostre laboratori es va proposar estudiar la rellevància dels miRNAs en la metamorfosi de *B. germanica*. Una aproximació per revelar la funció global dels miRNAs és impedir la generació de miRNAs madurs tot eliminant l'expressió de Dicer-1, un enzim clau en la biogènesi dels miRNAs (Giraldez *et al*. 2005). Aquesta tècnica va ésser realitzada exitosament a la panerola *B. germanica*; la supressió de Dicer-1 mitjançant RNAi va resultar en una síntesi baixa de miRNA madurs que derivà en una inhibició de la metamorfosi (Gomez-Orte and Belles 2009). Els insectes no van realitzar la metamorfosi i van mudar a una nimfa supernumerària, nimfa 7, amb una combinació de caràcters juvenils i adults (Figura 1.6). La pregunta que obria el resultat d'aquest experiment era doble: d'una banda quin era el miRNA responsable d'aquest efecte, i d'altra, sobre quin mRNA diana actuava. Una aproximació per a repondre aquestes dues preguntes portada a terme en el nostre laboratori ha estat l'estudi de miRNAs candidats, tot eliminant la seva expressió i caracteritzant la funció durant la metamorfosi de la panerola. L'estratègia seguida va consistir en suprimir els miRNAs més abundants a la darrera fase nimfal : miR-8 i let-7, miR-100, miR-125, els quals van presentar funcions modestes relacionades majoritàriament amb el desenvolupament de l'ala (Rubio and

Belles 2013, Rubio *et al*. 2013). Cap d'aquests miRNAs estudiats podia explicar el fenotip global obtingut després del tractament amb RNAi de Dicer-1, és a dir, la supressió total de la metamorfosi. En aquesta tesi vam canviar l'aproximació i vam seleccionar un possible mRNA candidat a ser regulat per miRNAs, i vam escollir Kr-h1 com a candidat, ja que és un factor clau que ha de desaparèixer abans de l'inici de la metamorfosi. L'mRNA de Kr-h1 és present en les diferents fases de la nimfa i sobtadament desapareix a la darrera fase nimfal, i aquesta caiguda tant abrupta és difícilment explicable només per l'absència de l'HJ**.** En anàlisis preliminars vam comprovar que els individus amb baixos nivells de miRNAs deguts a la interferència de Dicer-1 presentaven nivells alts de Kr-h1.

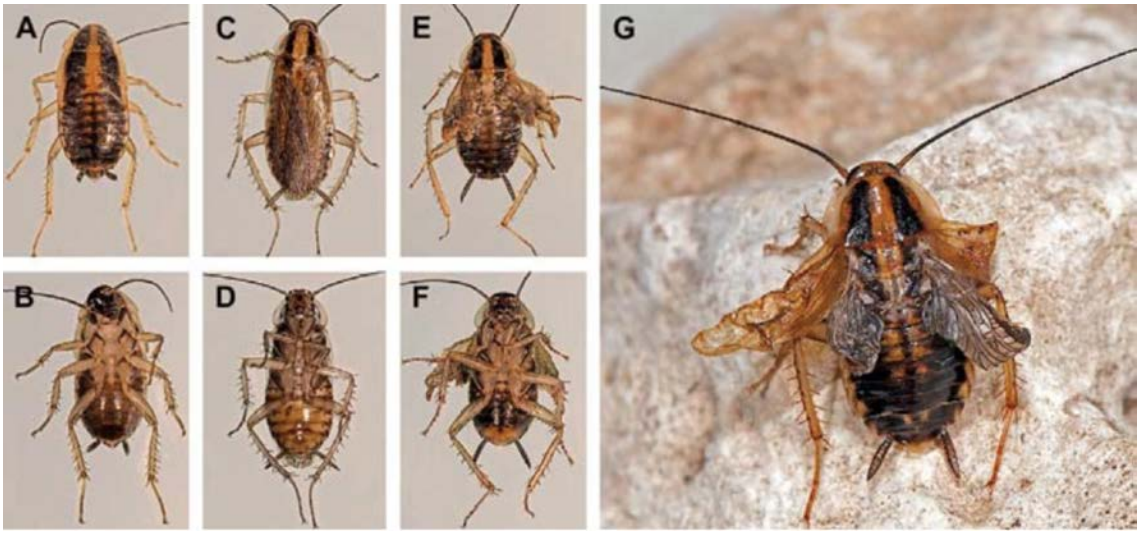

Figura 1.6: Inhibició de la metamorfosi a *Blattella germanica* després de disminuir l'expressió de Dicer-1 mitjançant RNAi a la darrera fase nimfal (sisena). Visió dorsal i ventral d'una femella a sisena fase normal (A, B), adult normal (C, D) i setena fase nimfal supernumerària (E, F) com a resultat de la inhibició de la metamorfosi. Els nimfoids s'assemblen a aquells obtinguts després de tractar la sisena fase nimfal amb HJ (G). Fotografies d'Albert Masó, les dades són extretes de Gomez-Orte and Belles (2009).

2. Objectius

2. Objectius

L'objectiu general d'aquesta tesi doctoral és ajudar a comprendre els mecanismes moleculars pels quals l'hormona juvenil (HJ) reprimeix la metamorfosi, utilitzant com a model la panerola *Blattella germanica*. Els objectius específics serien els següents.

(1) Confirmar que Methoprene-tolerant (Met) actua en la senyalització de l'HJ en la metamorfosi del nostre insecte model.
(2) Estudiar la funció de Taiman (Tai) en el mateix context.
(3) Estudiar la funció de factors clau en la senyalització de l'HJ per sota de Met i Tai, com Broad-Complex (BR-C), el qual havia estat prèviament demostrat com a especificador de pupa en insectes holometàbols.
(4) En la línia de l'objectiu 3, estudiar la funció de Krüppel-homolog 1 (Kr-h1), factor de transcripció que s'havia prèviament caracteritzat com a repressor de la metamorfosi en insectes holometàbols.
(5) Estudiar si l'expressió de Kr-h1, en particular la davallada que es produeix abans de l'inici de la metamorfosi, està modulada per microRNAs.

3. Informes sobre les publicacions

3. Informe del director de tesi del factor d'impacte dels articles publicats derivats d'aquesta tesi

La següent secció està formada per 3 articles publicats, 1 article acceptat i en premsa, i un altre sotmès a una revista i en procés d'anàlisi pels àrbitres de la mateixa.

Capítol 4

Lozano, J., & Belles, X. (2014). Role of Methoprene-Tolerant (Met) in Adult Morphogenesis and in Adult Ecdysis of *Blattella germanica*. *PloS One*, 9(7), e103614.

Aquest article ha estat publicat a la revista científica *PloS One*. Aquesta és una revista dins la categoria 'multidisciplinar', amb un Factor d'Impacte a 2013 de 3,534 i situada en el primer quartil, i la vuitena posició dins de les revistes multidisciplinàries.

Capítol 5

Lozano, J., Kayukawa, T., Shinoda, T., & Belles, X. (2014). A Role for Taiman in Insect Metamorphosis. *PLoS Genetics*, in press.

Aquest article està acceptat i en premsa a la revista científica *PLoS Genetics*. Aquesta és una revista de l'àmbit de 'Genètica i Herència' i posseeix un Factor d'Impacte a 2013 de 8,167, situant-se en el primer quartil i la catorzena posició de les revistes de la seva àrea.

Capítol 6

Huang, J., **Lozano, J.**, & Belles, X. (2013). Broad-complex functions in postembryonic development of the cockroach *Blattella germanica* shed new light on the evolution of insect metamorphosis. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830:2178–2187.

Aquest article ha estat publicat a la revista científica *Biochimica et Biophysica Acta (BBA) - General Subjects*. Aquesta és una revista dins la categoria 'Bioquímica i Biologia Molecular', amb un Factor d'Impacte a 2013 de 3,829 i situada en el segon quartil i la noranta-tresena posició dins de la seva àrea.

Capítol 7

Lozano, J., & Belles, X. (2011). Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. *Scientific Reports* 1:163.

Aquest article ha estat publicat a la revista científica *Scientific Reports*. Aquesta és una revista de la categoría 'multidisciplinar' amb un Factor d'Impacte a 2013 de 5,078, situada en el primer quartil de revistes multidisciplinars i a la cinquena posició d'aquestes.

Capítol 8

Lozano, J., Montañez, R., & Belles, X. (2014) miR-2 regulates insect metamorphosis by controlling juvenile hormone signalling pathway.

Aquest manuscrit ha estat sotmés a la revista científica *Proceedings of the National Academy of Sciences of the United States of America*, i es troba en procés de primer anàlisi pels àrbitres de la mateixa. Aquesta és una revista dins la categoria 'multidisciplinar', amb un Factor d'Impacte a 2013 de 9,809 i situada en el primer quartil, i la quarta posició dins de les revistes multidisciplinàries.

Xavier Bellés i Ros

3. Informe del director de tesi sobre la contribució del doctorand a cada un dels articles publicats derivats d'aquesta tesi

Capítol 4

Lozano, J., & Belles, X. (2014). Role of Methoprene-Tolerant (Met) in Adult Morphogenesis and in Adult Ecdysis of Blattella germanica. *PloS One*, 9(7), e103614.

Van concebre i dissenyar els experiments: XB JL. Va realitzar els experiments: JL. L'anàlisi de dades va ser portat a terme per: JL XB. Van contribuir a la escriptura del manuscrit: XB JL. Tots els autors han llegit i aprovat el manuscrit.

Capítol 5

Lozano, J., Kayukawa, T., Shinoda, T., & Belles, X. (2014) A Role for Taiman in Insect Metamorphosis. Acceptat a *PLoS Genetics*.

Van concebre i dissenyar els experiments: XB, JL, TK, TS. Van realitzar els experiments: JL, TK. L'anàlisi de dades va ser portat a terme per: JL, XB, TK, TS. Va contribuir a la escriptura del manuscrit: XB. Tots els autors han llegit i aprovat el manuscrit.

Capítol 6

Lozano, J., & Belles, X. (2011). Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. *Scientific Reports* 1:163.

Va concebre i dissenyar els experiments: XB. Va realitzar els experiments i anàlisi filogenètics: JL. L'anàlisi de dades va ser portat a terme per: JL, XB. Va contribuir a la escriptura del manuscrit: XB i JL. Tots els autors han llegit i aprovat el manuscrit.

Capítol 7

Huang, J., **Lozano, J.**, & Belles, X. (2013). Broad-complex functions in postembryonic development of the cockroach Blattella germanica shed new light on the evolution of insect metamorphosis. *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1830:2178–2187.

Va concebre i dissenyar els experiments: XB. Va realitzar els experiments i anàlisi filogenètics: JH, JL. L'anàlisi de dades va ser portat a terme per: JH, XB. Va contribuir a la escriptura del manuscrit: XB. Tots els autors han llegit i aprovat el manuscrit.

Capítol 8

Lozano, J., Montañez, R., & Belles, X. (2014) miR-2 regulates insect metamorphosis by controlling juvenile hormone signalling pathway.

Van concebre i dissenyar els experiments: XB, JL. Van realitzar els experiments: JL, RM. L'anàlisi de dades va ser portat a terme per: JL, XB, RM. Va contribuir a la escriptura del manuscrit: XB. Tots els autors han llegit i aprovat el manuscrit.

Confirmo que cap dels coatours d'aquests treballs ha utilitzat, implícitament o explícitament aquests treballs per a l'elaboració d'una tesi doctoral.

Xavier Bellés i Ros

4. Funcions de Methoprene-tolerant (Met) en la morfogènesi adulta i en l'ècdisi adulta a *Blattella germanica*

Funcions de Methoprene-tolerant (Met) en la morfogènesi adulta i en l'ècdisi adulta a *Blattella germanica*

Jesús Lozano[1], Xavier Bellés[1]

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Resum

L'hormona juvenil (HJ) reprimeix la metamorfosi durant els estadis juvenils dels insectes. Un dels principals participants en la senyalització hormonal és Methoprene-tolerant (Met), que actua com a receptor d'HJ. Usant l'insecte polineòpter *Blattella germanica* com a model d'estudi i RNA d'interferència per fer davallar el transcrit, hem confirmat que Met transdueix la senyal antimetamòrfica de l'HJ en nimfes joves a més de tenir funcions en la darrera muda nimfal d'aquesta espècie. Anteriorment, la funció del Met com a receptor d'JH s'havia demostrat en el clade Eumetabola, amb experiments en Holometabola (l'escarabat *Tribolium castaneum*) i en el seu llinatge germà, el clade Paraneoptera (a l'hemípter *Pyrrhocoris apterus*). El nostre resultat mostra que la funció de Met com a receptor d'HJ també es conserva als polineòpters més basals. La funció del Met com a transductor de l'HJ, per tant, podria ser anterior la innovació evolutiva de la metamorfosi. A més, l'expressió de Met també es va trobar en últim estadi nimfal de *B. germanica*, quan l'HJ està absent. La interferència d'RNA de Met en aquesta etapa va provocar deficiències en el creixement de l'ala i problemes d'ècdisi a la muda imaginal. Vam quantificar davallada del l'mRNA de E75A, un gen induïble per ecdisona, i baixada d'Insulin-Like-Peptide 1 en aquests exemplars interferits de Met, suggerint que aquest podria estar implicat en les vies de senyalització de l'ecdisona i de la insulina en l'últim estadi nimfal, quan l'HJ es troba virtualment absent.

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PLOS ONE

Role of Methoprene-Tolerant (Met) in Adult Morphogenesis and in Adult Ecdysis of *Blattella germanica*

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Abstract

Juvenile Hormone (JH) represses metamorphosis of young instars in insects. One of the main players in hormonal signalling is Methoprene-tolerant (Met), which plays the role of JH receptor. Using the Polyneopteran insect *Blattella germanica* as the model and RNAi for transcript depletion, we have confirmed that Met transduces the antimetamorphic signal of JH in young nymphs and plays a role in the last nymphal instar moult in this species. Previously, the function of Met as the JH receptor had been demonstrated in the Eumetabola clade, with experiments in Holometabola (in the beetle *Tribolium castaneum*) and in their sister group Paraneoptera (in the bug *Pyrrhocoris apterus*). Our result shows that the function of Met as JH receptor is also conserved in the more basal Polyneoptera. The function of Met as JH transducer might thus predate the evolutionary innovation of metamorphosis. Moreover, expression of Met was also found in last nymphal instar of *B. germanica*, when JH is absent. Depletion of Met in this stage provoked deficiencies in wing growth and ecdysis problems in the imaginal moult. Down-regulation of the ecdysone-inducible gene E75A and Insulin-Like-Peptide 1 in these Met-depleted specimens suggest that Met is involved in the ecdysone and insulin signalling pathways in last nymphal instar, when JH is virtually absent.

Citation: Lozano J, Belles X (2014) Role of Methoprene-Tolerant (Met) in Adult Morphogenesis and in Adult Ecdysis of *Blattella germanica*. PLoS ONE 9(7): e103614. doi:10.1371/journal.pone.0103614

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

In essence, insect metamorphosis is regulated by the interplay of two hormones, 20-hydroxyecdysone (20E), which triggers the successive moults throughout the life cycle, and juvenile hormone (JH), which represses the transition to the adult stage [1]. From an evolutionary point of view, it is worth noting that this essential endocrine regulation is conserved in the species closest to the ancestral state, where juvenile stages are similar to the adult (hemimetabolan insects), as well as in more derived species, where juvenile stages can be extremely divergent with respect to the adult stage (holometabolan insects) [1].

While molecular mechanisms regulating the action of 20E have largely been unveiled, those underlying the action of JH have remained almost a complete mystery until recent years, when the transcription factor Methoprene-tolerant (Met) has been proclaimed as the JH receptor [2]. In 1986, Met was discovered to be a factor that determines resistance to the toxic effects of Methoprene, a JH analogue, in some *Drosophila melanogaster* strains [3]. Later, Met was identified as a member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors [4], which are critical regulators of gene expression networks underlying many essential physiological and developmental processes [5]. Subsequently, Miura et al. [6] showed that *D. melanogaster* Met protein could bind JH with a very high affinity (Kd of 5.3 nM), providing the first clear indication that Met might play the role of JH receptor. More recently, it has been observed that depletion of Met mRNA levels with RNAi in early larval stages of the holometabolan species *T. castaneum* triggers precocious pupal morphogenesis [7,8], which showed that Met is involved in antimetamorphic JH signal transduction. RNAi studies also demonstrated the JH-transducing role of Met in the hemimetabolan species *Pyrrhocoris apterus* [9]. Using Met of *T. castaneum*, Charles et al. [10] confirmed that the JH binding affinity is high (Kd of 2.9 nM in this case), that the PAS-B motif of Met is both necessary and sufficient to bind JH, and that when JH binds to a Met moiety in a Met-Met homodimer, the homodimer dissociates and JH+Met binds to another bHLH-PAS protein called Taiman.

In the meantime, it was found that the JH signal downstream of Met is transduced by Krüppel-homolog 1 (Kr-h1), a transcription factor with a DNA binding domain consisting of eight zinc fingers. The function of Kr-h1 as a transducer of the antimetamorphic action of JH was demonstrated through RNAi experiments in holometabolan species, such as *D. melanogaster* [11] and *T. castaneum* [12], as well as in hemimetabolan species, such as the cockroach *Blattella germanica* [13] and the bugs *P. apterus* and *Rhodnius prolixus* [9]. In all cases, depletion of Kr-h1 mRNA

levels in pre-final juvenile stages triggered precocious metamorphosis.

From a mechanistic point of view, results obtained in NIAS-Bm-aff3 cells from the silkworm *Bombyx mori* [14] are specially relevant because they revealed the presence of a JH response element, which was named *k*JHRE, in the promoter region of *B. mori* Kr-h1. Interestingly, *k*JHRE contains an E-box to which bHLH-PAS proteins may bind, and reporter assays in mammalian HEK293 cells (which presumably lack JH signalling genes) showed that while *k*JHRE-specific reporter activity was triggered in the presence of JH when the complete open reading (ORF) frame of *B. mori* Met was expressed, this activation was higher when Taiman ORF was coexpressed with Met. Therefore, these results suggest that Met and Tai jointly interact with the *k*JHRE located in the Kr-h1 promoter region and that this interaction is JH-dependent [14]. Equivalent experiments carried out on the Tc81 embryonic cell line of *T. castaneum* [15] led to the identification of the same *k*JHRE, this time located in both the promoter region and in the first intron of *T. castaneum* Kr-h1. Moreover, reporter assays performed with HEK293 cells also showed that *k*JHRE reporter activity was triggered when Met was expressed in the presence of JH and the activation was enhanced when Taiman was coexpressed with Met [15]. Collectively, the data presents clear evidence that Met plays the role of JH receptor and, therefore, in metamorphosis repression, at least in Paraneopteran insects such as the bug *P. apterus* [9] and in Holometabola such as the beetle *T. castaneum* [7,8].

The present work aims to extend the investigation into the JH receptor role of Met in metamorphosis to Polyneopteran insects, taking the cockroach *B. germanica* as model. Moreover, our preliminary experiments showed that Met is also expressed in last nymphal instar of this cockroach, intriguingly when JH is absent. A second purpose of the present work was, then, to study the function of Met in this stage.

Materials and Methods

Insects

The *B. germanica* specimens used in the experiments were from a colony reared in the dark at 30±1°C and 60–70% relative humidity. Freshly ecdysed female nymphs were selected and used at the chosen ages. Prior to injection treatments, dissections and tissue sampling, the specimens were anaesthetized with carbon dioxide.

RNA Extraction and retrotranscription to cDNA

We carried out total RNA extraction from the whole body (excluding the digestive tube to avoid intestine parasites) or specific tissues using the miRNeasy extraction kit (QIAGEN). A sample of 500-ng from each RNA extraction was treated with DNase (Promega) and reverse transcribed with first Strand cDNA Synthesis Kit (Roche) and random hexamers primers (Roche). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies).

Cloning and sequencing of BgMet

BgMet mRNA was obtained from a *B. germanica* transcriptome obtained with RNA extracts from whole body of penultimate instar female nymphs and sequenced with a 454 Junior sequencer (Roche, Barcelona, Spain) at the Technical and Scientific Services of the Biomedical Research Park of Barcelona (PRBB). Transcriptome sequences were validated with RT-PCR using specific primers and cDNA from penultimate instar female nymphs of *B. germanica* as a template. Further 3′ and 5′ rapid amplification of cDNA ends (RACE, Ambion) allowed us to obtain a practically complete full length ORF of BgMet. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced.

Determination of mRNA levels by quantitative real-time PCR

Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using SYBR Green (Power SYBR Green PCR Master Mix; Applied Biosystems). A template-free control was included in all batches. The primers used to detect mRNA levels are detailed in Table S1. The efficiency of each set of primers was first validated by constructing a standard curve through four serial dilutions. Levels of mRNA were calculated relative to BgActin-5c (Accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA or as standardized relative expression, setting controls levels to 1.0.

Treatments in vivo with juvenile hormone III

Freshly emerged last instar female nymphs of *B. germanica* were topically applied in dorsal abdomen with JH III (Sigma-Aldrich), which is the native JH of B. *germanica* [16], at a dose of 20 µg diluted in 1 µL of acetone. Controls received 1 µL of acetone alone. The commercial JH III is a mixture of isomers containing *ca*. 50% of the biologically active (10*R*)-JH III, thus the active dose applied was around 10 µg per specimen.

RNA interference

The detailed procedures for RNAi experiments were as described previously [17]. The two dsRNAs used for BgMet targeting as well as the primers to generate the corresponding templates are summarized in Table S1. The fragments were amplified by PCR and cloned into the pSTBlueTM-1 vector. A 307-bp sequence from *Autographa californica* nucleopoyhedrovirus was used as control dsRNA (dsMock). The dsRNAs were prepared as reported elsewhere [17]. A volume of 1 µL of dsRNA solution (3 µg/µL, unless stated otherwise) was injected into the abdomen of specimens at chosen ages and stages with a 5-µL Hamilton microsyringe. Control specimens were treated with the same dose and volume of dsMock.

Results

B. germanica has a conserved orthologue of Met

Combining BLAST search in *B. germanica* transcriptomes available in our laboratory and PCR strategies, we obtained a cDNA of 1783 bp (GenBank accession number HG965209) whose conceptual translation rendered a 594 amino acid protein with sequence similarity to Met proteins that we called BgMet. The BgMet sequence contains the bHLH, PAS A, PAS B and PAC (PAS-associated C-terminal region) motifs that are typical of Met proteins (Figure 1A) and top BLAST scores were obtained from Met orthologues of other insects. The highest level of overall amino acid sequence identity (47%) was found with the Met orthologue of the firebrat *Thermobia domestica*, whereas the lowest level (29%) was with Met1 of *B. mori* (Figure S1).

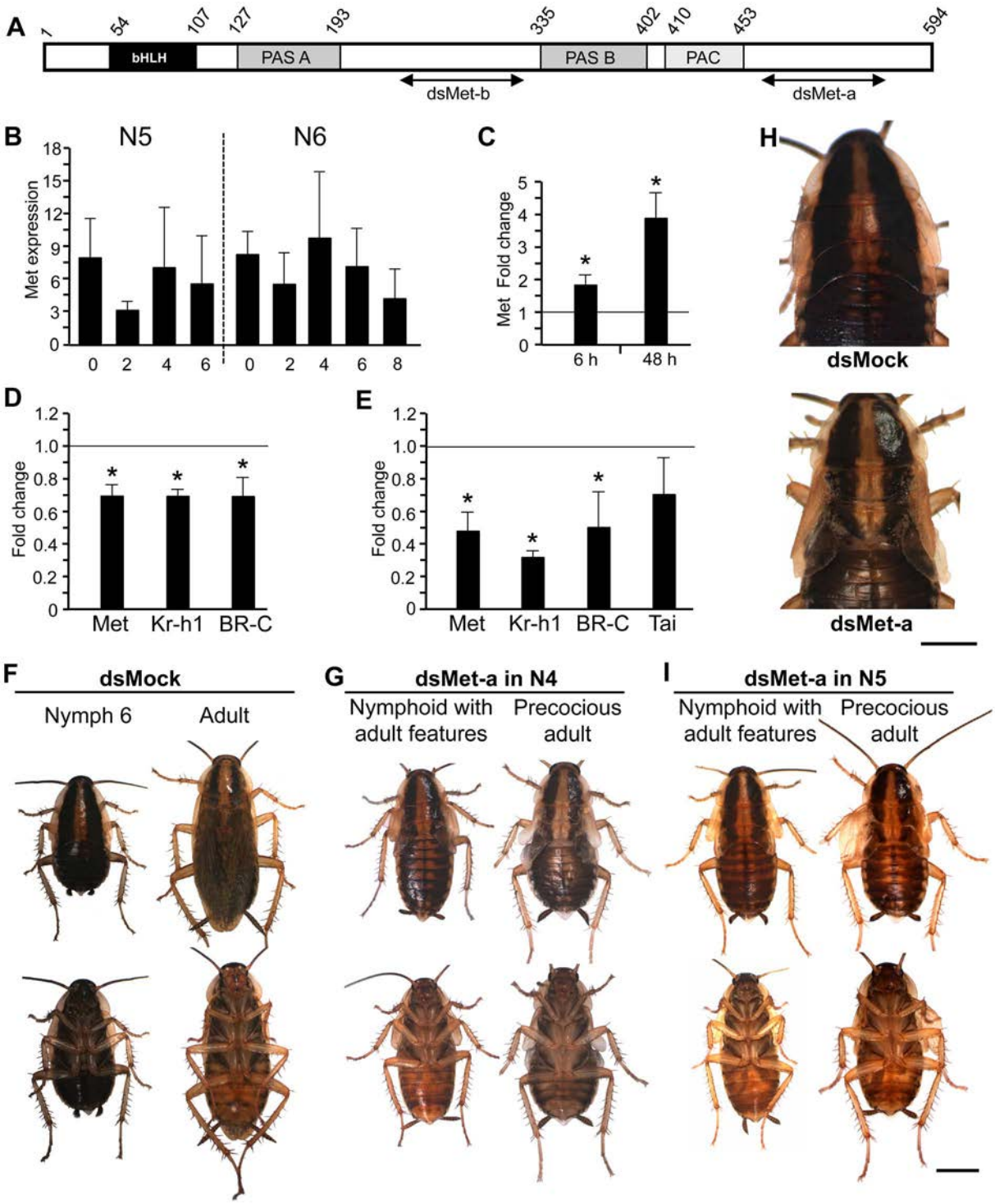

Figure 1. Structure, expression and function of BgMet in *Blattella germanica* metamorphosis. (A) Organization of BgMet protein in different domains; also indicated are the regions were the two dsMet used in RNAi studies were designed. (B) Expression of BgMet mRNA in whole body of female nymphs in penultimate (N5) and last (N6) instar. (C) Effect of JH III treatment (20 μg) on BgMet expression; JH was topically applied in freshly emerged N6, and BgMet mRNA levels were measured 6 and 48 h later. (D) Effects, at transcript level, of dsMet-a treatment in N4; N4 females received two 3-μg doses of dsMet-a, one on N4D0 and the other on N4D3, and transcript levels (of Met, Kr-h1 and BR-C) were measured on N4D5; controls received an equivalent treatment with dsMock. (E) Effects, at transcript level, of dsMet-a treatment in N5; the experimental design was equivalent to that used in N4, with a double treatment, one on N5D0 and the other on N5D3; transcript levels (of Met, Kr-h1, BR-C and Tai) were measured on N5D6. (F–I) Dorsal and ventral view of specimens resulting from dsMet-a treatment in N4 and N5; normal last nymphal instar and adult obtained from dsMock treatments, either in N4 or N5 (F); nymphoid (instead of N6) with adult features, and precocious adult obtained from dsMet-a treatments in N4 (G), detail of the enlarged lateral expansions in T2 and T3 of a nymphoid with adult features compared with a control N6 (H), nymphoid with adult features, and precocious adult obtained from dsMet-a treatments in N5 (I). Each point of quantitative data in histograms represents 4 biological replicates and results are expressed as the mean ± SEM; data in A represent copies of BgMet mRNA per 1000 copies of

BgActin-5c mRNA; data in C, D and E are normalized against the dsMock-treated samples (reference value = 1), and the asterisk indicates statistically significant differences with respect to controls ($p<0.05$), according to the REST software tool [29]. Photomicrographs in F–I were taken with a Zeiss DiscoveryV8 Stereo microscope with an AxioCam MRc digital camera; scale bars in F, G, I = 3 mm and in H = 2 mm.
doi:10.1371/journal.pone.0103614.g001

Expression of Met is maintained during the last two nymphal instars of *B. germanica*

BgMet transcript levels fluctuate between 3 and 10 mRNA copies per 1000 copies of actin mRNA, either in the penultimate (N5) or last (N6) nymphal instars (Figure 1B). Expression in N5 is not surprising because JH, which represses metamorphosis in young nymphs, is produced at significant levels throughout this stage [16]. However, BgMet is also expressed in N6 when there is no JH [16] to be transduced. To test whether BgMet is JH-inducible, we treated freshly emerged sixth instar female nymphs with JH III, the naturally occurring JH homologue in *B. germanica* [16], and measured BgMet transcript levels 6 and 48 h after treatment. Results showed that JH up-regulates BgMet expression and that the stimulatory effect increases with time (Figure 1C). JH-treated specimens not used for transcript measurements moulted into supernumerary nymphs, as observed in previous studies [18].

Depletion of Met in young nymphal instars triggers precocious metamorphosis

Involvement of Met in *B. germanica* metamorphosis was studied by RNAi approaches using a double-stranded RNA (dsRNA) which targets BgMet (dsMet-a) (Figure 1A). In a first set of experiments, we injected two 3-μg doses of dsMet-a in antepenultimate (N4) nymphal instar females, one when freshly emerged (N4D0) and the other on day 3 (N4D3). Controls received an equivalent treatment with dsMock. Transcript measurements carried out on N4D5 indicated that BgMet mRNA levels were already significantly reduced (*ca.* 30%) in dsMet-a-treated specimens (Figure 1D). At the same time, we measured the expression of Kr-h1 and BR-C, two JH-dependent genes, and both were also down-regulated at similar levels than Met (Figure 1D). In a second set of experiments we treated N5 female instar following an experimental design equivalent to that used for N4 nymphs, with a double 3-μg treatment, one on N5D0 and the other on N5D3. Transcript levels of Met were measured on N5D6, and resulted to be significantly reduced (*ca.* 50%) (Figure 1E). We also measured the expression of Kr-h1, BR-C and that of Tai, as a putative partner of Met for JH reception and signal transduction. Kr-h1 and BR-C expression was significantly down-regulated (*ca.* 70 and 50%, respectively), whereas Tai mRNA levels did tend to be lower, the differences with respect to dsMock-treated specimens were not statistically significant (Figure 1E).

At the phenotypic level, we first analysed the specimens that received a double treatment of dsMet-a or dsMock in N4. All dsMock-treated specimens (n = 27) moulted to N5, then to N6 and to adult, all stages showing normal morphological features (Figure 1F). As regards to the dsMet-a-treated specimens (n = 54), 9 of them (17%) died during N4 or N5, often during moulting, just before or while ecdysing. The remaining 45 specimens that survived N4 and N5 stages produced the following different phenotypes after the next moult: 8 specimens (18%) died during the next ecdysis, presenting fundamentally nymphal features, but with flexible and somewhat enlarged lateral expansions in the mesonotum (T2) and metanotum (T3) (Figure S2A); 7 specimens (16%) showed a phenotype that we categorised as nymphoid with adult features, having a general shape similar to an N6 nymph but exhibiting adult yellowish colouration and enlarged and membranous wing-like lateral expansions in T2 and T3 (Figure 1 G and H); 11 specimens (24%) that we categorised as precocious adults, exhibiting a general shape and yellow colouration similar to an adult but with tegmina and wings that were membranous and flexible yet underdeveloped (Figure 1G); and 19 specimens (42%) showing the morphology of a normal N6. In turn, these 19 specimens underwent the imaginal moult; 10 of them died between apolysis and ecdysis, 4 moulted to an adult with the tegmina and wings correctly patterned but not well extended, and also with deformed hind tibiae (Figure S2B), and the remaining 5 moulted to normal adults (results summarised in Table S2).

Then, we analysed the specimens that received a double treatment of dsMet-a or dsMock in N5. All dsMock-treated specimens (n = 19) moulted to normal N6 and then to normal adults. None of the dsMet-a-treated specimens (n = 35) died during N5 or N6. After the moult from N5, 5 out of the 35 specimens (14%) were nymphoids with adult features (Figure 1I), 1 specimen (3%) had a precocious adult morphology (Figure 1I) and 29 specimens (83%) were normal N6 nymphs. Of the 29 N6 females that underwent the imaginal moult, 5 of them died between apolysis and ecdysis, 12 moulted to adults with the tegmina and wings correctly patterned but not well extended (as in Figure S2B), and the remaining 12 moulted to normal adults (Table S2).

To assess the specificity of dsMet-a, we tested an alternative Met-targeting dsRNA (dsMet-b) designed in another region of the BgMet sequence (Figure 1A). We carried out experiments equivalent to those performed with dsMet-a in N4 and N5 nymphs, and the results were equivalent in phenotypical terms (Figure S3A and Table S2). We mistakenly included a male nymph in the experiments in N4, which moulted to N5, and then to a practically perfect precocious adult, including correctly patterned tergal glands, similar to those obtained after Kr-h1 depletion [13] (Figure S3B). Depletion of Met expression and the effects on other transcripts (Kr-h1, BR-C and Taiman) in N5 treatments were similar to those obtained with dsMet-a (Figure S3C).

Depletion of Met in the last nymphal instar delays the adult moult and provokes ecdysis deficiencies

The expression of Met in N6 (Figure 1B), when JH is absent, lead us to consider Met functions in this stage. To address this question, we administered two doses of dsMet-a to N6 females, one on N6D0 and the other on N6D3, whereas controls received an equivalent treatment with dsMock. Transcript measurements performed on N6D6 revealed that BgMet mRNA levels were significantly reduced (*ca.* 65%) in dsMet-a-treated specimens (Figure 2A). At the phenotypic level, all specimens treated with dsMock (n = 14) moulted to normal adults (Figure 2B), with the tegmina and membranous wings well extended (Figure 2C), and they spent 8 days in N6, as usual in our rearing conditions. The dsMet-a-treated specimens (n = 28) spent an average of 9 days in N6 and a number of deficiencies arose at the imaginal moult. Five specimens (18%) died between apolysis and ecdysis. Ten (36%) moulted to adults with abnormalities in the tegmina and membranous wings, ranging from relatively mild (both slightly smaller and the tegmina somewhat curled at the tip) to severe (both tegmina and membranous wings severely wrinkled and reduced) (Figure 2B). Finally, 13 specimens (46%) moulted to normal adults (Table S2).

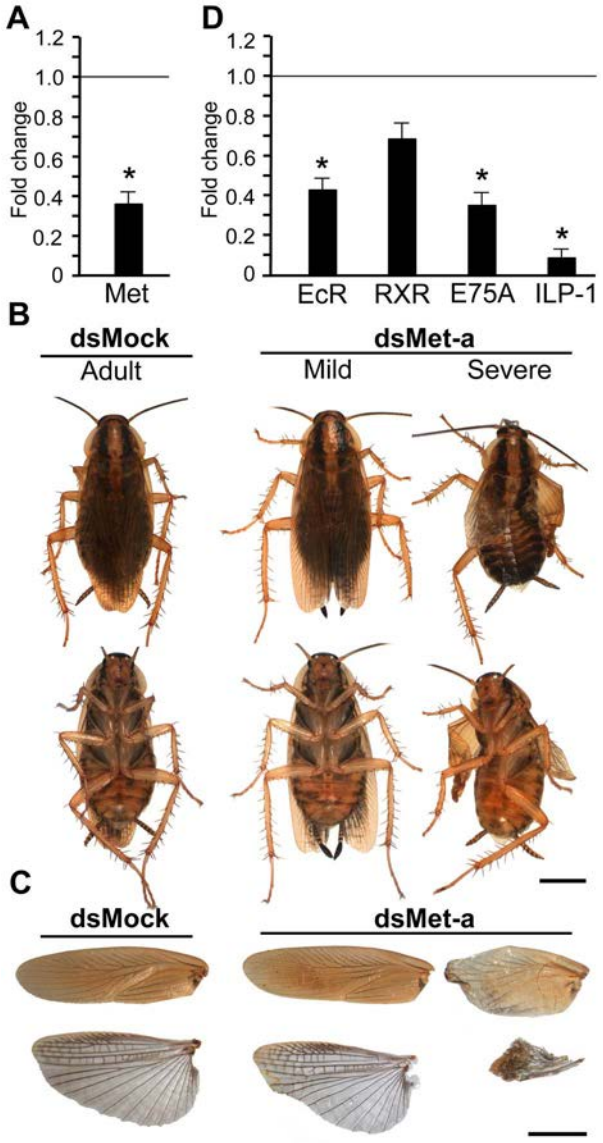

Figure 2. Effects of BgMet depletion in last nymphal stage of *Blattella germanica*. (A) Effects at transcript level; N6 females received two 3-μg doses of dsMet-a, one on N6D0 and the other on N6D3, and Met mRNA levels were measured on N6D6; controls received an equivalent treatment with dsMock. (B) Dorsal and ventral view of specimens resulting from dsMet-a treatment in N6; normal adult obtained from dsMock treatments; mild and severe adult phenotypes obtained after Adult N6 with adultoid features, mild and severe, obtained from dsMet-a treatments. (C) Tegmina and membranous wings corresponding to normal adult obtained after dsMock treatments, and to adults treated with dsMet-a showing the mild and the severe phenotype. (D) Effects of Met depletion on N6 on the expression of EcR, RXR, E75A and ILP-1 measured on N6D6. In A and D, each point represents 4 biological replicates and results are expressed as the mean ± SEM; data are normalized against the dsMock-treated samples (reference value = 1), and the asterisk indicates statistically significant differences with respect to controls ($p<0.05$), according to the REST software tool [29]. Photomicrographs in B–C were with a Zeiss DiscoveryV8 Stereo microscope with an AxioCam MRc digital camera; scale bars = 3 mm.
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Given that the phenotypes obtained pointed to moulting problems, we wondered whether the expression of genes of the ecdysone signalling pathway could be affected. We measured mRNA levels of the components of the ecdysone receptor in dsMet-a-treated specimens; results showed that those of EcR were significantly lower (*ca*. 60%) than controls, whereas those of RXR tended to be lower but differences with respect to controls were not statistically significant. The ecdysone inducible gene E75A was significantly down-regulated (*ca*. 65%) (Figure 2D). As moulting and wing size problems also suggested that insulin pathway might be affected, we measured the *B. germanica* orthologue of Insulin-Like-Peptide 1 (ILP-1), which is the most highly expressed ILP in *B. germanica* brain (J. L. Maestro, unpublished data). ILP-1 mRNA levels in dsMet-a-treated specimens were significantly, and dramatically, lower (*ca*. 90%) than in control specimens (Figure 2D).

Discussion

B. germanica Met is the first Met sequence reported in Dictyoptera, a Polyneopteran within the Pterygota subclass of hemimetabolan insects. In terms of overall percentage of identity, BgMet is closer to *T. domestica* (an Apterygota ametabolan species) than to the phylogenetically closer Pterygota hemimetabolan *L. migratoria*. As expected, the homologous sequences of Diptera and Lepidoptera, the most modified orders of Pterygota Holometabola, are the most divergent with respect to BgMet.

Expression of BgMet in the penultimate (N5) nymphal instar, when JH is present, is not surprising. In this instar, we propose that Met transduces the JH signal that represses adult morphogenesis, as Met depletion during this stage triggers precocious metamorphosis at the next moult. When dsMet was administered in the antepenultimate (N4) nymphal instar the adult characters appeared after two moults. Similar findings relating to the precocious apparition of adult characters after depleting Met in pre-final juvenile stages have been reported in both hemimetabolan (*P. apterus*) and holometabolan (*T. castaneum*) species [7–9]. These data suggest that insects must attain a minimum critical size to activate adult morphogenesis, and that in our study the *B. germanica* N4 did not attain such a size and therefore moulted to N5 despite dsMet treatment. Moreover, adult features triggered by Met depletion were more apparent and with higher penetrance when the specimens were treated in earlier stages, N4, compared to N5, possibly because the experimental design allowed more time for tissue growth and development and for more effective Met depletion. These observations are in line with the recently proposed concept that insects acquire competence to metamorphose only in late nymphal or larval instars [19].

Met depletion in *B. germanica* provoked significant mortality and the formation of precocious, although imperfect, adults in what should be N6. This contrasts with results obtained after depleting Kr-h1 in young instars which did not provoke mortality and induced the precocious formation of practically perfect adults [13]. The precocious imperfect adult morphogenesis and higher mortality obtained after depleting Met may be due to the fact that Met is upstream of Kr-h1 and its depletion could also affect other pathways that do not directly involve Kr-h1. Secondly it may also be because the level of Met depletion, and the concomitant decrease in Kr-h1 mRNA levels, was less than that achieved when directly depleting Kr-h1 [13].

Intriguingly, BgMet was also expressed in the last (N6) nymphal instar of *B. germanica*, when JH is absent [16], and this led us to study its possible functions in this instar. When Met mRNA levels were depleted in N6, the length of the instar was extended by one day with respect to the controls and ecdysis to the adult stage was deficient in approximately half the cases. This occurred especially because the insects were incapable of fully shedding the exuvium,

which resulted in deficiencies in wing extension and other, mainly mechanical problems. Previous studies have shown that Met depletion in late larval stages of *T. castaneum* and *B. mori* provokes a certain level of mortality and ecdysis complications [7,8,20,21]; in *B. mori*, moreover, Met-depleted specimens moulted on average one day later than controls [20]. In our experiments, expression of genes involved in the ecdysone signalling pathway, such as EcR, RXR and E75A, was reduced in Met-depleted specimens, suggesting that Met is involved in this pathway. Indeed, the phenotypes obtained are reminiscent of the less affected phenotypes obtained after depleting EcR-A in the last nymphal instar of *B. germanica* [22]. Bitra and Palli [23] provided other cues that point to this involvement when they demonstrated that Met physically interacts with EcR-USP in *D. melanogaster*, while Guo et al. [20] reported that Met is required for maximum activity of the ecdysone signalling pathway during metamorphosis of *B. mori*. The malformed adult phenotypes observed after moulting problems, especially in terms of wrinkled wings and deformed tibiae and which were obtained when depleting Met in N4 and N5, suggest the hypothesis that Met is also involved in the ecdysone signalling pathway in *B. germanica*, a hypothesis that would require further study to elucidate the mechanistic aspects.

The delayed onset of moulting and ecdysis plus wing size complications, might also be caused by metabolic and growth deficiencies related to the Insulin/IGF-1 signalling (IIS) pathway. Our experiments showed that expression of *B. germanica* ILP-1 was dramatically reduced in Met-depleted specimens, which suggests that Met enhances ILP-1 transcription without the contribution of JH. JH and Met involvement in the IIS pathway has already been reported in *T. castaneum* adults [24]. Depletion of Met in adult females reduced the expression of two of the four *T. castaneum* ILPs (ILP-2 and -3, although *T. castaneum* ILPs cannot be homologated to *B. germanica* ILP-1) in brain and fat body. In the fat body, vitellogenin transcription, which is JH-dependent, was also reduced in Met-depleted specimens and the authors showed that JH-induced vitellogenin expression in the fat body is mediated by ILP signalling [24]. More recently, the same research group has shown that depletion of JH-acid-methyltransferase (a key enzyme for JH synthesis), Met or ILP-2 mRNA in starved adult males of *T. castaneum* decreased lipid and carbohydrate metabolism and extended their survival [25]. In any case, the possible involvement of Met in the insulin pathway and in the last instar nymph of *B. germanica*, when JH is absent, would merit further study to elucidate the underlying mechanisms.

As a main conclusion, our results show that Met transduces the JH signal and, therefore, represses metamorphosis in the cockroach *B. germanica*, a representative of Polyneoptera. This function of Met had previously been demonstrated in Holometabola (*T. castaneum*) and Paraneoptera (*P. apterus*), thus meaning that it is present across Eumetabola, according the phylogeny of the extant hexapod orders reported by Wheeler et al. [26]. Our results extend this function to the Polyneoptera, thereby encompassing all Neoptera. Met is also found in the firebrat *T. domestica* [9] but its function in this basal Zygentoma species is yet unknown. Given that *T. domestica* is ametabolan, Met function in this species cannot be related to metamorphosis, but it could, however, be related to JH transduction for vitellogenin production and oocyte growth, as occurs in the hemipteran *P. apterus*, according to the study of Smykal et al. [27]. If so, then the function of Met as JH signal transducer would predate the emergence of winged insects and the evolutionary innovation of metamorphosis [28].

Supporting Information

Figure S1 Comparison of BgMet with other insect Met protein sequences. In addition to the percentage of overall identity, we indicate the percentage of identity for each of the characteristic domains of the protein. The species included (and the respective code and accession number in GenBank) are: *Thermobia domestica* (TdMet, AEW22978), *Rhodnius prolixus* (RpMet, AEW22977), *Locusta migratoria* (LmMet, AHA42531), *Tribolium castaneum* (TcMet, BAG71980), *Acyrtosiphon pisum* (ApMet, XP_003246905), *Aedes aegypti* (AaMet, AAW82472), *Pyrrhocoris apterus* (PaMet, AEW22976), *Drosophila melanogaster* Germ cell-expressed (DmGCE, NP_511160), *Bombyx mori* (BmMet 2, BAJ05086), *Drosophila melanogaster* Methoprene-tolerant (DmMet, NP_511126), *Bombyx mori* (BmMet 1, NP_001108458). * indicate that the sequence lacks the region between the initial Met and the bHLH domain and a part of the latter, and ** that the sequence lacks the region between the initial Met and the bHLH domain, but the latter is complete.
(TIF)

Figure S2 Mild phenotypes obtained after treating *Blattella germanica* N4 with dsMet-a. (A) Dorsal and ventral view of a specimen that died during the ecdysis to N6; the detail shows that the lateral expansions in the mesonotum and metanotum are somewhat enlarged and apparently flexible (indicated with an arrow in the detail). (B) Dorsal and ventral view of a specimen that moulted from N6 to adult with the tegmina and wings correctly patterned but not well extended, and with the hind tibiae deformed.
(TIF)

Figure S3 Effects of Met depletion with dsMet-b in *Blattella germanica*. (A) Dorsal and ventral view of a precocious adult obtained after treating with dsMet-b in N5. (B) Dorsal and ventral view of a male precocious adult obtained after treating with dsMet-b in N4. (C) Effects, at transcript level, of dsMet-b treatment in N5; insects received a 3-μg dose in N5D0 and another on N5D3; transcript levels (of Met, Kr-h1, BR-C and Tai) were measured on N5D6. Each point represents 4 biological replicates and results are expressed as the mean ± SEM; data are normalized against the dsMock-treated samples (reference value = 1), and the asterisk indicates statistically significant differences with respect to controls ($p<0.05$), according to the REST software tool [29].
(TIF)

Table S1 Primers used to detect transcript levels by qPCR and those used to prepare the dsRNAs for RNAi experiments.
(PDF)

Table S2 Summary of the effects of Met depletion at phenotypic level in the experiments treating with dsMet-a or dsMet-b and at the stages N4, N5 or N6. In all cases the corresponding dsRNA was administered in two 3 μg-doses, one on day 0 and the other on day 3 of the given instar. Controls were equivalently treated with dsMock. Methodological details are described in the main text.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: XB JL. Performed the experiments: JL. Analyzed the data: JL XB. Contributed to the writing of the manuscript: XB JL.

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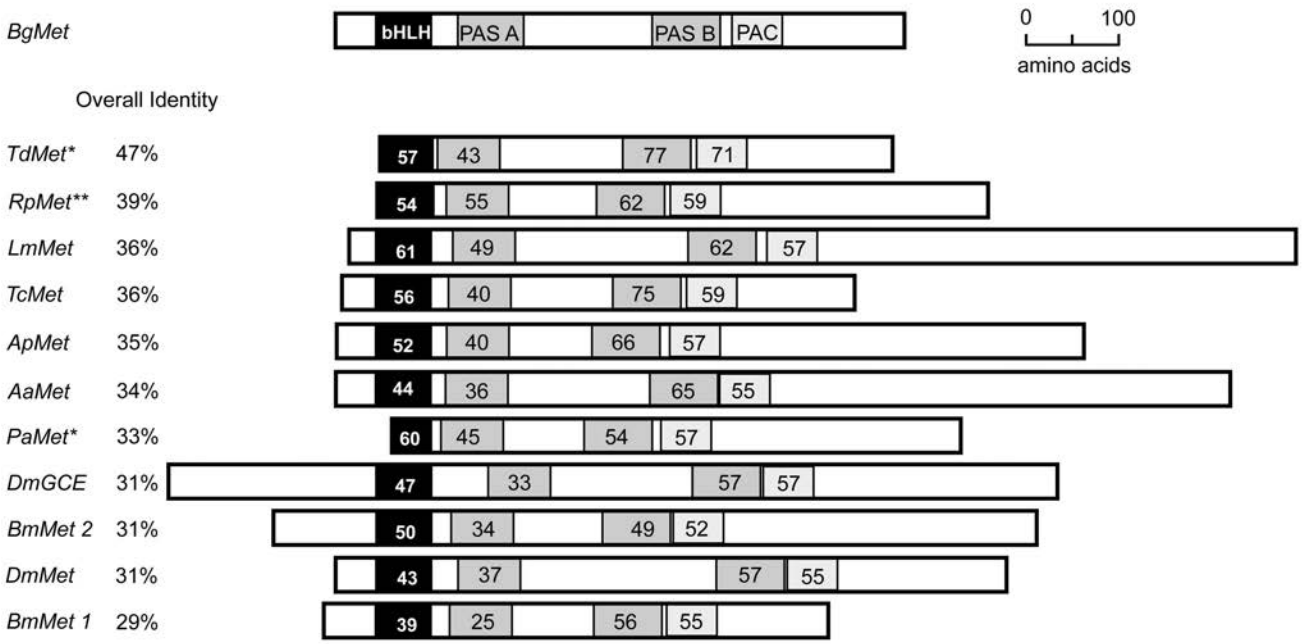
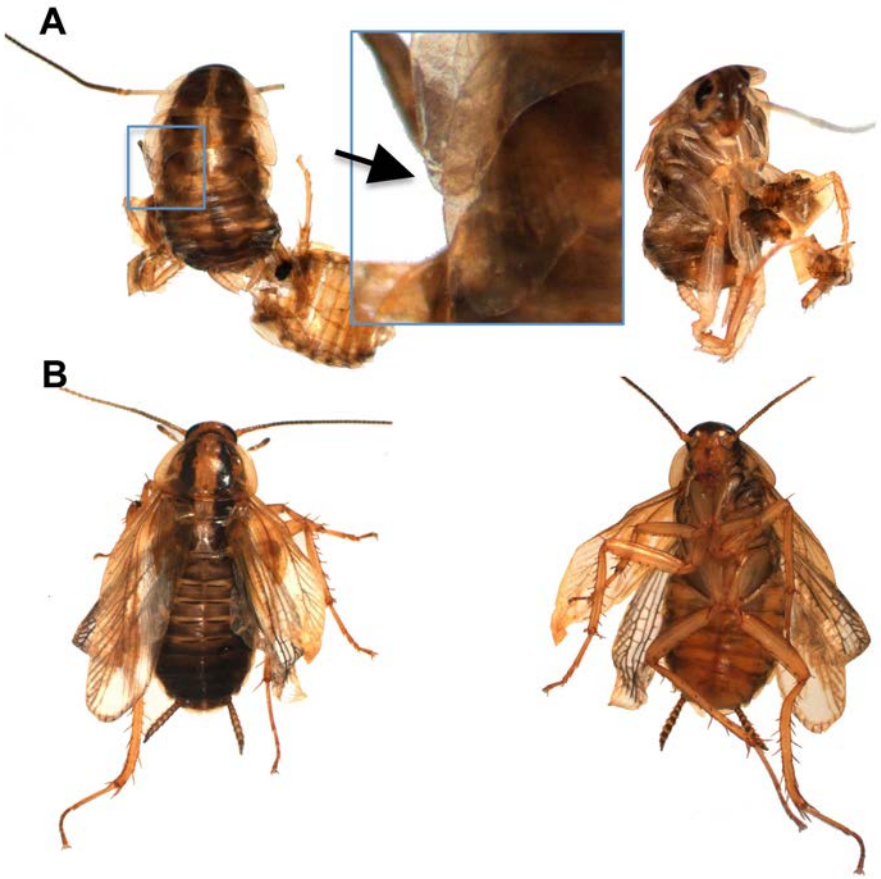
Supporting Information

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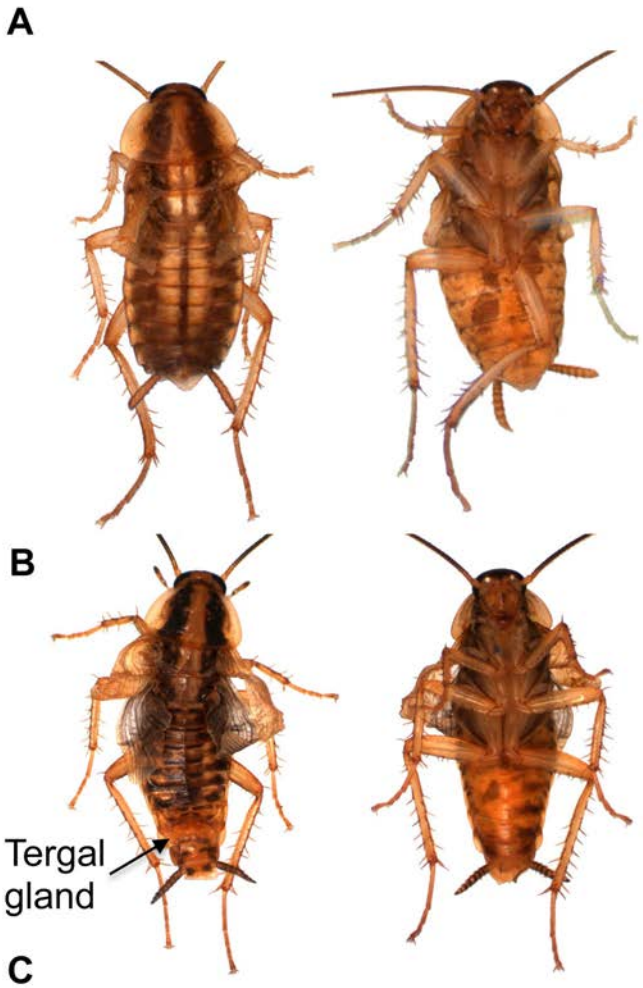
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Fold change
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Met
Kr-h1
BR-C
Tai

Table S1 – Primers used to detect transcript levels by qPCR and those used to prepare the dsRNAs for RNAi experiments.

Primer set	Length (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Encompassed Region
Met	58 bp	CTGTTGGGACATCAGCAGAA	GGCAGGTGATGGAGTGAAGT	Nucleotide 470 to 527 of HG965209
Kr-h1	77 bp	GCGAGTATTGCAGCAAATCA	GGGACGTTCTTTCGTATGGA	Nucleotide 493 to 569 of HE575250
BR-C	76 pb	CGGGTCGAAGGGAAAGACA	CTTGGCGCCGAATGCTGCGAT	Nucleotide 699 to 774 of FN651774
EcR	163 bp	GACAAACTCCTCAGAGAAGATCAAA	CTCCCAATCCTGCCAGACTA	Nucleotide 1472 to 1634 of AM039690
RXR	86 pb	ATAATTGACAAGAGGCAGAGGAA	TGAACAGCCTCCCTCTTCAT	Nucleotide 527 to 612 of AJ854490
E75A	101 bp	GTGCTATTGAGTGTGCGACATGAT	TCATGATCCCTGGAGTGGTAGAT	Nucleotide 58 to 158 of AM238653
ILP-1	96 bp	AGAAGCAGAATTCCCTTTCCG	TCATCGACAATGCCTCCGT	Nucleotide 343 to 438 of HG972850
Actin	213 pb	AGCTTCCTGATGGTCAGGTGA	TGTCGGCAATTCCAGGGTACATGGT	Nucelotide 96 to 308 of AJ862721
dsMet-a	458 pb	GCAAATTGTATCCTTCATCTGC	TGACAGACTCGCGCTTTATG	Nucelotide 1309 to 1766 of HG965209
dsMet-b	394 pb	GCAGGCGACCAAGAGTCTAC	CCACGGCAATCAGAACAGTA	Nucelotide 581 to 974 of HG965209
dsMock	307 pb	ATCCTTTCCTGGGACCCGGCA	ATGAAGGCTCGACGATCCTA	Nucelotide 370 to 676 of K01149

Table S2 –Summary of the effects of Met depletion at phenotypic level in the experiments treating with dsMet-a or dsMet-b and at the stages N4, N5 or N6. In all cases the corresponding dsRNA was administered in two 3µg-doses, one on day 0 and the other on day 3 of the given instar. Controls were equivalently treated with dsMock. Methodological details are described in the main text.

	Instar of treatment	**N**	**Died before N6**	**Died during the ecdysis to N6**	**Nymphoid with adult features in N6**	**Precocious adult in N6**	**Died in ecdysis from N6 to adult**	**Adult with wrinkled wings after N6**	**Normal adult after N6**
dsMet-a	N4	54	9	8	**7**	**11**	10	4	5
dsMet-b	N4	10	1	2	**2**	**1**	1	2	1
dsMock	N4	27	0	0	**0**	**0**	0	0	27
dsMet-a	N5	35	0	0	**5**	**1**	5	12	12
dsMet-b	N5	25	0	0	**5**	**0**	1	12	7
dsMock	N5	19	0	0	**0**	**0**	0	0	19
dsMet-a	N6	28	-	-	**-**	**-**	5	10	13
dsMock	N6	14	0	0	**0**	**0**	0	0	14

5. Un paper per a Taiman en la metamorfosi dels insectes

Un paper per a Taiman en la metamorfosi dels insectes

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Resum

Estudis recents han mostrat que el complex format per Methoprene-tolerant (Met) i Taiman (Tai) és el receptor funcional de l'hormona juvenil (HJ). Els experiments suprimint Met han confirmat el seu paper en la transducció del senyal d'JH en la repressió de la metamorfosi, però no hi ha dades equivalents respecte Tai perquè els experiments reduint l'expressió d'aquest (en l'escarabat *Tribolium castaneum* i l'hemipter *Pyrrhocoris apterus*) van resultar en una mortalitat del 100%.

Hem descobert que la panerola *Blattella germanica* posseeix quatre isoformes de Tai resultants de la combinació de dues insercions/delecions (indels) a la regió C-terminal de la seqüència. La presència d'almenys un equivalent a l'indel-1 en seqüències de Tai de *T. castaneum* i d'altres espècies suggereix que les isoformes de Tai poden ser comunes en els insectes. L'eliminació concomitant de les quatre isoformes de Tai a *B. germanica* dóna lloc a una mortalitat del 100%, però quan només es suprimeixen les isoformes que contenen la inserció 1, la mortalitat es redueix significativament i aproximadament la meitat dels exemplars tractats experimenten un desenvolupament precoç a adult, demostrant que Tai està implicat en la repressió de la metamorfosi. A més, assajos amb reportador van indicar que tant la isoforma de Tai de *T.castaneum* que conté la inserció-1 com la que no la conté presenten la capacitat, en conjunció amb Met i HJ, d'activar un element de resposta a HJ (kJHRE) de Krüppel-homolog 1.

Els resultats reforcen la hipòtesi que el receptor d'HJ per a la repressió metamorfosi és el complex HJ + Met - Tai i posa en relleu la importància de distingir les isoformes de Tai en l'estudi de funcions d'aquest factor de transcripció.

A Role for Taiman in Insect Metamorphosis

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Abstract

Recent studies in vitro have reported that the Methoprene-tolerant (Met) and Taiman (Tai) complex is the functional receptor of juvenile hormone (JH). Experiments *in vivo* of Met depletion have confirmed this factor's role in JH signal transduction, however, there is no equivalent data regarding Tai because its depletion in larval or nymphal stages of the beetle *Tribolium castaneum* and the bug *Pyrrhocoris apterus* results in 100% mortality.

We have discovered that the cockroach *Blattella germanica* possesses four Tai isoforms resulting from the combination of two indels in the C-terminal region of the sequence. The presence of at least one equivalent indel-1 in Tai sequences in *T. castaneum* and other species suggests that Tai isoforms may be common in insects and that this particular indel may have functional importance. Concomitant depletion of all four Tai isoforms in *B. germanica* resulted in 100% mortality, but when only the insertion 1 (IN-1) isoforms were depleted mortality was significantly reduced and about half of the specimens experienced precocious adult development. This shows that Tai isoforms containing IN-1 are involved in transducing the JH signal that represses metamorphosis. Reporter assays indicated that both *T. castaneum* Tai isoforms, one that contains the IN-1 and another that do not (DEL-1) activated a JH response element (*k*JHRE) in *Krüppel homolog 1* in conjunction with Met and JH.

The results indicate that Tai is involved in the molecular mechanisms that repress metamorphosis, at least in *B. germanica*, and highlight the importance of distinguishing Tai isoforms when studying the functions of this transcription factor.

Author Summary

Insect metamorphosis is one of the most fascinating processes of animal development. However, the mechanisms governing metamorphosis only started to be unveiled in the last century, when physiological research revealed that the main factor involved is juvenile hormone (JH), which represses metamorphosis in juvenile stages. Further steps to elucidate the molecular mechanisms underlying the action of JH remained elusive until recently, when the transcription factor Methoprene-tolerant (Met) was reported to be the JH receptor in the context of metamorphosis. Further experiments in vitro suggested that Met did not act alone as the JH receptor, but had to heterodimerise with another protein, Taiman (Tai). Unfortunately, Tai depletion experiments to demonstrate this protein's involvement in metamorphosis proved unsuccessful because they resulted in 100% mortality. We have discovered that Tai is expressed in a number of isoforms, and selective depletion has shown that at least some of these are involved in transducing the JH signal that represses metamorphosis. Moreover, our results show that the whole range of isoforms should be considered when studying Tai functions.

Introduction

Insect metamorphosis, either through gradual morphogenesis (hemimetaboly) or abrupt morphogenesis mediated by the pupal stage (holometaboly), is essentially regulated by two hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH). 20E triggers the successive moults throughout the life cycle, whereas JH represses metamorphosis [1-3]. A great deal of information has been obtained on the 20E signalling pathway and corresponding transcription factors, many of which belong to the nuclear receptor superfamily [4,5]. Conversely, the molecular mechanisms underlying the action of JH remained enigmatic until recently, when the transcription factor Methoprene-tolerant (Met) was reported as the JH receptor [6], and a number of components of the JH signalling pathway were identified [7,8].

Met is a member of the basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family of transcription factors that was discovered in 1986 as a factor determining resistance towards the toxic effects of the JH analogue methoprene in some *D. melanogaster* strains [9,10]. Years later, Miura *et al*. [11] showed that the *D. melanogaster* Met protein could bind JH with a very high affinity (Kd of 5.3 nM), providing the first hint that Met might play a role in JH reception. More recently, it has been observed that depleting Met mRNA levels with RNAi in early larval stages of the holometabolan species *T. castaneum* triggers precocious pupal morphogenesis [12,13], showing that Met is involved in the transduction of the anti-metamorphic JH signal. RNAi studies have also demonstrated the JH-transducing role of Met in the metamorphosis of the bug *Pyrrhocoris apterus* [14] and the cockroach *Blattella germanica* [15], both hemimetabolan species. Using Met from *T. castaneum*, Charles *et al*. [6] confirmed that the JH binding affinity is high (Kd of 2.9 nM in this case), that the PAS-B motif of Met is required and is sufficient to bind JH, and that when JH binds to a Met moiety in a Met-Met homodimer, the homodimer dissociates and JH+Met binds to another bHLH-PAS transcription factor named Taiman (Tai).

Tai was originally discovered in *Drosophila melanogaster* when searching for factors regulating border cell migration during oogenesis [16]. Later, Zhu *et al*. [17] used a yeast two-hybrid system to identify proteins interacting with the transcription factor βFtz-F1, a nuclear receptor associated with ecdysone signalling in the fat body of the mosquito *Aedes aegypti*, and they recovered a Tai homolog which they called FISC

(βFtz-F1 Interacting Steroid receptor Coactivator). In a similar yeast two-hybrid screening, Tai/FISC was again found in *A. aegypti* [18] as well as in the beetle *Tribolium castaneum* [19], this time interacting with Met. To further complicate the nomenclature, the Tai homolog of *T. castaneum* was named SRC due to its homology with mammal SRCs.

It was subsequently found that downstream of Met, the JH signal is transduced by Krüppel-homolog 1 (Kr-h1), a transcription factor with a DNA binding domain of eight zinc fingers. The function of Kr-h1 as a transducer of the anti-metamorphic action of JH was first demonstrated by ectopic expression in *D. melanogaster* [20]. Soon after, RNAi experiments in holometabolan species like *T. castaneum* [21], as well as hemimetabolan species, such as the cockroach *B. germanica* [22] and the bugs *P. apterus* and *Rhodnius prolixus* [14], revealed that depleting Kr-h1 mRNA levels in early juvenile stages triggered precocious metamorphosis in all cases.

While there is robust data available showing that Met plays a role in JH reception in metamorphosis and that Kr-h1 transduces the anti-metamorphic JH signal downstream of Met, the possible role of Tai in this process has remained unclear. Using approaches *in vitro*, Tai-dependent expression of Kr-h1 has been reported in the Aag-2 line of *A. aegypti* cells [19], in NIAS-Bm-aff3 cells of the silkworm *Bombyx mori* [23] and in Tc81 cells of *T. castaneum* [24]. The results obtained by Kakuyawa *et al*. [23] in NIAS-Bm-aff3 cells of *B. mori* are relevant because they demonstrated the occurrence of a new JH response element, *k*JHRE, in the promoter region of *B. mori* Kr-h1that contains an E-box to which bHLH-PAS proteins may bind. Reporter assays in mammalian HEK293 cells, which presumably lack JH signalling elements, showed that *k*JHRE-specific reporter activity was weakly activated in the presence of JH when the complete open reading frame (ORF) of *B. mori* Met was expressed, although reporter activity significantly increased when Tai ORF was coexpressed with Met. The results therefore suggested that Met and Tai jointly interacted with the *k*JHRE found in the Kr-h1 promoter and that this interaction was JH-dependent [23]. Equivalent work carried out on the new Tc81 embryonic cell line of *T. castaneum* [24] allowed the identification and functional characterisation of a *k*JHRE, found in the promoter region and the first intron of *T. castaneum* Kr-h1, which was identical to that reported in *B. mori*. Experiments with mammalian HEK293 cells and *Drosophila* S2 cells showed that *k*JHRE reporter activity was weakly activated when the complete ORF of *T. castaneum*

Met was expressed in the presence of JH, but when Tai ORF was coexpressed with Met, reporter activity increased [24].

Data demonstrating the involvement of Tai in metamorphosis is practically non-existent for approaches *in vivo*, not because Tai depletion experiments have been not attempted, but because these experiments, carried out on *T. castaneum* larvae [25] and *P. apterus* nymphs [26], resulted in 100% mortality. The only information *in vivo* derives from assays carried out on *T. castaneum* larvae which showed that treatment with hydroprene (a JH analogue) stimulates Kr-h1 expression, whereas depletion of Tai overrides this stimulatory effect [19]. The paradox is that current models explaining the mechanisms repressing insect metamorphosis consider Tai to be the partner of Met in the JH heterodimer receptor at the top of the repressor pathway [1,2,7,8], but, unfortunately, no convincing evidence *in vivo* of Tai's contribution to metamorphosis has been forthcoming. The results reported herein show that Tai is involved in repressing metamorphosis. The path to this conclusion involved working with the cockroach *B. germanica*, which is an especially favourable model for carrying out functional RNAi experiments, and in which different isoforms of Tai can be examined.

Results

B. germanica has alternative splicing isoforms of Tai

BLAST search of Tai in transcriptomes of *B. germanica* followed by PCR amplifications gave an ORF sequence of 4914 bp whose conceptual translation rendered a 1638 amino acid protein with sequence similarity to insect Tai/FISC/SRC proteins and that we called BgTai. The sequence contained the bHLH, PAS A and PAS B domains, as well as seven LxxLL motifs and several glutamine-rich regions characteristics of Tai/FISC/SRC proteins (Figure 1A), and top BLAST scores were obtained from Tai/FISC/SRC orthologues of other insects. The phylogenetic analysis of vertebrate SRC and insect Tai/FISC/SRC shows that insect proteins cluster a part, as a sister group of the vertebrate SRCs that is formed by three separated nodes corresponding to SRC-1, SRC-2 and SRC-3 subgroups (Figure S1). Therefore, it seems appropriate to use a single name, Taiman, for all insect representatives of the SRC superfamily of proteins. BgTai protein shows the highest amino acid identity with the Tai orthologue of the

hemimetabolan species *Pediculus humanus*, both in the overall protein sequence and in the bHLH domain (57 and 82% identity, respectively); identity values decreased for *D. melanogaster* (25 and 49%) and *A. aegypti* (32 and 51%) (Figure S2).

Four different transcripts of BgTai corresponding to four variants of the protein were amplified from RNA extracts obtained from last instar nymphs. The four variants result from the combination of two indels localized towards the C-terminal region of the sequence. The first insertion (IN-1) starts at nucleotide 4293 from the first Met triplet and has a length of 276 nucleotides, whereas the second (IN-2) starts at nucleotide 4771 and is 74 nucleotides long. The deletion corresponding to IN-2 (DEL-2) produces a frame-shift and an early in-frame stop codon that slightly truncates the protein in the C-terminal region. We named the four variants as follows: BgTai-A (which has IN-1 and IN-2, GenBank accession number HG965205), BgTai-B (has only IN-1, GenBank accession number HG965206), BgTai-C (has only IN-2, GenBank accession number HG965207), and BgTai-D (has neither IN-1 nor IN-2, GenBank accession number HG965208) (Figure 1B). The common regions of the four BgTai variants have identical nucleotide sequence, which suggests that they are alternative splicing products of the same gene.

According to public database information, an indel-1 equivalent to that found in BgTai occurs in the Tai sequences of *T. castaneum*, *D. melanogaster* and the honey bee *Apis mellifera* (Figure S3). In the case of *B. mori*, the available Tai sequence reported by Kayukawa *et al*. [23] is likely to be a DEL-1 isoform. Using *T. castaneum* specific primers and adult whole body cDNA of *T. castaneum* as a template, we amplified the region around the indel-1 and we obtained two sequences, one containing the insertion and the other without it (Figure S4), thus confirming that both IN-1 and DEL-1 variants exist in this species.

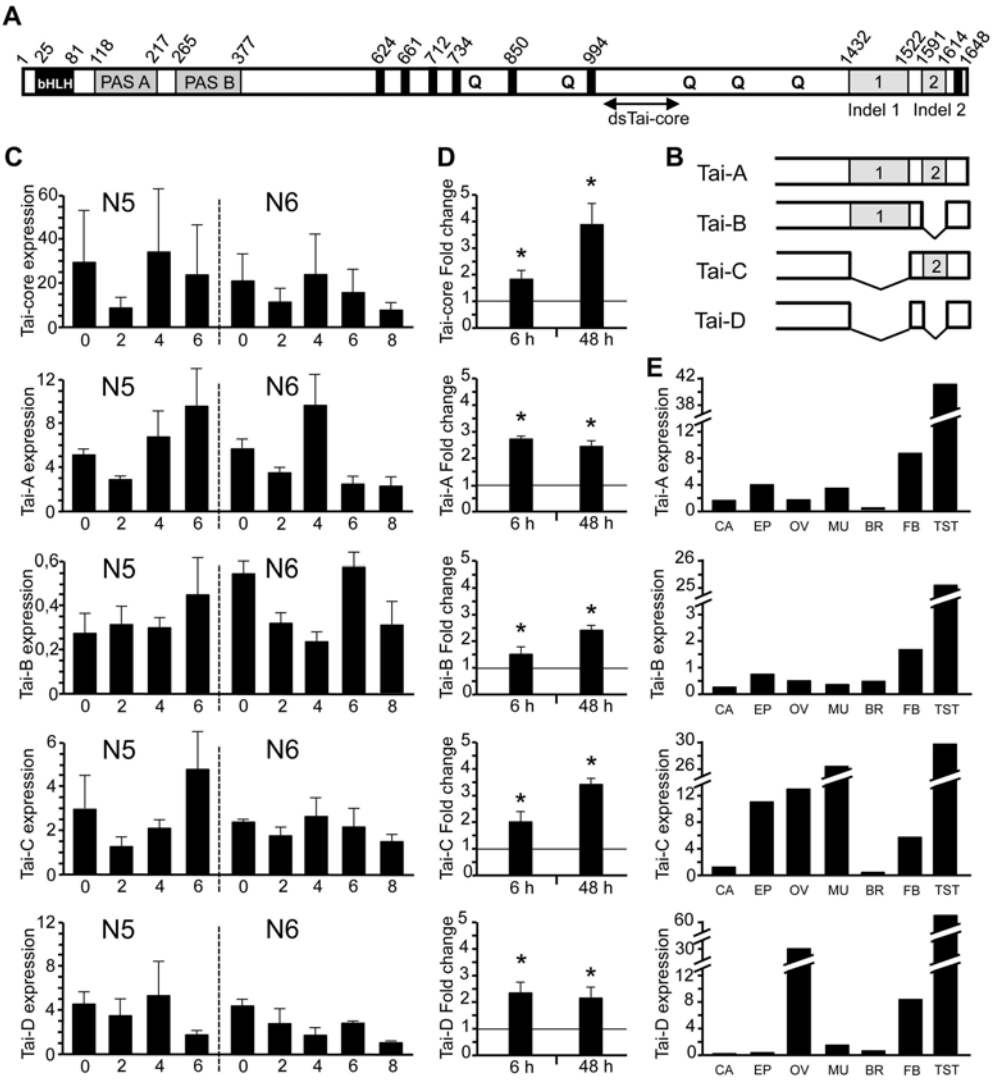


Figure 1. Structure and expression of BgTai and their isoforms in *Blattella germanica*. (A) Organization of BgTai protein in different domains; in addition to the bHLH region and the two PAS domains, it contains seven LxxLL motifs indicates as black bars, five regions rich in Glutamine, indicated with "Q", and two Insertions/Deletions towards the 3' region one (IN-1) of 276 bp and the other (IN-2) of 74 bp; also indicated is the region were the dsTai-core used in RNAi studies was designed. (B) Scheme representing the C-terminal region of the four isoforms of BgTai. (C) Expression of BgTai mRNA in whole body of female nymphs in penultimate (N5) and last (N6) instar; from top to down it is showed the expression of the ensemble of isoforms (amplified with primers designed to regions common to all them), and that of each isoform A, B, C and D (amplified with respective specific primers). (D) Effect of JH III treatment (20 µg) on BgTai expression, on the ensemble of isoforms, and specifically on each one; JH was topically applied in freshly emerged N6, and BgMet mRNA levels were measured 6 and 48 h later. (E) Expression of each BgTai isoform in different tissues of females in N6D0: *corpora allata* (CA), epidermis of the thoracic discs (EP), ovaries (O), muscle (M), brain (B), fat body (FB), and in testicles (TST) from males of the same age. Each point in C and D represents 4 biological replicates and results are expressed as the mean ± SEM, whereas those in E represent a pool of 5 specimens; data in C and E are expressed as copies of BgTai mRNA per 1000 copies of BgActin-5c mRNA; data in D are normalized against the dsMock-treated samples (reference value=1), and the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), according to the REST software tool [38].

Tai isoforms are expressed with slightly different patterns during the last nymphal instars of *B. germanica* and expression is unevenly distributed among tissues

We firstly studied the expression of BgTai in fifth (penultimate, N5) and sixth (last, N6) nymphal instars using primers designed in a region common to the four BgTai isoforms, which gave the expression pattern of the four-isoform ensemble. Values fluctuate between 10 and 30 mRNA copies per 1000 copies of actin mRNA (Figure 1C). Using isoform-specific primer pairs, we determined the expression patterns of each isoform to reveal that BgTai-A is the most abundantly expressed, followed by BgTai-C, BgTai-D and BgTai-B. The pattern of BgTai-A was similar to that of the four-isoform ensemble (denoted as "Tai-core" in Figure 1C), but the other isoforms' patterns showed slight differences amongst themselves (Figure 1C). We then determined that BgTai expression is up-regulated upon JH treatment and that the stimulatory effect is induced in all isoforms and, in general, increases with time (Figure 1D). Expression studies in different tissues revealed that all isoforms are abundantly expressed in testes; clear differential expression of the different isoforms was observed in ovaries (BgTai-D being the most expressed) and muscle (where BgTai-C is the most expressed) (Figure 1E).

Depletion of all Tai isoforms has lethal effects

To test whether BgTai has a role in *B. germanica* metamorphosis, we designed a dsRNA based on a region common to all isoforms (dsTai-core, Figure 1A) in order to deplete the four-isoform ensemble. Firstly, we injected two 3-µg doses of dsTai-core into N5 females, one when freshly emerged (N5D0) and the other on day 3 (N5D3). Controls were equivalently treated with dsMock. Transcript measurements carried out on N5D6 indicated that the whole ensemble of Tai mRNAs was significantly down-regulated (*ca.* 70%) in dsTai-core-treated specimens in comparison with controls (Figure 2A). In these specimens, mRNA levels of Met, Kr-h1 and Broad complex (BR-C, an ecdysone- and JH-dependent transcription factor that promotes wing primordia growth in *B. germanica* [27] were also significantly reduced (*ca.* 70, 65 and 70%, respectively) (Figure 2A).

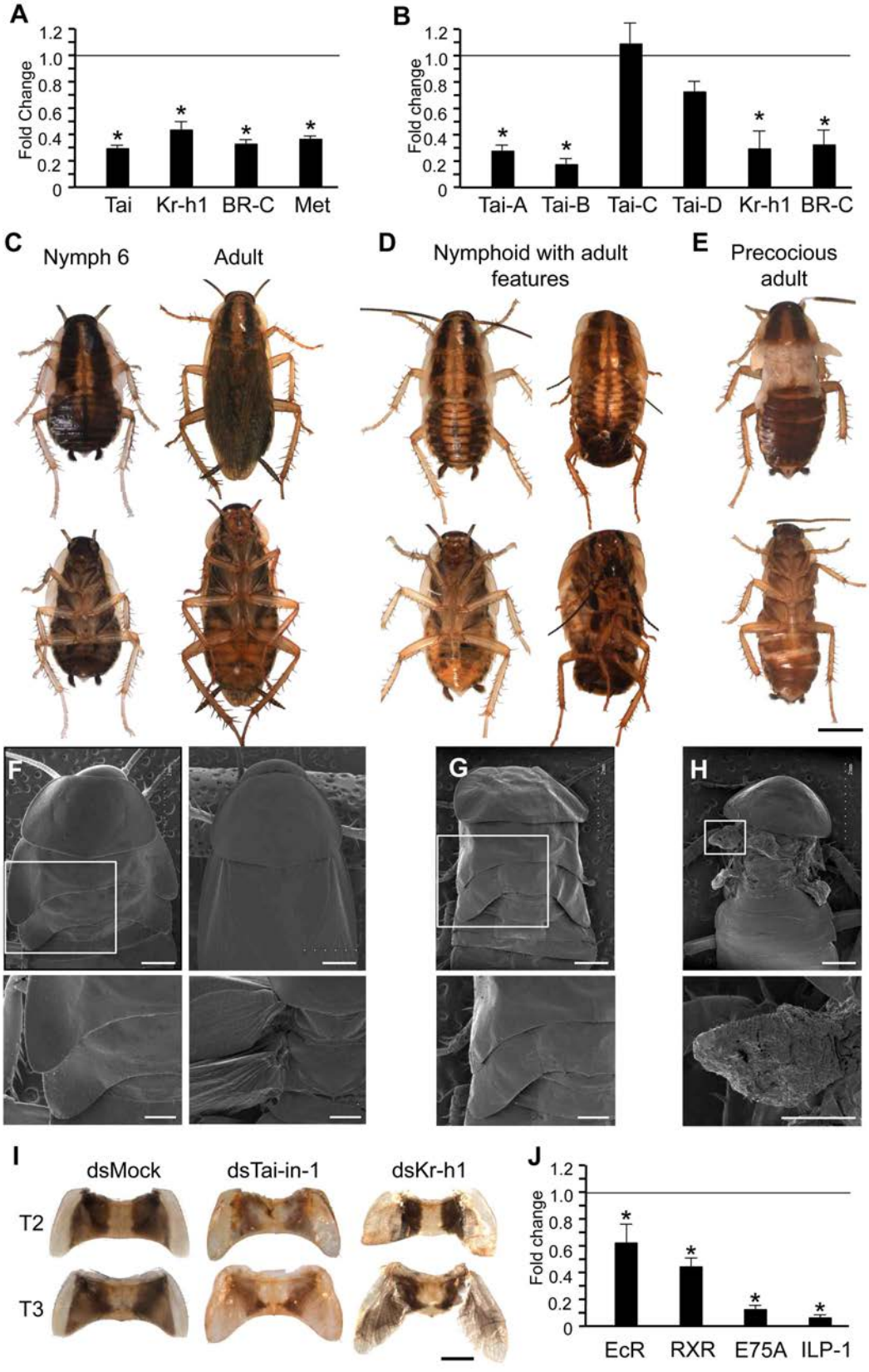
At the phenotypic level, the dsMock group (n = 14) moulted to N6 and then to normal adults, but specimens treated with dsTai-core (n = 18) died within the same instar (Table S1). These nymphs showed reduced motility, stopped growing, became darker (Figure S5A) and finally died 7 - 10 days after the last treatment. Equivalent experiments carried out on N4, injecting two 3-µg doses of dsTai-core in N4D0 and in N4D3 (n = 12), produced 100% mortality, while equivalently dsMock-treated specimens (n = 10) developed normally until the adult stage (Table S1). To reduce mortality, we administered a single 0.5-µg dose of dsTai-core in N4D0 (n = 7) or in N5D0 (n = 16), which still provoked almost 100% mortality before reaching N6 (Table S1). Treatments in N4D0 with a single 0.3-µg dose (n = 16) reduced nymphal mortality to *ca*. 40% but the survivors moulted to normal adults, in few cases with slightly wrinkled wings. Finally, treatments with 0.2 µg of dsTai-core in N4D0 (n = 12) did not trigger mortality and all specimens moulted to adults, in most cases (ca. 70%) with the wings wrinkled (results summarised in Table S1); intriguingly, when they were in N6 the lateral expansions of the mesothorax (T2) and metathorax (T3) looked somewhat longer and more transparent than normal (Figure S5B), however they all finally moulted to adults.

Depletion of BgTai isoforms containing the insertion 1 is not lethal and triggers precocious formation of adult features

Considering the possibility that each Tai isoform assumes different specific roles, we designed a dsRNA based on the 276 nucleotides of insertion 1 (dsTai-in-1), which should specifically deplete the two longer isoforms, Tai-A and Tai-B (Figure 1B). We injected two 3-µg doses of dsTai-in-1 in N4 females, one in N4D0 and the other in N4D3. Controls received an equivalent treatment with dsMock. Transcript measurements carried out on N5D6 showed that the treatment specifically targeted the long isoforms Tai-A and Tai-B, which were effectively depleted (*ca*. 75 and 85%, respectively), whereas Tai-C and Tai-D transcripts were unaffected (Figure 2B). dsTai-in-1-treatment resulted in a significant reduction of Met, Kr-h1 and BR-C mRNA levels (*ca*. 50, 70 and 65%, respectively) (Figure 2B).

At the phenotypic level, only 5 out of the 27 (19%) specimens treated with dsTai-in-1died before N6. A total of 19 out of the 22 survivors (86%), instead of moulting to normal N6, moulted to nymphoids with adult features such as a general yellowish colouration and enlarged lateral expansions in T2 and T3 that appeared flexible in the distal part. About 40% of these 19 specimens completed the ecdysis (Figure 2D, left) and remained in this stage for three to six weeks without moulting again, whereas the other ~60% did not complete the ecdysis, thus parts of the exuvium remained attached to the abdomen (Figure 2D, right); these specimens died within 3-4 days after attempting ecdysis. Finally, 3 out of the initial 22 survivors (14%) underwent apolysis to N6 but did not start ecdysis. Manual removal of the exuvium revealed that they had the general shape and colouration of a precocious adult, showing wing-like although heavily folded structures at both sides of T2 and T3 (Figure 2E, Table S1).

Figure 2. Function of BgTai in *Blattella germanica* metamorphosis. (A) Effects, at transcript level, of dsTai-core treatment in N5; N5 females received two 3-µg doses of dsTai-core, one on N5D0 and the other on N5D3, and transcript levels (of Tai, Met, Kr-h1 and BR-C) were measured on N5D6; controls received an equivalent treatment with dsMock. (B) Effects, at transcript level, of dsTai-in-1 treatment in N4; the experimental design was equivalent to that used in N5, with a double treatment, one on N4D0 and the other on N4D3; transcript levels (of Tai-A, Tai-B, Tai-C, Tai-D, Met, Kr-h1 and BR-C) were measured on N5D6. (C-E) Dorsal and ventral view of specimens resulting from dsTai-in-1 treatment in N4; normal last nymphal instar and adult obtained from dsMock treatments (C); nymphoids with adult features obtained (instead of N6) from dsTai-in-1 treatments (D); precocious adult obtained (instead of N6) from dsTai-in-1 treatments (E). (F-H) SEM images of T2 and T3 (general and detail of the lateral parts) from specimens resulting from dsTai-in-1 treatment in N4; normal last nymphal instar and adult obtained from dsMock treatments (F); nymphoid of the figure D, right (G); precocious adult from the figure E (H). (I) Dissected T2 and T3 of: N6 control (dsMock-treated), a nymphoid with adult features obtained after dsTai-in-1 treatment, a nymphoid with adult features obtained after dsKr-h1 treatment. (J) Effects of dsTai-in-1 treatment on N4 on the expression of EcR, RXR, E75A and ILP-1 measured on N5D6. Each point of quantitative data in histograms A, B and J represents 4 biological replicates and results are expressed as the mean ± SEM; data are normalized against the dsMock-treated samples (reference value=1), and the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), according to the REST software tool [38]. Scale bars in C, D, E = 3 mm, in F, G, H = upper and bottom respectively = 1 and =0.5 mm, and in I = 1 mm.


A
Fold Change
1.2
1.0
0.8
0.6
0.4
0.2
0
Tai
Kr-h1
BR-C
Met
B
Fold change
Tai-A
Tai-B
Tai-C
Tai-D
Kr-h1
BR-C
C
Nymph 6
Adult
D
Nymphoid with adult features
E
Precocious adult
F
G
H
I
dsMock
dsTai-in-1
dsKr-h1
T2
T3
J
Fold change
EcR
RXR
E75A
ILP-1

Scanning electron microscopy examination of T2 and T3 in dsMock-treated specimens (Figure 2F), compared with dsTai-in-1-treated specimens (Figures 2G and H), revealed more details of the enlarged and partially flexible lateral expansions of the "nymphoids with adult features" (Figure 2G) and the wing-like structures articulated to the pleura of the specimens categorised as "precocious adults" (Figure 2H). The wing-like structures of these precocious adults are reminiscent of the heavily folded wings forming within the wing pocket of T2 and T3 lateral expansions in last instar nymphs when observed just before the imaginal ecdysis (Figure S6). The features of T2 and T3 lateral expansions of the nymphoids with adult features obtained after dsTai-in-1 treatment (Figure 2I) are similar to those observed in nymph-adult intermediates obtained by depleting Kr-h1 with a single dsRNA treatment, as described by Lozano and Belles [22].

The recurrent moulting problems and wing size deficiencies of the specimens treated with dsTai-in-1 led us to measure transcript levels of genes related to the ecdysone signalling pathway, such as EcR, RXR and E75A, as well as those of ILP-1. In all genes, mRNA levels were significantly lower than those of the controls, especially in the case of E75A and ILP-1 (Figure 2J).

Depletion of BgTai isoforms containing the insertion 2

As a next step, we attempted the depletion of the isoforms containing the insertion 2, using a dsRNA encompassing the 74 nucleotides of this insertion (dsTai-in-2). This dsRNA should specifically deplete the isoforms Tai-A and Tai-C (Figure 1B). We injected two doses of 3 µg each of dsTai-in-2 in N4 females, one in N4D0 and the other in N4D3, whereas controls received an equivalent treatment with dsMock. On N5D6, transcript measurements indicated that dsTai-in-2 targeted the isoforms Tai-A and Tai-C, as expected, which resulted in a rather modest depletion (approximately 55 and 60% in the case of Tai-A and Tai-C, respectively), whereas Tai-B and Tai-D transcripts were unaffected (Figure 3A). These treatments with dsTai-in-2 did not reduce the mRNA levels of Met, Kr-h1 and BR-C (Figure 3A). In the same samples, we also measured mRNA levels of the ecdysone pathway genes (EcR, RXR and E75A), as well as those of ILP-1, and also in these genes, dsTai-in-2-treatment did not reduce

transcript levels; only Kr-h1 showed somewhat lower average levels, although differences with respect to controls were not statistically significant (Figure 3B).

A total of 33 dsTai-in-2-treated specimens were left alive, and 3 of them (9%) died in N4, 5 (15%) in N5, 4 (12%) in N6 and 4 (12%) during the adult molt. A total of 17 (52%) survived during all the experiment and molted to normal adults (Table S1). In all cases, the morphology of N5, N6 and adult of these specimens was identical to that of controls, and they did not experience ecdysis problems, neither in the nymphal nor in the imaginal molts. These results must be considered with caution due the modest depletion of Tai-A and Tai-C reached in dsTai-in-2-treated specimens.

Finally, we depleted the isoforms containing both insertions 1 and 2, by injecting dsTai-in-1 (3 µg) and dsTai-in-2 (3 µg) in N4D0 in a single injection, and repeating the treatment in N4D3. These treatments should target Tai-A, Tai-B and Tai-C isoforms (Figure 1B). Controls received two 6-µg doses of dsMock, one in N4D0 and the other in N4D3. Transcript measurements performed on N5D6, indicated that treatment with dsTai-in-1 plus dsTai-in-2 significantly reduced transcript levels of Tai-A (ca. 70%) and Tai-B (ca. 50%), and tended to reduce (ca. 40%) those of Tai-C. Tai-D transcripts were unaffected (Figure 3C). Transcript levels of Met and BR-C were higher with respect to controls, whereas those of Kr-h1 tended to be lower (Figure 3C). Transcript levels of EcR were not modified, and those of RXR, E75A and ILP-1tended to be higher than in controls (Figure 3D).

With respect to the phenotype, 7 out of the 20 specimens treated with dsTai-in-1 plus dsTai-in-2 died shortly after the second injection. The remaining 13 specimens molted to N5 normally (Figure 3E) and then moulted to nymphoids with adult features like a general yellowish colouration and enlarged and more or less flexible lateral expansions in T2 and T3 (Figure 3F, Table S1). The morphology of these specimens reminded that of the 86% of nymphoids with adult features obtained in the treatments with dsTai-in-1 (cf. Figure 2D). These 13 specimens completed the ecdysis normally and remained in this N6 nymphoid stage for three to four weeks without moulting again and then died. The dsMock-treated controls (n = 15) moulted to N5 and then to N6 (Figure 3G) and to adult normally.

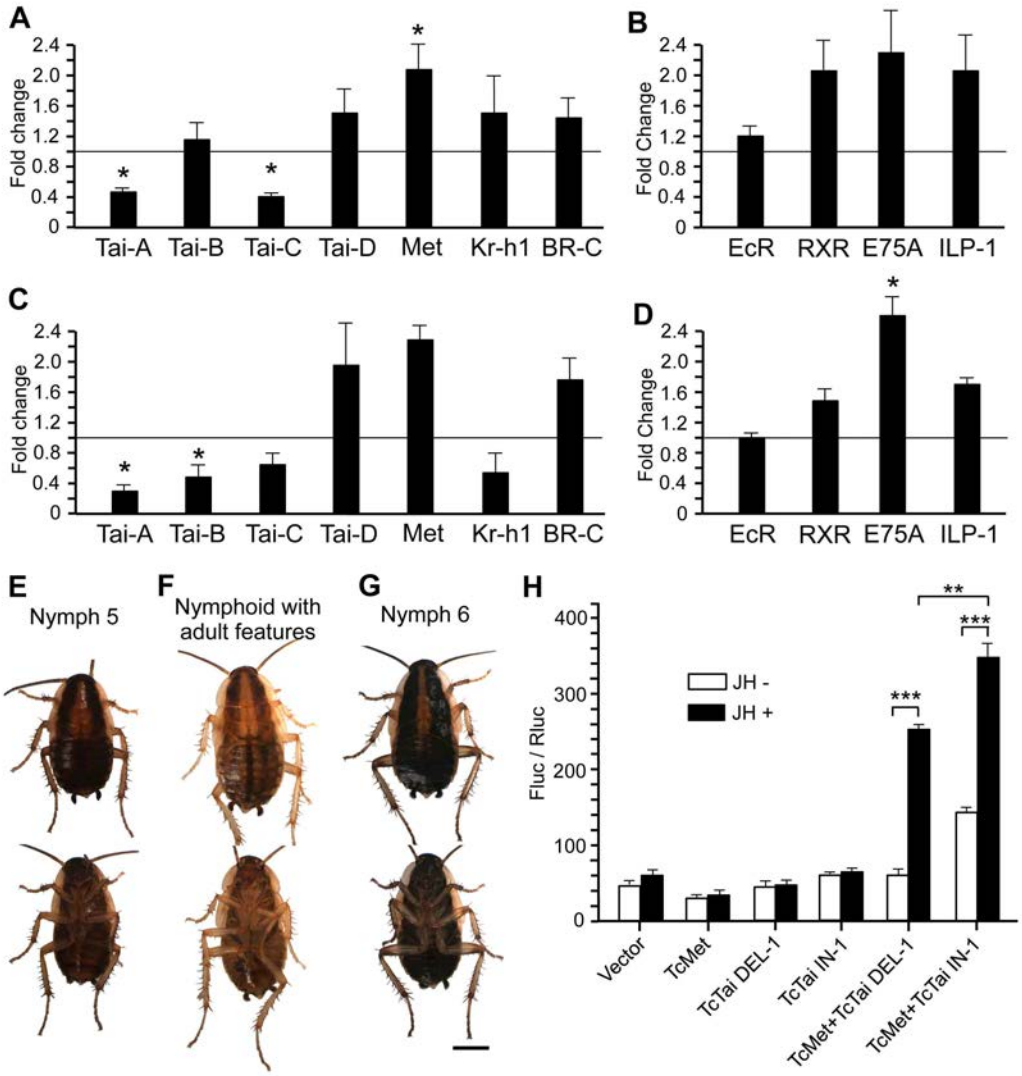


Figure 3. Functional study of IN-2-containing isoforms of BgTai and reporter assays to assess the contribution of IN-1 of TbTai to transduce the JH signal. (A) Effects, at transcript level, of dsTai-in-2 treatment in N4; N4 females received two 3-µg doses of dsTai-in-2, one on N4D0 and the other on N4D3, and transcript levels (of Tai-A, Tai-B, Tai-C, Tai-D, Met, Kr-h1 and BR-C) were measured on N5D6; controls received an equivalent treatment with dsMock. (B) Effects of the dsTai-in-2 treatment on the expression of EcR, RXR, E75A and ILP-1 measured also on N5D6. (C) Effects, at transcript level, of dsTai-in-1 plus dsTai-2 treatment in N4; specimens received an injection of dsTai-in-1 (3 µg) plus dsTai-in-2 (3 µg) in N4D0, and the treatment was repeated in N4D3; controls received an equivalent treatment with dsMock. (D) Effects of the dsTai-in-1 plus dsTai-in-2 treatment on the expression of EcR, RXR, E75A and ILP-1 measured also on N5D6. (E-G) Dorsal and ventral view of specimens resulting from dsTai-in-1 plus dsTai-in-2 treatment; normal N5 obtained from this or from dsMock treatments (E); nymphoids with adult features obtained (instead of N6) from dsTai-in-1 plus dsTai-in-2 treatments (F); normal N6 obtained from dsMock treatments (G). Each point of quantitative data in histograms A to D represents 4 biological replicates and results are expressed as the mean ± SEM; data are normalized against the dsMock-treated samples (reference value=1), and the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), according to the REST software tool [38]. Scale bars in E-G = 3 mm. (H) Reporter assays to study the JH-dependent interaction of TcMet and TcTai DEL-1 or TcTai IN-1 with *k*JHRE in *Drosophila* S2 cells. The cells were transfected with a *k*JHRE-reporter vector (-477 to +1883, pGL4.14), a reference reporter plasmid carrying *Renilla* luciferase (pIZT-Rluc) and a plasmid expressing the full ORF of TcMet and/or TcTai IN-1 or TcTai DEL-1. Cells were treated with 1 µM of JH III for 24 h. Reporter activity was measured using a Dual-Luciferase Reporter Assay System. Each bar indicates the mean ± SEM (n=6). The asterisks indicate statistically significant differences between measurements (***$p < 0.001$; **$p < 0.01$) using a two-tailed t-test (n=6).

Transduction of the JH signal of *Tribolium* Tai isoforms *in vitro*

We then wondered about possible differences of signal transduction efficiency in different Tai isoforms. Given the availability of tools for JH signalling analysis in *T. castaneum* [24], these mechanistic studies were conducted using the transcription factors of this species. There is no indel-2 equivalent in the two known *T. castaneum* sequences of Tai (TcTai), but there is a homologous indel 1. Therefore, we carried out the reporter assays in *Drosophila* S2 cells, using a kJHRE reporter vector [24] combined with TcMet and TcTai DEL-1 or TcTai IN-1, in the presence or absence of JH.

Results (Figure 3H) showed that the wild type reporter did not elicit any JH-dependent induction in cells whether neither TcMet nor TcTai DEL-1 or TcTai IN-1 were expressed, or in cells where only one construct, either TcMet, or TcTai DEL-1 or TcTai IN-1 was expressed, as expected. Conversely, significant JH-dependent induction was detected in cells in which both, TcMet plus TcTai DEL-1 or TcMet plus TcTai IN-1 were expressed. Comparing the results in the presence of JH, the combination TcMet plus TcTai IN-1 elicited a significantly higher response with respect to that of TcMet plus TcTai DEL-1 (Figure 3H). However, and intriguingly, TcTai IN-1 also increased a basal, JH-independent reporter activity, thus the specific activation by JH became reduced. Comparing JH-dependent activation relative to activity without JH, and separately for each set of transfected proteins, it can be estimated that TcTai DEL-1 mediates *ca*. 4-fold activation, whereas Tai IN-1 achieves *ca*. 2.5-fold activation in response to JH. Therefore, both Tai isoforms are functional in transducing the JH signal, although TcTai DEL-1 appears to elicit a higher JH-specific response than the TcTai IN-1.

Discussion

Tai isoforms

We have found a Tai homologue in *B. germanica*, as shown by sequence comparisons and phylogenetic analysis. The latter included not only insect sequences but also vertebrate homologues (SRC sequences, some of which are also known by the names AIB1, ACTR, RAC3, TRAM-1 and p160/NCoA-1) and showed that insect Tai

sequences (some of which are also known by the names FISC, SRC, NRC3 and NcoA) cluster a part, as a sister group of the vertebrate SRC cluster. Considering the above, we propose using the name Taiman (Tai) for all insect representatives of the SRC superfamily of proteins, given that they cluster as a well differentiated monophyletic group within the SRC superfamily tree and because Taiman was the first name coined for an insect SRC [16].

Previous studies on insect Tai homologues, whether in *D. melanogaster* [16], *A. aegypti* [17,18], *T. castaneum* [19] or *P. apterus* [26], did not provide evidence of isoforms in the C-terminal region of the molecule. In *B. germanica*, we report four isoforms resulting from the combination of two indels in the C-terminal region of Tai. The presence of at least one equivalent indel-1 in Tai sequences of other insects, such as *T. castaneum*, suggests that this kind of Tai isoforms might be common in insects, and that this particular IN-1 can be of functional importance. Our observations that Tai isoforms are expressed with slightly different patterns during the last nymphal instars of *B. germanica* and at different levels depending on the tissue indicate that they may have specific functions. In the fat body, for example, isoforms A, C and D are well expressed, which can be related with the vitellogenic role of JH in this species [28], as occurs in *P. apterus*, where Tai is involved in the transduction of the JH vitellogenic signal [26]. In the ovary, the high expression of the C and D isoforms can be related with oogenesis processes, as occurs in *D. melanogaster* [16]; high expression of Tai in the ovaries has been also observed in *A. aegypti* [17]. The dramatically high expression of all BgTai isoforms in testes is interesting and merits a functional study.

Tai and metamorphosis

Depletion of the four Tai isoforms (Tai-A to Tai-D) in *B. germanica* with effective doses of dsTai-core resulted in 100% mortality. However, we were able to determine the expression levels of the JH-dependent gene Kr-h1, which were lower than in controls, thus affording initial indications of possible functions of Tai in metamorphosis. Totally lethal effects were also observed after Tai depletion in larval stages of *T. castaneum* [25] and nymphs of *P. apterus* [26], which precluded any conclusion about Tai function in metamorphosis at the phenotypic level. In *T.*

castaneum, Tai depletion in larvae affected lipid metabolism, which might contribute to the growth arrest and ultimately the mortality observed in these specimens [25].

When only the longer Tai-A and Tai-B isoforms were selectively and efficiently depleted (*ca*. 75 and 95%, respectively) in antepenultimate nymphal instar of *B. germanica*, mortality was only 18%, Kr-h1 expression was again reduced and, more interestingly, the experimental specimens underwent precocious morphogenesis of adult features after two moults (in what should be the last nymphal instar). These observations suggest that at least Tai-A or Tai-B or both are involved in transducing the JH signal that prevents metamorphosis in *B. germanica*. When the isoforms Tai-A and Tai-C were depleted (*ca*. 55 and 60%, respectively), mortality was higher (48%), Kr-h1 levels were not affected, and survivors moulted to N6 and to adult normally. These negative results at phenotypic levels might be due to the modest levels of depletion reached by the dsRNA used, limited to a length of 74 nucleotides. Finally, when Tai-A, Tai-B and Tai-C were simultaneously depleted (*ca*. 70, 50 and 40%, respectively), mortality was intermediate (35%), Kr-h1 mRNA levels tended to decrease (by 40%), but with no statistically significant differences in comparison with controls, and insects moulted to N5 normally and then to N6 that showed faint adult features (yellowish colouration and enlarged and flexible lateral expansions in T2 and T3). Intriguingly, whereas the expression of Met and ecdysone and insulin signalling pathway genes are down-regulated in specimens where IN-1 Tai isoforms were depleted, the expression of these genes increased when IN-2 Tai isoforms were depleted, which suggests that different isoforms may have different effects. Effects of double depletion, of IN-1 and IN-2 isoforms at transcript levels were relatively similar to those observed when depleting IN-2 isoforms, but the phenotypes obtained reminded the mild ones observed when depleting IN-1 isoforms.

It is difficult to interpret the combination of all results, especially because the RNAi of isoforms containing the IN-2 (Tai-A and Tai-C) was rather inefficient probably due to the short length of the dsRNA used, based on this IN-2. The more robust results were obtained when depleting the isoforms containing the IN-1 (Tai-A and Tai-B), which showed that at least these isoforms are involved in transducing the anti-metamorphic signal of JH to Kr-h1. These results, however, do not rule out the possibility that the other isoforms are also involved in this role (as well as in other more vital functions), as our experiments cannot discard some degree of functional

redundancy between all them. Thus, the main conclusion derived from these RNAi studies *in vivo* is that one or more isoforms of Tai participate in the transduction of the JH signal that represses metamorphosis, which stands in itself as an innovative information.

According to the RNAi information, coupled with the available data in vitro that demonstrates the interaction of Met with Tai, and the functionality of the heterodimer Met-Tai as JH receptor [6,18,19,23], we propose that one or more isoforms of Tai function as partners of Met in the reception of JH in metamorphosis repression in *B. germanica*. The JH signal would then be transduced to Kr-h1, which plays the role of adult morphogenesis master repressor, as its specific depletion triggers a clear-cut precocious metamorphosis, as evidenced elsewhere [22]. As there are no robust genetic information and tools in *B. germanica* to examine functional differences of different isoforms in the transduction of the JH signal to Kr-h1, we used *T. castaneum* for this purpose as TcTai sequences present an indel-1 (Figure S3) homologous to that of BgTai. Reporter assays indicated that both TcTai IN-1 and TcTai DEL-1 activated the kJHRE reporter when co-expressed with Met in the presence of JH. Intriguingly, TcTai DEL-1 was more efficient than TcTai IN-1, although the differences are minor and support the suggestion that all indel Tai isoforms may be involved in transducing the anti-metamorphic signal of JH to Kr-h1, although the biological context and the transduction efficiency can determine the use of either isoform.

Tai versatility beyond metamorphosis

Moreover, Tai proteins can heterodimerise with Met and play roles in processes other than metamorphosis, like in the induction of vitellogenin transcription [26,29]. They can bind to other partners, i.e., to other bHLH proteins or to nuclear receptors, like βFtz-F1 [17], thus contributing to modulate the expression of other genes in other pathways. Indeed, our results depleting BgTai indicate that in *B. germanica* it functions in the ecdysone signalling pathway, which has been previously reported in *D. melanogaster* [16] and *A. aegypti* [17], as well as in the insulin signalling pathway, a function that would deserve further research. Indeed, the information available indicates that Tai proteins may have different partners, which allows the possibility to perform a variety of tasks, and the data reported herein suggest that Tai isoforms may be

influential in determining which partners are chosen and which roles are played. In mammals, SRC1 has at least 5 isoforms that differ in the C-terminal region [30] and which affect the interaction with nuclear receptors in different hormone signalling pathways [31-33]. In light of such versatile interaction capabilities and actions, vertebrate SRCs have been qualified, admittedly rather pompously, as masters of systems biology [34]. By extension, the Tai subfamily of SRC proteins may also play central roles in the circuitries regulating homeostasis and developmental processes in insects. Although this is a world yet to be explored, we predict that the Tai gene will fulfil a variety of roles in most cases taking advantage of the possibilities for versatility and promiscuity provided by the splicing isoforms.

Materials and methods

Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 ºC and 60–70% relative humidity. Freshly ecdysed female nymphs were selected and used at the appropriate ages. They were anaesthetized with carbon dioxide prior to injection treatments, dissections and tissue sampling.

RNA Extraction and retrotranscription to cDNA

We performed total RNA extraction from the whole body or specific tissues if indicated, using the miRNeasy extraction kit (QIAGEN). A 500-ng sample from each RNA extraction was treated with DNase (Promega) and reverse transcribed with first Strand cDNA Synthesis Kit (Roche) and random hexamers primers (Roche). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies).

Cloning and sequencing of BgTai and indel 1 fragment of TcTai cDNA

BgTai and ILP-1mRNAs were obtained from a *B. germanica* transcriptome prepared with RNA extracts from whole body of penultimate instar female nymphs and sequenced with a 454 Junior sequencer (Roche, Barcelona, Spain) at the Technical and Scientific Services of the Biomedical Research Park of Barcelona (PRBB). Transcriptome sequences were validated with RT-PCR using specific primers and cDNA from penultimate instar female nymphs of *B. germanica* as a template. Further 3' and 5' rapid amplification of cDNA ends (RACE, Ambion) allowed obtaining the complete ORF of BgTai and ILP-1. In the case of BgTai, 3'-RACE rendered four different isoforms based on two indels in the C-terminal region of the sequence. To amplify the fragment including the indel 1 of TcTai, we used primers based on the flanking motifs of this indel in the TcTai sequence (GenBank Accession number AB762694) (Table S2) and cDNA from last instar larvae of *T. castaneum* as a template. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced.

Determination of mRNA levels by quantitative real-time PCR

Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems). A template-free control was included in all batches. The primers used to detect mRNA levels are described in Table S2. The efficiency of each set of primers was first validated by constructing a standard curve through four serial dilutions. Levels of mRNA were calculated relative to BgActin-5c (Accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA or as standardized relative expression, setting controls levels to 1.0.

Treatments *in vivo* with juvenile hormone III

Freshly emerged last instar female nymphs of *B. germanica* were topically applied in dorsal abdomen with JH III (Sigma-Aldrich), which is the native JH of B. *germanica* [35], at a dose of 20 µg diluted in 1 µL of acetone. Controls received 1 µL of acetone alone. The commercial JH III is a mixture of isomers containing *ca*. 50% of the biologically active (10*R*)-JH III, thus the active dose applied was around 10 µg per specimen.

RNA interference

The detailed procedures for RNAi experiments were as described previously [36]. The different dsRNAs used for BgTai targeting as well as the primers to generate the corresponding templates are summarized in Table S2. The fragments were amplified by PCR and cloned into the pSTBlueTM-1 vector. A 307-bp sequence from *Autographa californica* nucleopoyhedrovirus was used as control dsRNA (dsMock). The dsRNAs were prepared as reported elsewhere [36]. A volume of 1 µL of dsRNA solution (3 µg/µL, unless stated otherwise) was injected into the abdomen of specimens at chosen ages and stages with a 5-µL Hamilton microsyringe. Control specimens were treated with the same dose and volume of dsMock.

Micrographs and Scanning Electron Microscopy

Photomicrographs were taken under bright field using a Zeiss DiscoveryV8 Stereo microscope with an AxioCam MRc digital camera. For Scanning Electron Microscopy, samples were dried at room temperature for 2 weeks, posteriorly silver coated, and observed under a Hitachi S-57 scanning electron microscope.

Reporter assays

Reporter plasmid carrying *k*JHRE and basal promoter of *TcKr-h1* (-477 to +1883) was used as described previously [24]. Expression plasmids of TcMet and TcTai DEL-1 for *Drosophila* S2 cells were constructed using the Gateway system (Invitrogen). The full ORFs of these cDNAs were amplified by PCR with Table S2 primer and pBIND_TcMet, pBIND_TcTai DEL-1 (=TcSRC) [24] as the templates, and subcloned into pIZT vector (Invitrogen). To construct expression plasmid of TcTai IN-1, the IN-1 region was amplified by PCR using Table S2 primer and pSTBlue-1_TcTai IN-1 as a template, and pIZT_TcTai DEL-1 was amplified by inverse PCR using the KOD -Plus- Mutagenesis Kit (Toyobo) with Table S2 primer and pIZT_ TcTai DEL-1 as a template. The amplified IN-1 was inserted to pIZT_TcTai DEL-1 by blunt-end ligation (TOYOBO). S2 cells were seeded at a density of 2×10^5 cells/well in 200 μl medium in a 96-well plate (Iwaki) one day before transfection. Transfections were performed by using the Fugene HD (Promega). The pIZT_RLuc vector containing the *Renilla* luciferase gene [37] was used as the reference. The cells were incubated for 48 h after transfection and treated with 1 μM of JH III for 24 h. They were then processed by using the Dual-Luciferase reporter assay system (Promega) in accordance with the manufacturer's instructions and analyzed with a luminometer (ARVO, PerkinElmer).

Acknowledgements

Thanks are due to Jose Luis Maestro for sharing data on ILPs expression in *Blattella germanica* and for helpful discussions, and Guillem Ylla for helping with the analysis of *B. germanica* transcriptomes, and Jose Manuel Fortuño, for technical assistance in Scanning Electron Microscopy examinations.

Supporting information

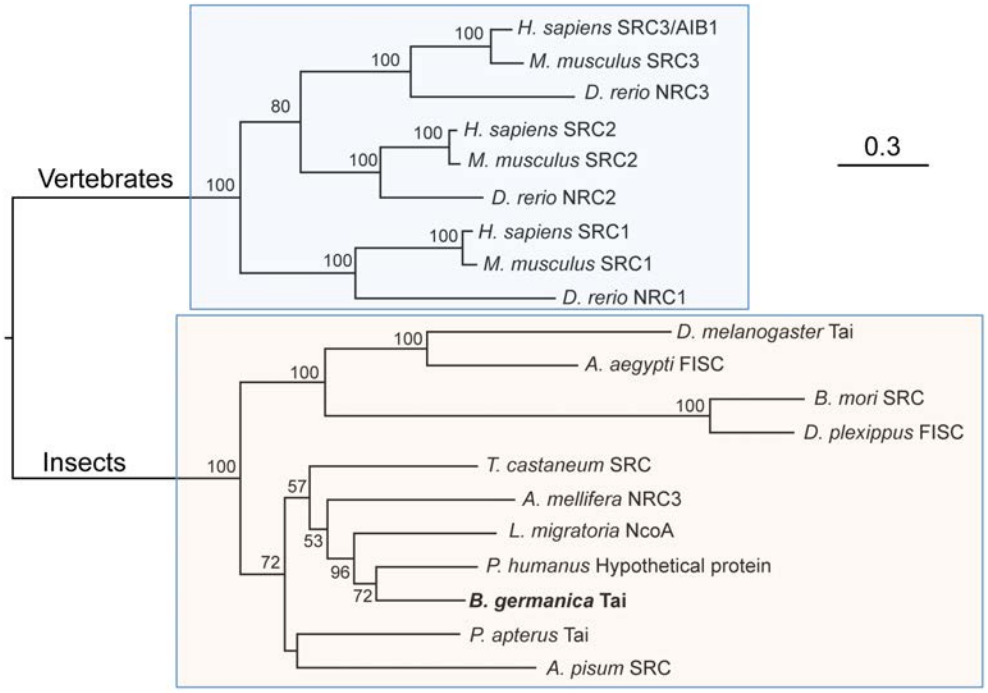


Figure S1. Phylogenetic analysis of insect Taiman/SRC/FISC proteins and vertebrate SRC proteins using maximum likelihood. The species and protein sequences included in the analysis were the following (the accession number is indicated in parenthesis; the protein name is the one used in the literature or in GenBank). Insects: *Acyrtosiphon pisum* SRC (XP_001944363); *Aedes aegypti* FISC (ABE99837); *Apis mellifera* NRC3 (XP_006563176); *Blattella germanica* Tai (CDO33883); *Bombyx mori* SRC (BAM17304); *Drosophila melanogaster* Tai (AAG16637); *Locusta migratoria* NcoA (AHA42532); *Pediculus humanus* hypothetical protein (assembly XP_002430185+XP_002430186); *Pyrrhocoris apterus* Tai/FISC (AGI17570); *Tribolium castaneum* SRC (XP_967666) and *Danaus plexippus* FISC (EHJ64466). Vertebrates: *Danio rerio* NRC 1 (XP_691744), NRC 2 (NP_571852) and NRC 3 (XP_692938); *Homo sapiens* SRC1 (NP_003734), SRC2 (NP_006531) and SRC3/AIB1 (AAC51677); *Mus musculus* SRC1 (XP_006515071), SRC2 (NP_032704) and SRC3 (NP_032705). The protein sequences were aligned using the MAFFT program (http://mafft.cbrc.jp/alignment/software), with the E-INS-I parameter. The model of protein evolution that best fits the data, determined using ProtTest 2.4 (http://darwin.uvigo.es/ software/prottest.html), was the LG+I+G+F, which was the one implemented in the maximum likelihood analyses. These were carried out with the PHYML version 3.0 program (http://www.atgc- montpellier.fr/phyml/). Data were bootstrapped for 100 replicates using the same program. Bootstrap values >50 are indicated on the corresponding node. The scale bar represents 0.3 substitutions per position.

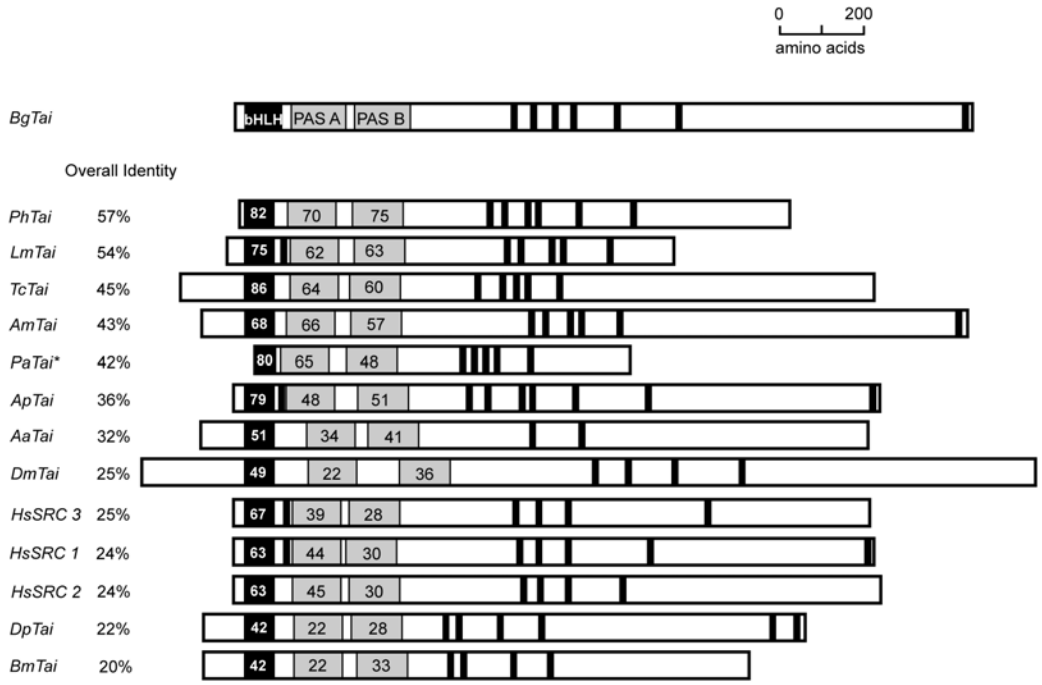


Figure S2. Comparison of BgTai with other insect Tai and human SRC protein sequences. In addition to the bHLH region and PAS domains, the LxxLL motifs are also indicated (black bars). We indicate the percentage of overall identity and the percentage of identity for each of the characteristic domains of the protein. The species included are *Pediculus humanus* (Ph), *Locusta migratoria* (Lm), *Tribolium castaneum* (Tc), *Apis mellifera* (Am), *Pyrrhocoris apterus* (Pa), *Acyrtosiphon pisum* (Ap), *Aedes aegypti* (Aa), *Drosophila melanogaster* (Dm), *Homo sapiens* (Hs), *Danaus plexippus* (Dp) and *Bombyx mori* (Bm). The original protein names and the GenBank accession number of the sequences are indicated in Figure S1. * indicates that the sequence is incomplete in the region comprised between the initial Met and the bHLH domain and towards the C-terminal region.

Figure S3. Alignment of Taiman sequences of insect species having the indel-1 found in GenBank. We included the sequences of *Blattella germanica* (Bg) (with the insertion: CDO33883 and without the insertion CDO33885), *Tribolium.castaneum* (Tc) (with the insertion: XP_967666 and without the insertion: BAN62669), *Apis mellifera* (Am) (with the insertion: XP_006563176 and without the insertion: XP_006563185) and *Drosophila melanogaster* (Dm) (with the insertion, Tai-C: NP_001188746 and without the insertion, Tai-F: NP_001188748). The protein sequences were aligned using the MAFFT algorithm following the procedure described in Figure S1 and visualized in Geneioius Software. Positions with 100% of identity are indicated in black, 80 to 99% in dark grey, 60 to 79% in bright grey and in white with less than 59%. The blue and red square frame the insertion and the deletion, respectively.

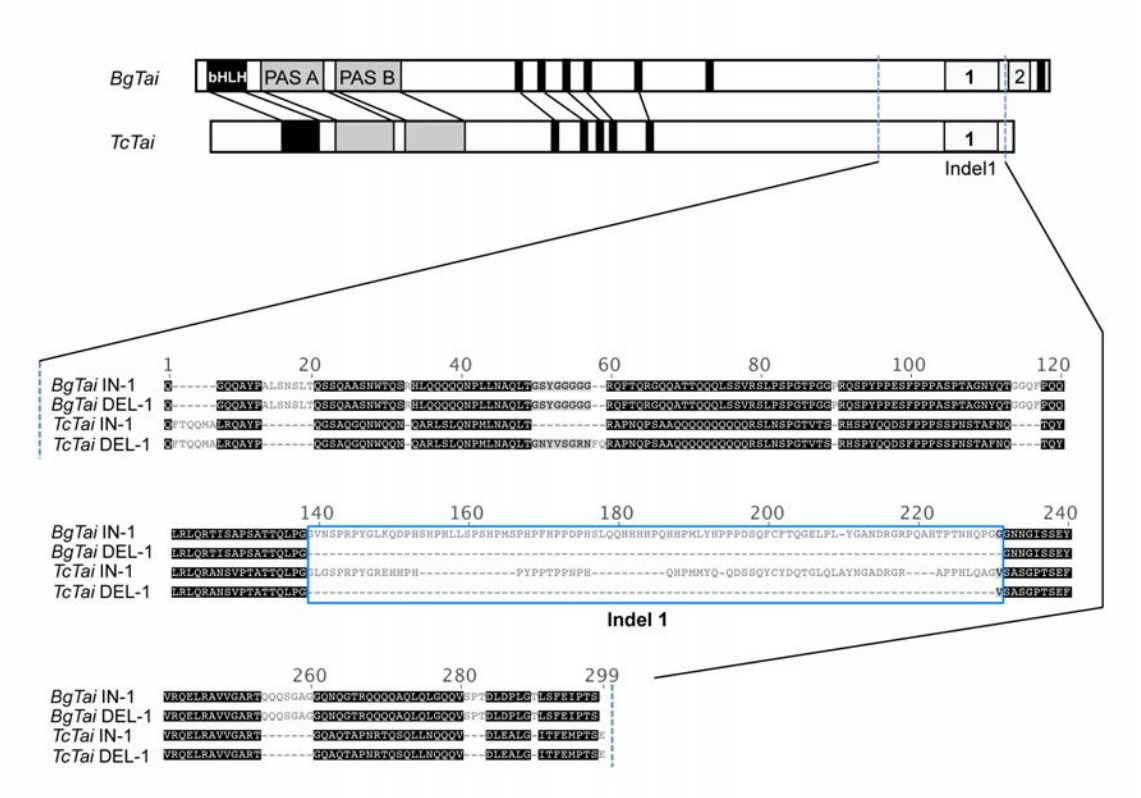


Figure S4. Sequences obtained after amplifying the region around the indel-1 in *Tribolium castaneum* Tai (TcTai) using the specific primers described in Table S2, compared with the equivalent sequences of *Blattella germanica* Taiman (BgTai). Two types of amplicons were obtained, one showing the insertion-1 (TcTai-IN-1), and the other without it (TcTai-DEL-1). Alignment and visualization was carried out as in Figure S3. The blue square frames the stretch of amino acids corresponding to the indel-1.

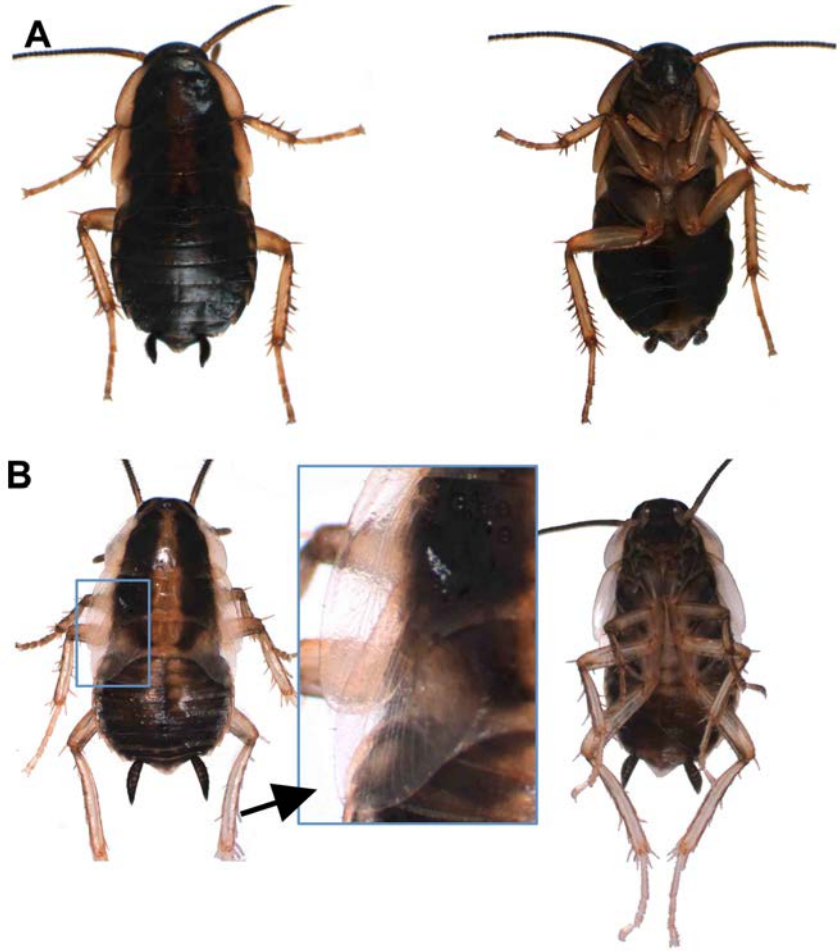


Figure S5. Phenotypes obtained after depleting the ensemble of *Blattella germanica* Tai isoforms with dsTai-core. (A) Dorsal and ventral view of a specimen that had been treated with high doses of dsTai-core (2 doses of 3 µg each, one on N5D0 and the other on N5D3) and photographed on N5D11; in general, these specimens showed reduced motility, stopped growing, became darker and finally died 7-10 days after the administration of the second dose on N5D3. (B) Dorsal and ventral view of a specimen that had been treated with a low dose of dsTai-core (1 dose of 0.2 µg on N5D0) and photographed on N6; this specimen shows the lateral expansions of T2 and T3 slightly longer and apparently more transparent than controls (indicated with an arrow in the detail), although it subsequently moulted to a normal adult.

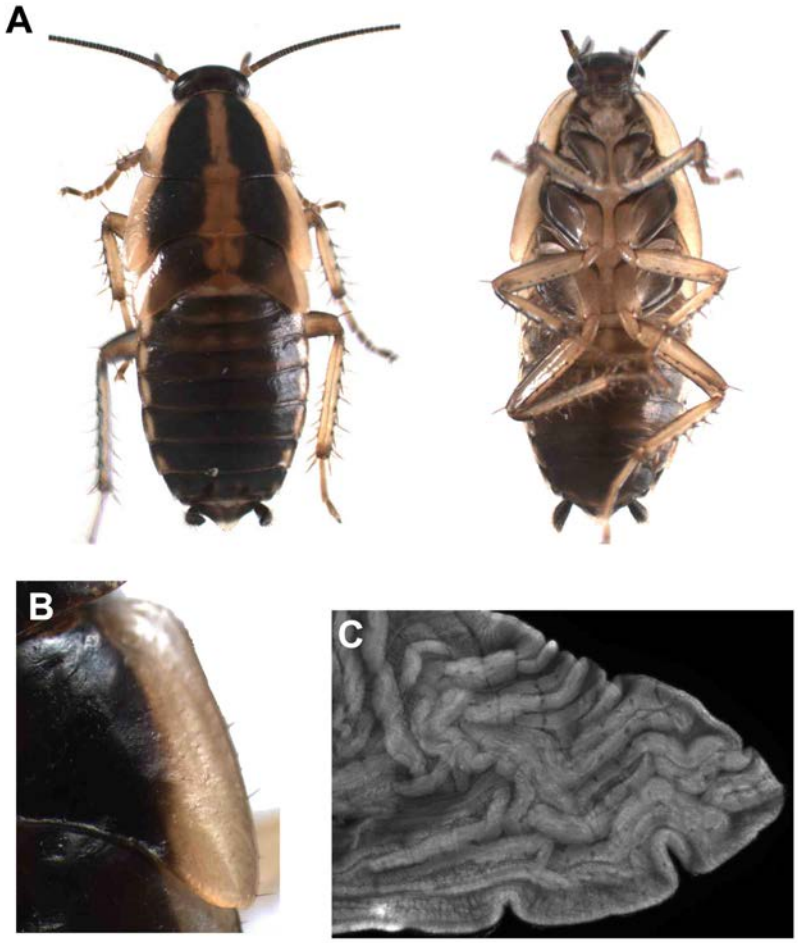


Figure S6. Wing morphology just before the imaginal ecdysis in *Blattella germanica*. (A) Normal N6 female on day 7, thus just before starting the ecdysis. (B) Detail of the right lateral expansion of T2 forming a pocket that contains the developing wing (tegmina). (C) Heavily folded developing wing (tegmina) dissected out from the lateral T2 pocket.

Table S1. Summary of the effects of Tai depletion at phenotypic level in the experiments treating with dsTai-core, dsTai-in-1 and dsTai-in-2 at the instar N4 and/or N5. The corresponding dsRNA was administered in one or two doses depending on the experiment. Controls were equivalently treated with dsMock as indicated. Methodological details are described in the main text.

Instar of treatment	Dose	dsRNA day 0	dsRNA day 3	N	Died before N6	Died N6 and Adult	**Nymphoid with adult features in N6**	**Precocious adult in N6**	Adult with stretched wings after N6	Normal adult after N6
N5	3 µg	dsTai-core	dsTai-core	18	18	0	**0**	**0**	0	0
N5	3 µg	dsMock	dsMock	12	0	0	**0**	**0**	0	12
N5	0.5 µg	dsTai-core	-	16	11	0	**0**	**0**	1	1
N4	3 µg	dsTai-core	dsTai-core	12	12	0	**0**	**0**	0	0
N4	0.5 µg	dsTai-core	-	7	7	0	**0**	**0**	0	0
N4	0.3 µg	dsTai-core	-	16	6	0	**0**	**0**	6	4
N4	0.2 µg	dsTai-core	-	12	0	0	**0**	**0**	8	4
N4	3 µg	dsTai-IN-1	dsTai-IN-1	27	5	0	**19**	**3**	0	0
N4	3 µg	dsTai-IN-2	dsTai-IN-2	33	8	8	**0**	**0**	0	17
N4	3 µg	dsTai-IN-1 + dsTai-IN-2	dsTai-IN-1 + dsTai-IN-2	20	7	0	**13**	**0**	0	0
N4	3 µg	dsMock	dsMock	15	0	0	**0**	**0**	0	15

Table S2. Primers used to detect transcript levels by qPCR in *Blattella germanica* tissues, to amplify the indel-1 region of *Tribolium castaneum* Taiman, to prepare the dsRNAs for RNAi experiments, and to amplify the full ORF of TcTai IN-1 and TcTai DEL-1 from *T. castaneum* tissues.

Primer set	Length (bp)	Forward primer (5'-3')	Reverse primer (5'-3')	Encompassed Region
Met	58 bp	CTGTTGGGACATCAGCAGAA	GGCAGGTGATGGAGTGAAGT	Nucleotide 470 to 527 of HG965209
Kr-h1	77 bp	GCGAGTATTGCAGCAAATCA	GGGACGTTCTTTCGTATGGA	Nucleotide 493 to 569 of HE575250
BR-C	76 pb	CGGGTCGAAGGGAAAGACA	CTTGGCGCCGAATGCTGCGAT	Nucleotide 699 to 774 of FN651774
Tai	50 pb	GCAGCGAGTAATTGGACACA	TTTTGCTGTTGCTGTTGGAG	Nucleotide 4288 to 4337 of HG965205
EcR	163 bp	GACAAACTCCTCAGAGAAGATCAAA	CTCCCAATCCTGCCAGACTA	Nucleotide 1472 to 1634 of AM039690
RXR	86 pb	ATAATTGACAAGAGGCAGAGGAA	TGAACAGCCTCCCTCTTCAT	Nucleotide 527 to 612 of AJ854490
E75A	101 bp	GTGCTATTGAGTGTGCGACATGAT	TCATGATCCCTGGAGTGGTAGAT	Nucleotide 58 to 158 of AM238653
ILP-1	96 bp	AGAAGCAGAATTCCCTTTCCG	TCATCGACAATGCCTCCGT	Nucleotide 343 to 438 of BgILP-1 (unpublished)
Tai-A	260 bp	CACACACCAACCAATCACCA	CCCCAAAGTTGCTTGCTATC	Nucleotide 4870 to 5129 of HG965205
Tai-B	241 bp	CACACACCAACCAATCACCA	CATAGTATTCAGAGGTAGGAA	Nucelotide 4870 to 5110 of HG965206
Tai-C	241 bp	AGTTGCCAGGTGGTAACAATG	CCCCAAAGTTGCTTGCTATC	Nucleotide 4613 to 4852 of HG965207
Tai-D	222 pb	AGTTGCCAGGTGGTAACAATG	CATAGTATTCAGAGGTAGGAA	Nucleotide 4613 to 4834 of HG965208
Actin	213 pb	AGCTTCCTGATGGTCAGGTGA	TGTCGGCAATTCCAGGGTACATGGT	Nucelotide 96 to 308 of AJ862721
dsTai-core	343 bp	CAACTTCAACAGCAGCAGCAA	AAGGACTCTTCATTACTGTT	Nucleotide 3169 to 3511 of HG965205
dsTai-IN-1	276 pb	TGGAGTAAACTCCCCGCGGC	CCACCGGGCTGGTGATTGGT	Nucleotide 4623 to 4789 of HG965205
dsTai-IN-2	74 pb	GGTGCCAGTGATAGCAAGCA	CTTGAGGAGGAGAGCCCGGG	Nucleotide 5101 to 5174 of HG965205
TcTai indel 1	551 bp	ACAGTTCACGCAACAGATGG	AACTCAGACGTGGGCATTTC	Nucleotide 3777 to 4490 of XM_962573
dsMock	307 bp	ATCCTTTCCTGGGACCCGGCA	ATGAAGGCTCGACGATCCTA	Nucleotide 370 to 676 of K01149

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6. Les funcions de Broad-complex en desenvolupament postembrionari de la panerola *Blattella germanica* proporcionen una visió més clara sobre l'evolució de la metamorfosi dels insectes

Les funcions de Broad-complex en desenvolupament postembrionari de la panerola *Blattella germanica* proporcionen una visió més clara sobre l'evolució de la metamorfosi dels insectes

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Resum

Antecedents: La metamorfosis dels insectes procedeix de dues maneres: hemimetàbola, canvi gradual al llarg del cicle de vida; i holometàbola, caracteritzada per un canvi sobtat de larva a adult mediat per una fase de pupa. Tots dos tipus de meatmorfosi estan regulats per la 20-hidroxiecdisona (20E), que promou la muda, i per l'hormona juvenil (HJ), que reprimeix la morfogènesi adulta. L'expressió de Broad-complex (BR-C) és induïda per la 20E i modulada per l'HJ. En les espècies holometàboles, com *Drosophila melanogaster*, l'expressió de BR-C és inhibida en larves joves per HJ i augmenta en larves madures, quan l'HJ declina i l'expressió de BR-C especifica l' estat de pupa.

Mètodes: Utilitzant *Blattella germanica* com a model hemimetàbol basal, i RT-PCR quantitativa, es van determinar els patrons d'expressió d'mRNA de les diferents isoformes de BR-C, i posteriorment es van estudiar les funcions de les mateixes amb RNA d'interferència.

Resultats: Vam trobar que l'expressió de BR-C es activada per l'HJ i es correlaciona amb la concentració d'HJ a l'hemolimfa. Els diferents factors de BR-C semblen estar implicats en la divisió cel·lular i el creixement del primordi alar, així com el patró de venació de l'ala.

Conclusions: A *B. germanica* , l'expressió de BR-C és regulada per l'HJ, i els factors de BR-C semblen promoure el creixement de l'ala per assolir la mida, forma i patró adequats, la qual cosa contrasta amb la regulació endocrina i funcions complexes observades en espècies holometàboles.

Significat General: Els nostres resultats aporten noves dades per clarificar l'evolució d'hemimetàbols a holometàbols respecte a BR-C, el qual se va veure afectat en regulació i funcions per dues innovacions en aquesta evolució: 1) un canvi en l'acció de l'HJ sobre l'expressió de BR-C durant les etapes joves, de estimulant a inhibidora, i 2) una expansió de funcions, des de la regulació del desenvolupament de l'ala, a la determinació de la morfogènesi de la pupa.

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Broad-complex functions in postembryonic development of the cockroach *Blattella germanica* shed new light on the evolution of insect metamorphosis

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ABSTRACT

Background: Insect metamorphosis proceeds in two modes: hemimetaboly, gradual change along the life cycle; and holometaboly, abrupt change from larvae to adult mediated by a pupal stage. Both are regulated by 20-hydroxyecdysone (20E), which promotes molts, and juvenile hormone (JH), which represses adult morphogenesis. Expression of Broad-complex (BR-C) is induced by 20E and modulated by JH. In holometabolous species, like *Drosophila melanogaster*, BR-C expression is inhibited by JH in young larvae and enhanced in mature larvae, when JH declines and BR-C expression specifies the pupal stage.
Methods: Using *Blattella germanica* as a basal hemimetabolous model, we determined the patterns of expression of BR-C mRNAs using quantitative RT-PCR, and we studied the functions of BR-C factors using RNA interference approaches.
Results: We found that BR-C expression is enhanced by JH and correlates with JH hemolymph concentration. BR-C factors appear to be involved in cell division and wing pad growth, as well as wing vein patterning.
Conclusions: In *B. germanica*, expression of BR-C is enhanced by JH, and BR-C factors appear to promote wing growth to reach the right size, form and patterning, which contrast with the endocrine regulation and complex functions observed in holometabolous species.
General significance: Our results shed new light to the evolution from hemimetaboly to holometaboly regarding BR-C, whose regulation and functions were affected by two innovations: 1) a shift in JH action on BR-C expression during young stages, from stimulatory to inhibitory, and 2) an expansion of functions, from regulating wing development, to determining pupal morphogenesis.

1. Introduction

The origin and evolution of insect metamorphosis poses one of the most enigmatic conundrums in evolutionary biology. In his "On the Origin of Species", Charles Darwin already complained about the difficulty of integrating insect metamorphosis (due to the striking difference between the morphologies and life styles of larvae and adults of the same species) into his theory of species evolution by natural selection [1]. However, it is clear that insect metamorphosis has been a key innovation in insect evolution as most of the present biodiversity on Earth is composed of metamorphosing insects, with approximately 1 million species described, and 10–30 million still to be discovered [2,3].

The first systematic studies on insect metamorphosis were carried out by Renaissance entomologists, who established that post-embryonic changes are most spectacular in insects like butterflies, beetles and flies, which undergo a dramatic morphological transformation from larva to pupa and adult, a phenomenon now known as holometaboly. Other insects, such as locusts and cockroaches, also metamorphose from the last nymphal instar to adult, although the change of form is not as radical given that the nymphs are similar to the adults. However, they undergo qualitative metamorphic changes, such as formation of mature wings and external genitalia in a type of metamorphosis known as hemimetaboly [4,5]. Metamorphosis evolved from hemimetaboly to holometaboly, and the latter innovation was most successful because more than 80% of present insects are holometabolous species (including the "big four" orders: Lepidoptera, Coleoptera, Diptera and Hymenoptera) [2,3]. Therefore, explaining the evolutionary transition from hemimetaboly to holometaboly may give a new look to explain how this amazing biodiversity originated, and the study of the processes regulating metamorphosis shall surely provide important clues for such a goal [6].

Insect metamorphosis is regulated by two hormones, the molting hormone, which promotes molting, and the juvenile hormone (JH), which represses metamorphosis and, thus determines the molt type: to an immature stage when it is present, or to the adult when it is absent [4,6,7]. Although the molecular action of JH is still poorly understood [8], we know that an important transducer of the JH signal is Methoprene tolerant (Met), a transcription factor that was discovered in *Drosophila*

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melanogaster and that plays an important role in JH reception [9]. Key functional evidence that Met is required for the repressor action of JH on metamorphosis was obtained from the beetle *Tribolium castaneum*, a basal holometabolous insect where depletion of Met expression induced larvae to undergo precocious metamorphosis [10,11]. More recently, the function of Met as an early JH transducer has been demonstrated in the hemimetabolous species *Pyrrhocoris apterus* [12], which established the first regularity in the signaling pathway of JH in hemimetabolous and holometabolous insects. Another important element in JH transduction in relation to metamorphosis is the transcription factor Krüppel homolog 1 (Kr-h1), whose antimetamorphic action was firstly demonstrated in *D. melanogaster* [13] and *T. castaneum* [14]. More recently, the role of Kr-h1 as a transducer of the JH signal has been reported in three hemimetabolous insects: the cockroach *Blattella germanica* [15] and the bugs *P. apterus* and *Rhodnius prolixus* [12]. RNAi studies in these species have shown that Kr-h1 represses metamorphosis and that it acts downstream of Met in the JH signaling pathway. Kr-h1 therefore appears to be the more distal transcription factor in the JH signaling cascade whose role as mediator of the antimetamorphic action of JH has been conserved from cockroaches to flies. The next challenge is to unveil the factor(s) specifying the adult stage that are repressed by Kr-h1.

Concerning the molecular action of molting hormones, the effect of 20-hydroxyecdysone (20E) is also mediated by a cascade of transcription factors that starts upon its binding to the heterodimeric receptor composed of the ecdysone receptor and the ultraspiracle, which belong to the nuclear receptor superfamily. This activates expression of a hierarchy of transcription factors generally belonging to the same superfamily, like E75, E78, HR3, HR4 and FTZ-F1, which regulate the genes that underlie the cellular changes associated to molting and metamorphosis [16,17]. Most of the information available on this cascade refers to *D. melanogaster* [18,19], but there are a good deal of data from hemimetabolous species, especially from the cockroach *B. germanica*. Factors involved in 20E signaling in *B. germanica* are generally the same as in *D. melanogaster*, although the functions of some of them and their epistatic relationships may differ with respect to those observed in the fly [20–22].

Among the most interesting 20E-dependent factors are the products of the Broad-complex (BR-C) gene, whose functions may have radically diverged in hemimetabolous and holometabolous species. BR-C encodes a group of C2H2 zinc-finger transcription factors [23,24] that, in holometabolous species, like the dipteran *D. melanogaster*, the lepidopterans *Manduca sexta* and *Bombyx mori*, and the coleopteran *T. castaneum*, are expressed in the final larval stage, and this transient expression is essential for the successful formation of the pupae [11,25–28]. Experiments carried out on the hemipterans *Oncopeltus fasciatus* [29] and *P. apterus* [12], which are phylogenetically distal hemimetabolous species, suggested that BR-C transcription factors only regulate gradual wing bud growth. This specific role, which is radically different from the morphogenetic functions involved in pupae formation in holometabolous species, prompted us to undertake a detailed functional study of BR-C in *B. germanica*, a basal polyneopteran insect representing a poorly modified hemimetabolous species [4]. In *B. germanica*, the BR-C gene encodes six zinc-finger isoforms (BR-C Z1 to Z6), which play important roles in embryogenesis [30]. The present work, based on functional studies in post-embryonic development, reveals ancestral functions of BR-C transcription factors related to cell division and of wing pad growth, as well as to wing vein patterning, and provides new clues that illuminate the evolution of insect metamorphosis.

2. Materials and methods

2.1. Insects

B. germanica specimens used in the experiments were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% r.h. They were carbon dioxide-anesthetized prior to dissections and tissue sampling.

2.2. RNA extraction and retrotranscription to cDNA

All RNA extractions were carried out with the Gen Elute Mammalian Total RNA kit (Sigma-Aldrich, Madrid, Spain). An amount of 400 ng from each RNA extraction was treated with DNase (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Promega). RNA quantity and quality were estimated by spectrophotometric absorption at 260 nm in a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies, Wilmington, DE, USA).

2.3. Determination of mRNA levels with quantitative real-time PCR

Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Madrid, Spain), using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems, Madrid, Spain). A control without a template was included in all batches. The primers used to detect all isoforms simultaneously or to detect each isoform specifically are described in Table S1 (see Supplementary data). The efficiency of each primer set was first validated by constructing a standard curve through four serial dilutions. mRNA levels were calculated relative to BgActin-5c (accession number: AJ862721) expression using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). The primers used to quantify BgActin-5c are indicated in Table S2 (see Supplementary data). We followed a method based in Ct (threshold-cycle) according to the Pfaffl mathematical model [31], simplifying to $2^{\Delta\Delta Ct}$ because the calculated efficiency values for studied genes and BgActin-5c amplicons were always within the range of 95 to 100%; therefore, no correction for efficiency was used in further calculations. Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA.

2.4. Treatments with juvenile hormone III in vivo

To study the effect of JH upon BR-C expression, JH III, which is the native JH of *B. germanica* [32,33], was applied topically to freshly emerged last instar nymphs at a dose of 20 μg per specimen in 1 μl of acetone. We used JH III from Sigma-Aldrich, which is a mixture of isomers containing about 50% of the biologically active (10R)-JH III. Thus, the active dose applied would be around 10 μg per specimen, which is an efficient dose to impair metamorphosis [34]. Controls received 1 μl of acetone.

2.5. RNA interference

B. germanica is very sensitive to RNA interference (RNAi) in vivo [35]. Detailed procedures for dsRNA preparation and RNAi experiments were as described previously [22,36,37]. Concerning BR-C, dsRNAs were prepared to deplete all isoforms simultaneously (dsBrCore) or specific isoforms BR-C Z1 to BR-C Z6 (dsBrZ1 to dsBrZ6). The primers used to generate templates with PCR for transcription of these dsRNAs are described in Table S3 (see Supplementary data). The fragments were amplified by PCR and cloned into the pSTBlue-1 vector (Novagen, Madrid, Spain). In all cases, we used a 307 bp sequence from *Autographa californica* nucleopolyhydrosis virus (accession number: K01149, from nucleotides 370 to 676) as control dsRNA (dsMock). A volume of 1 μl of each dsRNA solution (3 μg/μl) was injected into the abdomen of specimens at chosen ages and stages. The control specimens were treated with the same dose and volume of dsMock. RNAi of Kr-h1was carried out as recently reported [15].

2.6. Wing morphological studies

Development of mesonotum and metanotum wing primordia or wing buds was studied in 5th (N5, penultimate) and 6th (N6, last) nymphal instars. Wing buds were exposed out of the dorsal cuticular layer under Ringer's saline. Then, they were fixed in 4% paraformaldehyde and permeabilized in PBS-0.2% tween (PBT), incubated for 20 min in 300 ng/ml phalloidin–tetramethylrhodamine isothiocyanate (Sigma-Aldrich) in PBT, rinsed with PBS, and stained for 10 min in 1 μg/ml DAPI in PBT. After three washes with PBT, the wing buds were mounted in Mowiol 4–88 (Calbiochem), and were examined by epifluorescence microscopy AxioImager.Z1 (ApoTome System, Zeiss). Adult forewings (tegmina) and hindwings (membranous) were studied and photographed first in the intact animal, and then dissected out, mounted on a slide with Mowiol 4–88. In these cases, examinations and photographs were made with a stereomicroscope Zeiss DiscoveryV8. Biometrical measurements of wing size parameters were carried out with an ocular micrometer adapted to this stereomicroscope.

2.7. EdU experiments to measure cell proliferation in vivo

EdU (5-ethynyl-2′-deoxyuridine) is a thymidine analogue recently developed for labeling DNA synthesis and dividing cells in vitro [38,39], which is more sensitive and practical than the commonly used 5-bromo-2′-deoxyuridine, BrdU. We followed an approach in vivo, using the commercial EdU compound "Click-it EdU-Alexa Fluor® 594 azide" (Invitrogen, Molecular Probes), which was applied topically on the first abdominal tergites (10 μg in 1 μl of DMSO) of staged nymphs. The control specimens received 1 μl of DMSO. Wing buds from treated specimens were dissected 24 h later, and processed for EdU detection according to the manufacturer's protocol.

2.8. Statistics

In general, data are expressed as mean ± standard error of the mean (SEM). In qRT-PCR determinations, statistical analyses between groups were tested by the REST 2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research) [31]. This program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-wise Fixed Reallocation Randomization Test tool in REST [31]. Statistical analyses of wing biometrical measurements were carried out with the Student's *t*-test.

3. Results

3.1. Broad-complex isoforms are expressed along the entire nymphal life of B. germanica

As a first step in our work, we studied the temporal expression of BR-C during the nymphal life of the cockroach *B. germanica*. Firstly, we used whole body extracts and a primer set amplifying a fragment in the core region of BR-C, which is common to the six BR-C isoforms. Results obtained show that BR-C transcripts are present in the six nymphal instars (N1 to N6) at levels of some 100 copies per 1000 copies of actin mRNA. In the last nymphal instar (N6), BR-C mRNA levels steadily decreased until becoming practically undetectable just before molting to the adult stage (Fig. 1A).

Given that BR-C functions in hemimetabolous insects appeared to be associated to wing growth, we obtained expression patterns in the pooled wing pads, that is, the lateral expansions of the mesonotum and metanotum, which contain the wing buds, during the last two nymphal instars (N5 and N6). Results revealed that BR-C is highly expressed in wing pads in N5, the expression levels forming a peak on day 4. In N6 the expression levels are notably lower and steadily decreasing, although showing a small peak of expression on day 6 (Fig. 1B). The expression peaks in N5 and N6 correspond to the peaks of 20E, and the high levels of expression in N5 correlate with the presence of JH, while the levels decrease in N6 when JH vanishes (Fig. 1B, bottom).

The expression of the individual isoforms, BR-C Z1 to BR-C Z6 in the wing pads during N6 shows the same pattern in all isoforms: high levels at emergence, then a steady decrease although with a small peak of expression on day 6 (Fig. 1C). Moreover, the study shows that BR-C Z2 is the most abundant isoform, followed by Z3, Z6, Z4, Z1 and Z5. With the RNA extract used to study the individual isoforms (results in Fig. 1C) we also amplified the core region fragment as in Fig. 1A. The mRNA values obtained were similar to those resulting from the sum of values of all isoforms for each day (Supplementary Fig. 1), which suggests that the six BR-C isoforms known in *B. germanica* constitute the complete functional set.

As stated above, the dramatic differences of BR-C expression between N5 and N6 are probably due to JH, which is abundantly present in N5 and practically absent in N6. This suggests that JH enhances the expression of BR-C in *B. germanica*, and that the decline of BR-C mRNA levels in N6 may largely be due to the absence of JH. In agreement with this, topical treatment of freshly emerged N6 with 20 μg of JH III induced the re-expression of BR-C (Fig. 1D). Congruently, depletion of Kr-h1, a transcription factor involved in early JH signaling, decreases BR-C expression levels in N4 and in N5 (Fig. 1E).

3.2. BR-C depletion impairs wing development

To study the role of BR-C, cockroaches were treated with 3 μg dsRNA targeting the core region of BR-C (dsBrCore) at day 0 of N5. The controls were equivalently treated with dsMock. On day 6 of N5 and on day 0 of N6, the levels of BR-C mRNA in dsBrCore-treated specimens were significantly lower than those measured in the controls (Fig. 2A). dsBrCore-treated specimens (n = 34) molted normally to N6, but the adults emerging from the subsequent molt showed a number of differences with respect to dsMock-treated controls (n = 24). In dsBrCore-treated specimens, both wing pairs were well extended, but the forewings appeared to be somewhat broader and the hindwings were smaller than those of the controls, and showed a number of vein defects (Fig. 2B). Biometrical data supported the observations regarding form and size (Supplementary Fig. 2). And as to the hind wing vein patterning, 19 out of 34 specimens (56%) had the CuP vein shorter and an associated notch at the wing edge (defect A, Supplementary Fig. 3); four specimens (12%) showed vein/intervein pattern disorganization in the anterior part of the wing (defect B, Supplementary Fig. 3); six specimens (17%) showed the defects A and B; and five specimens (15%) had A and B defects and their A-veins were subdivided and incomplete (defect C, Supplementary Fig. 3). In all other respects, the external morphology of the adult of dsBrCore-treated specimens was normal. Control (dsMock-treated) specimens developed normally patterned wings, although a small percentage of the specimens (3 out of 24, 12.5%) showed the defect B (Supplementary Fig. 3).

We then increased the RNAi effectiveness using two doses of 3 μg of dsBrCore, administered respectively on days 0 and 3 of N5. Levels of BR-C mRNA measured on day 6 of N5 (Fig. 2C) were lower than those obtained with a single injection (Fig. 2A). The specimens treated twice with dsBrCore (n = 23) molted normally to N6, and then to the adult stage, which showed malformed wings. About 70% of the adult specimens (16 out of 23) showed a phenotype similar to that obtained with a single injection of dsRNA: the wings being well extended but with the typical notch produced by a shortening of the CuP vein, a similar diversity of vein patterning malformations, and with broader forewings and smaller hindwings than in the controls (Fig. 2D, left). The remaining 30% exhibited a wing phenotype more severe, with the forewings and hindwings heavily reduced and so wrinkled (Fig. 2D, right) that it was difficult to extend them on a slide; however, close examination indicated that all of them had the A, B and C vein defects observed in the dsBrCore-treated specimens that emerged with the

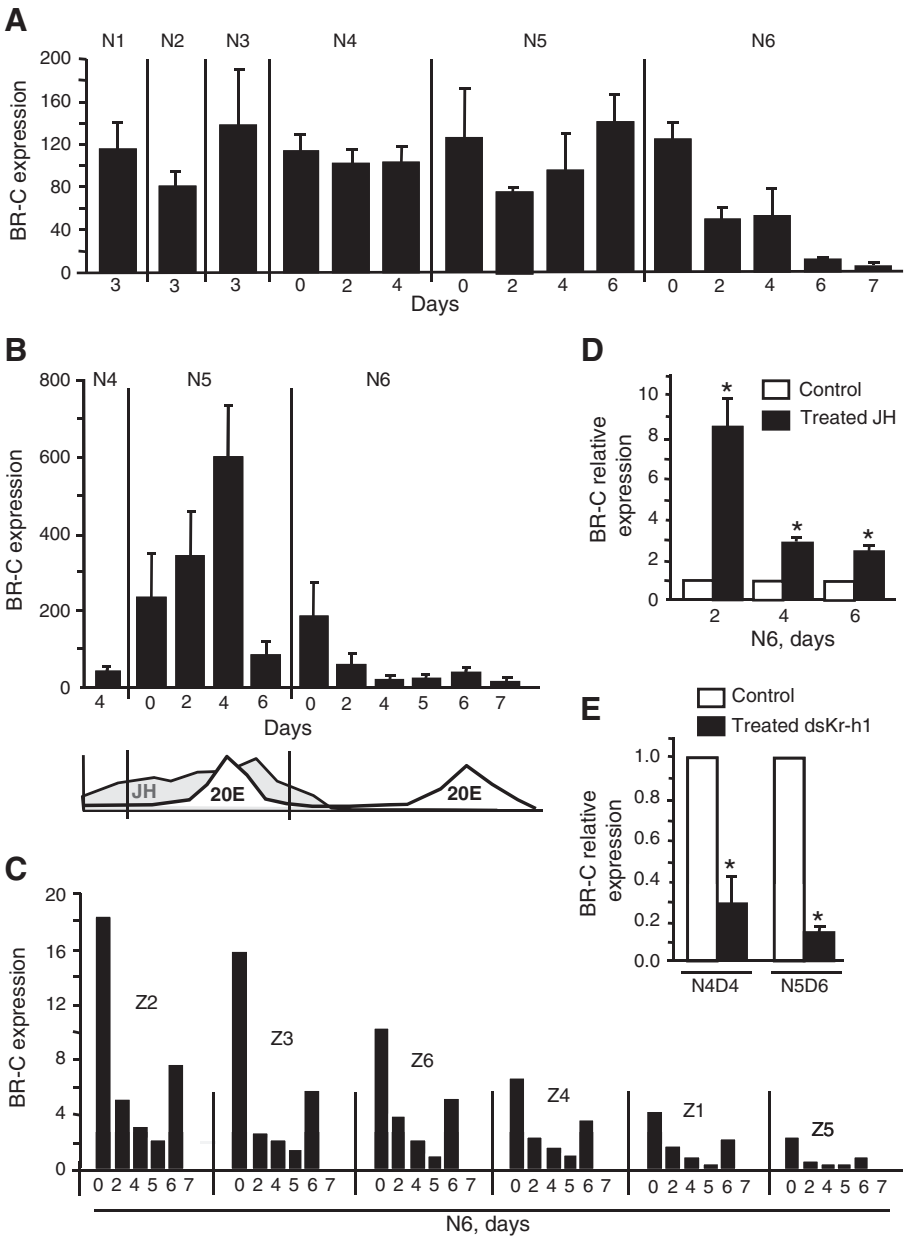

Fig. 1. Expression of BR-C mRNA in *Blattella germanica* female. (A) Joint expression of all isoforms in the whole body in selected days of the 6 nymphal instars: N1 to N6. (B) Joint expression of all isoforms in the mesonotum and metanotum wing pads in selected days of N4, N5 and N6. Concentration patterns of hemolymph juvenile hormone (JH) III and 20-hydroxyecdysone (20E) are indicated below, according to previously published data [33,40]. (C) Expression of individual BR-C isoforms (Z1 to Z6) in the wing pads on selected days of N6. (D) Effect of the application of 20 μg of JH III. The hormone was administered on freshly emerged N6 and BR-C mRNA levels were measured 2, 4 and 6 days later. (E) Effect of Kr-h1 depletion by RNAi on N4 and N5. Two doses (3 μg each) of dsKr-h1 (treated) or dsMock (control) were administered on N4 and N5 (on days 0 and 3 in both cases), and BR-C mRNA levels were measured on day 4 in the experiments of N4, and on day 6 in the experiments of N5. Data is represented as the mean ± SEM, and are indicated as copies of BR-C mRNA per 1000 copies of BgActin-5c. Each point represents 3–6 biological replicates, except in panel C, in which data represent one replicate per point. In panels D and E data are normalized against control females (arbitrary reference value = 1) and the asterisk indicates statistically significant differences with respect to controls ($p<0.05$), according to the REST software tool [31].

wings well extended. Arguably, differences in severity of the effects are due to differences of penetrance and intrinsic variability in these experiments. All the controls treated equivalently with two doses of dsMock (n = 18) emerged with normal wings in terms of size, form and vein patterning.

In order to assess whether BR-C could play a role on the wing development of younger instars, we administered two doses (3 μg-each) of dsBrCore on days 0 and 3 respectively of N4. On day 0 of N5, the levels of BR-C mRNA in dsBrCore-treated specimens were significantly lower than in the controls (dsMock-treated) (Fig. 2E). dsBrCore-treated specimens (n = 22) molted normally to N5 and to N6, but when molting to the adult stage they showed the wing malformations observed in the former experiments, with extended and wrinkled wing phenotypes, but with a higher proportion of the latter. Thus, only six out of 22 treated specimens (27%) showed the extended wing phenotype, and even these had the wings imperfectly extended (Fig. 2F, left); moreover, the wings exhibited the A, B and C defects and reduced size typical of BR-C knockdowns. The remaining 16 specimens (73%) showed the more severe wrinkled wing phenotype (Fig. 2F, right). The controls (dsMock-treated, n = 21), had normal wing patterning, except three specimens (14.3%) that exhibited the B defect.

Finally, we aimed at studying the effect of BR-C depletion in the last instar nymph (N6), where the transition to the adult stage occurs. Cockroaches were treated with two doses of 3 μg each of dsBrCore,

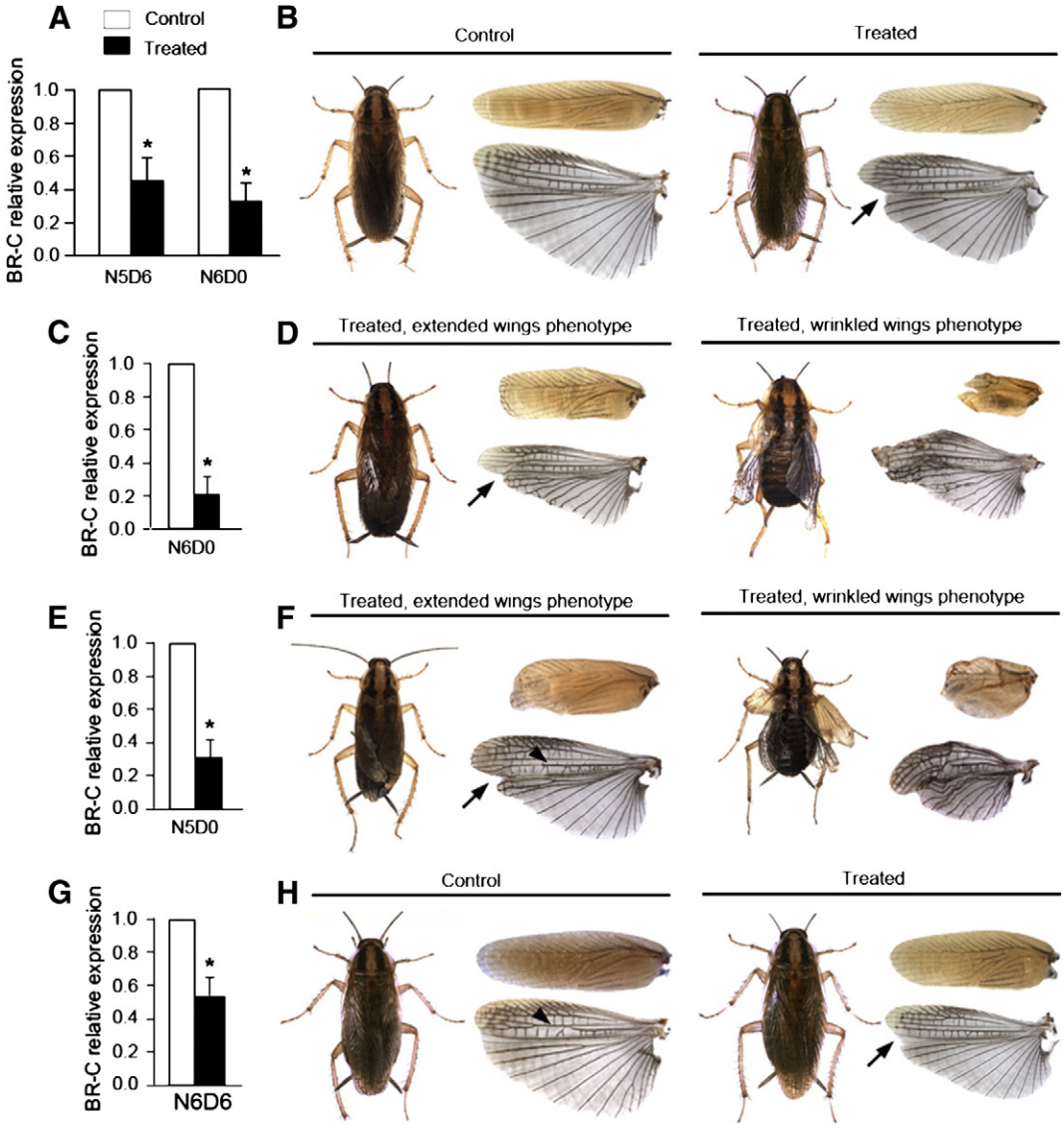

Fig. 2. Phenotype obtained after depleting all BR-C isoforms by RNAi in *Blattella germanica*. (A–B) Effect of a single 3 μg-dose of dsMock (control) or dsBR-core (treated) administered on day 0 of N5: BR-C mRNA levels on day 6 of N5 and on day 0 of N6 (A); adult phenotype (habitus, forewing and hindwing) (B). (C–D) Effects of two doses of 3 μg each of dsBR-core administered on day 0 and day 3 of N5: BR-C mRNA levels on day 0 of N6 (C); extended wings and wrinkled wings phenotype (D). (E–F) Effects of two 3 μg-doses of dsBR-core administered on day 0 and day 3 of N4: BR-C mRNA levels on day 0 of N5 (E); extended wings and wrinkled wings phenotype (F). (G–H) Effects of two 3 μg-doses of dsMock (control) or dsBR-core (treated) administered on day 0 and day 3 of N6: BR-C mRNA levels on day 6 of N6 (G); adult phenotype of controls and treated specimens (H). Data in (A), (C), (E) and (G) represent 3 biological replicates (mean ± SEM), are indicated as copies of BR-C mRNA per 1000 copies of BgActin-5c, and are normalized against control females (arbitrary reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p<0.05$), according to the REST software tool [31]. Arrows indicate the notch provoked by a shortening of the CuP vein, and the arrowhead indicates disorganized vein/intervein patterning in the anterior part of the hindwing. Note that the late defect appeared also in controls (H).

one administered on day 0 and the other on day 3 of N6. Three days later (day 6), the levels of BR-C mRNA were significantly lower in dsBrCore-treated insects than in the controls (Fig. 2G). All adults obtained after this treatment (n = 24) emerged with the wings well extended, and only 5 of them (21%) showed the A defect (Fig. 2H, right). Control (dsMock-treated) specimens (n = 23) had normal wing patterning, and only one specimen (4%) had the B defect (Fig. 2H, left).

3.3. BR-C depletion impairs cell division in wing buds

The decrease in wing size, especially in the hindwings, suggests that there was a problem of cell proliferation in dsBrCore-treated specimens. To test this possibility, we first studied cell division in the hindwing buds in N6 around the peak of 20E, which takes place between days 5 and 7, with maximum values on day 6 [40]. Cell division in the hindwing buds, which are located within a pocket in the lateral expansions of the metanotum, was labeled with EdU. On day 5, EdU labeling revealed intense cell division on the surface of the hindwing bud (Fig. 3A, day 5). On day 6, cell division on the surface practically vanished, while the wing bud started growing and folding (Fig. 3A, day 6). Towards the end of day 6 and during the whole of day 7, EdU labeling disappeared and a remarkable, general wing growth took place, that provoked multiple and thick folds on the hindwing surface (Fig. 3A, day 7). The same transition from cell division to wing growth between days 5 and 7 occurs in the forewing buds (Fig. 3B, control), which are located within the lateral expansions of the mesonotum.

In specimens treated with a double dose of 3 μg of dsBrCore administered respectively on days 0 and 3 of N5, EdU labeling in the forewing buds on day 5 of N6 was significantly reduced in comparison with

controls (dsMock-treated) (Fig. 3B, left panels, compare the control and the treated). On day 6, during the period of growth and folding in controls, remarkable surface reduction was noticed along the edges of the wing pad in dsBrCore-treated specimens (Fig. 3B, right panels, compare the control and the treated), which parallels the statistically significant reduction on wing size measured after the imaginal molt (Supplementary Fig. 2). Concerning the hindwing buds, EdU labeling on day 5 was also more reduced in the specimens treated with dsBrCore than in controls treated with dsMock. Differences were particularly obvious at the distal end of the CuP vein, were EdU labeling was practically absent in dsBrCore-treated specimens, in comparison with the controls (Fig. 3C). Interestingly, the distal end of the CuP vein, where there is no cell division, disappeared thereafter, on day 6, at the maximum peak of ecdysone, therefore forming the characteristic notch of the BR-C knockdowns (Fig. 2B, D, F, H and Supplementary Fig. 3). In the subsequent stage of tissue growth without cell division, from the end of day 6 to the ecdysis, on day 8, differences between dsBrCore-treated specimens and controls were not apparent in either the forewing bud or the hindwing bud. We also used EdU labeling to study wing bud cell division around the 20E peak of N5 in specimens that had been treated with two doses of dsBrCore on days 0 and 3 of N4. Results showed that cell division was lower in dsBRCore-treated specimens than in controls (dsMock-treated), both in the forewing and in the hindwing buds (results not shown), as occurred in N6.

3.4. Functions of individual BR-C isoforms in B. germanica

All BR-C isoforms, from Z1 to Z6, are expressed simultaneously, although at different levels, and show the same pattern (Fig. 1C), suggesting that all of them contribute to the same functions in post-embryonic development. However, we aimed to test this conjecture by carrying out isoform-specific RNAi experiments on all isoforms. Treatments were carried out in N5, by injecting two doses of 3 μg of the corresponding BR-C dsRNA (dsBrZ1 to dsBrZ6), one on day 0 and the other on day 3. The controls were equivalently treated with dsMock.

Transcript depletion of the specifically targeted isoform was measured on the wing pads on day 0 of N6. Transcript levels of all other non-targeted isoforms were also measured as a control of RNAi specificity. Results (Fig. 4A–F) show that, in general, individual isoform RNAi experiments were isoform-specific in terms of transcript depletion. Treatment with dsBrZ3 reduced BR-C Z3 mRNA levels, but differences with respect to the corresponding controls were not statistically significant. This treatment also tended to reduce BR-C Z2 mRNA levels and, intriguingly, tended to increase the mRNA levels of BR-C Z4, BR-C Z5 and BR-C Z6 (Fig. 4B). It is also worth noting that treatment with dsBrZ4 significantly reduced BR-C Z4 mRNA levels, as expected, but also those of BR-C-Z5 (Fig. 4D).

In terms of phenotype, all specimens in all isoform-specific RNAi experiments molted normally to N6 and then to adult. A detailed examination of the external morphology of the adult was then performed and differences with respect to controls were only noticed in the wings of specimens treated with dsBrZ2 and dsBrZ6. Concerning dsBrZ2-treated specimens (n = 27), 18 of them (67%) had only the A defect (Fig. 4G); 1 (4%) showed only the B defect; and 7 (26%) had no visible defects. No significant differences were observed in wing sizes between treated specimens and controls, neither in the hindwing nor in the forewing. The hindwing phenotype resembles that obtained when depleting all isoforms simultaneously, but less marked, with less penetrance and without the C defect (see Supplementary Fig. 3). All control (dsMock-treated) specimens (n = 12) showed normal wing patterning except one specimen (8%) that had the B defect. In the dsBrZ6-treated group (n = 25), 9 specimens (36%) showed the B defect (Fig. 4H), whereas the remaining 16 were perfectly patterned. In the control group (dsMock-treated) (n = 18), 2 specimens (11%) showed the B defect.

Specimens obtained from RNAi experiments targeting Z1 (dsBrZ1-treated, n = 18; dsMock-treated, n = 10), Z3 (dsBrZ3-treated, n = 24; dsMock-treated, n = 14), Z4 (dsBrZ4-treated, n = 16; dsMock-treated, n = 11) and Z5 (dsBrZ5-treated, n = 18; dsMock-treated, N = 10), had the wings well extended and correctly patterned (Fig. 4I), and only a

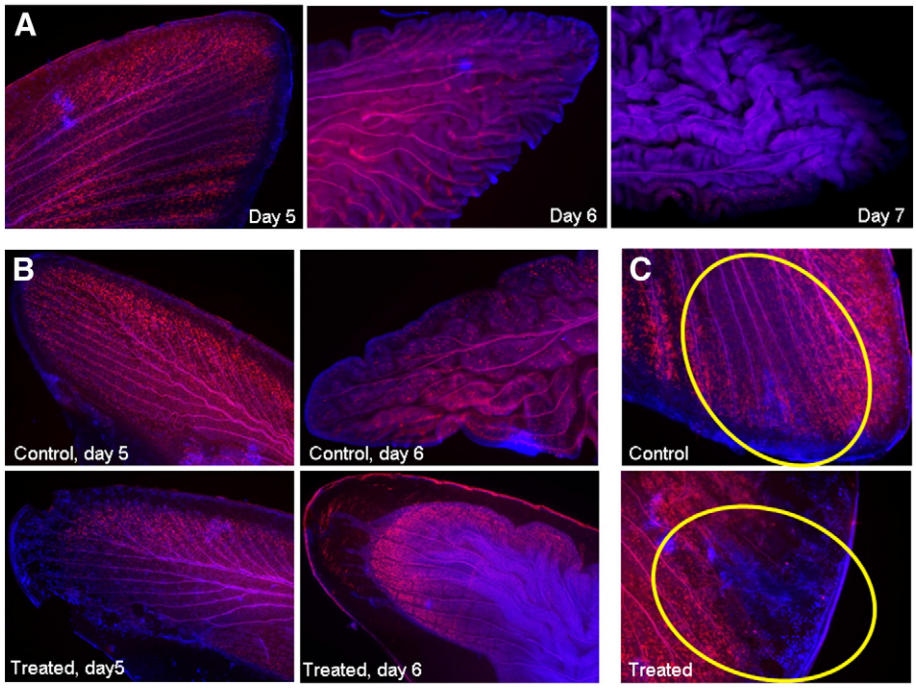

Fig. 3. Development of wing buds in control and BR-C knockdown specimens of *Blattella germanica*. (A) Double labeling EdU (discrete red spots) and DAPI (blue color) of a hindwing bud of untreated females on days 5, 6 and 7 of the last nymphal instar (N6). (B) EdU-DAPI double labeling of a forewing bud of females that were treated with two doses of dsBrCore (treated) or of dsMock (control) administered on days 0 and 3 of N5, and photographed on days 5 and 6 of N6. (C) EdU-DAPI double labeling of a hindwing bud of females that were treated as in (B) and photographed on day 5 of N6; the pictures show the region corresponding to the distal end of the CuP vein (yellow oval).

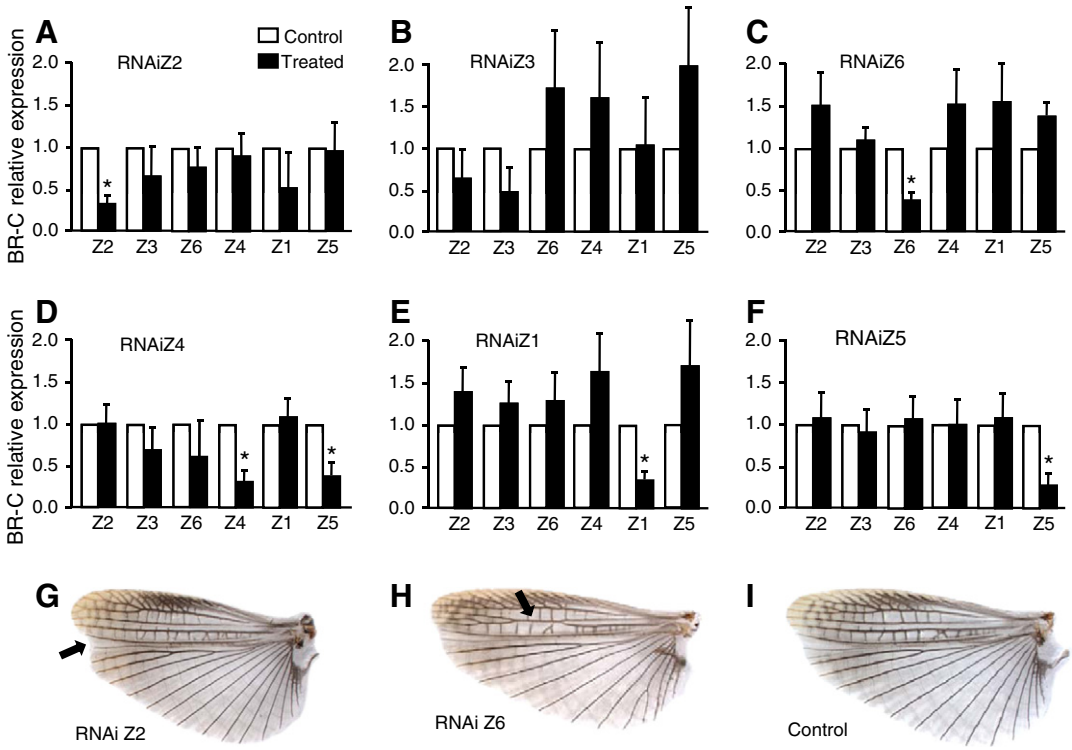

Fig. 4. Effects obtained after depleting each BR-C isoform, from Z1 to Z6, individually, by RNAi in *Blattella germanica*. (A–F) Transcript levels of the targeted and the other isoforms measured in the mesonotum and metanotum wing pads on the day 0 of N6. Two 3-μg doses of dsBrZ2 (A), dsBrZ3 (B), dsBrZ6 (C), dsBrZ4 (D), dsBrZ1 (E) or dsBrZ5 (F) administered on days 0 and 3 of N5; control received an equivalent treatment with dsMock; data represent 3 biological replicates (mean ± SEM) and are indicated as copies of the individual BR-C isoforms mRNA per 1000 copies of BgActin-5c; the asterisk indicates statistically significant differences with respect to controls ($p<0.05$), according to the REST software tool [31]. (G–I) Adult hindwing phenotype studied 3 days after the imaginal molt, from a dsBrZ2-treated specimen (G), a dsBrZ6-treated specimen (H), and from a control specimen treated with dsMock. The arrow indicates the main defects: the notch at the end of the CuP vein in G and the vein pattern disorganized in the anterior part of the wing in H.

few specimens (between 0 and 14%, irrespective of the group, either in the treated or the control) had the B defect.

4. Discussion

4.1. The patterns and the hormonal environment

In *B. germanica*, BR-C isoforms are expressed in all nymphal stages, and expression only declines in the last, pre-imaginal stage. The expression pattern is similar in all individual isoforms of BR-C, although the respective abundances differ, Z2 being the most abundant, followed by Z3, Z6, Z4, Z1 and Z5. Expression is more intense in the wing pads, and the pattern appears to be determined by the hormonal environment: maximum BR-C mRNA levels coincide with bursts of 20E production in the presence of high levels of JH. The coincidence of BR-C expression peaks and those of 20E reflects cause–effect relationships, as expression of BR-C is 20E-dependent [41]. Then, BR-C mRNA levels decline in the last nymphal instar (N6), in parallel to JH vanishing, although a small burst of expression is still observed on day 6 coinciding with a peak of 20E. The correspondence of BR-C and JH patterns, the induction of BR-C expression by exogenous JH III, and their down-regulation after depletion of Kr-h1, suggest that JH enhances BR-C expression during young nymphal instars. Therefore, the steady decline observed in N6 must be due, at least in part, to the decrease of JH titer. In the hemipteran *P. apterus*, treatment with a JH analogue induces ectopic expression of BR-C in last nymphal instar, whereas depletion of Met expression led to a significant reduction of BR-C expression [12]. These observations are in agreement with our present results as Met is an early transducer of the JH signal and seems to play a role in JH reception [9], also in *B. germanica* (our unpublished results).

Significant expression of BR-C in young nymphal instars has been reported in the hemimetabolous species *O. fasciatus* [29] and *P. apterus* [12]. Conversely, BR-C expression in holometabolous species is quantitatively relevant only in the larva–pupa transition. This includes *D. melanogaster* [25], *M. sexta* [25], *B. mori* [42] and *T. castaneum* [11,27,28]. Data from holometabolous species suggest that the onset of BR-C expression occurs after a small burst of 20E produced in the absence of JH at the end of the last larval instar. In *M. sexta*, BR-C transcripts appear at the end of the feeding stage (beginning of wandering behavior) in the epidermis of the last instar larvae, when the insect becomes committed to pupal differentiation. Administration of JH in this stage prevents the 20E-induced expression of BR-C [43]. Later, levels of BR-C mRNA decrease during the pupal stage and the pupae transform into the adult stage. In the pupae, exogenous JH induces the re-expression of BR-C, and the insect undergoes a second pupal molt [25].

The expression patterns of BR-C isoforms in the thysanopterans *Frankliniella occidentalis* and *Haplothrips brevitubus* [44], are especially interesting. Thysanopterans follow an essentially hemimetabolous development, in the sense that nymphs are morphologically similar to adults, but the life cycle includes 1 to 3 quiescent stages, called propupae and pupae, where wing buds develop considerably and which are reminiscent of the holometabolous pupal stage. This particular cycle has been distinguished as neometabolan development [5]. *F. occidentalis* has two nymphal stages, a propupal and a pupal stage, whereas *H. brevitubus* has two nymphal stages, a propupal and two pupal stages. In both species, expression of BR-C is low in the first instar nymph, peaks towards the end of the second instar nymph and decreases in the propupae. Moreover, treatment of propupae with a JH analogue induces the re-expression of BR-C in the pupae [44]. The BR-C expression pattern, showing a peak just before the transition from nymph to propupae, and the stimulatory effect of JH on BR-C expression in the

propupae, are reminiscent of the endocrine determinism of the pupal stage in holometabolous species.

4.2. The functions

RNAi experiments in nymphs of *B. germanica* have shown that wing buds experience intense cell proliferation, which is hampered in BR-C knockdowns. This suggests that BR-C isoforms regulate progressive growth of wing buds during nymphal life by promoting cell division. In the last nymphal instar there is a phase of cell proliferation encompassed by the increasing levels of 20E that lead to the peak on day 6, followed by a phase of cell growth and wing metamorphosis encompassed by the decreasing levels of 20E that occur after the peak. In this metamorphic instar, cell proliferation is also hampered in BR-C knockdowns, but the subsequent phase of cell growth and metamorphosis is generally not affected by BR-C RNAi. Only some details of vein patterning in the hindwing are affected by BR-C RNAi, including the length of the CuP vein, the organization of the longitudinal and small transversal veins in the anterior part of the hindwing, and the linear growth of the A-veins in the posterior part.

Therefore, the function of BR-C isoforms in postembryonic development of *B. germanica* appears to be restricted to sustaining cell division in the wing buds, which contributes to the final size and morphology of the adult wings, and regulation of a number of details of vein patterning and length and in the hindwing. While the latter function had not been described before in hemimetabolous insects, our observations on wing size are equivalent to those obtained in *O. fasciatus* and *P. apterus*, where RNAi treatments of BR-C hampered wing bud development in nymphs and resulted in adults with reduced and wrinkled wings [12,29]. Our observations refer to the external morphology, as no anatomical examinations were carried out during the present work. Thus, we cannot rule out the possibility of a possible role of BR-C factors in the nervous system during metamorphosis of *B. germanica*, as what occurred in *D. melanogaster* [45,46]. However, the primitive hemimetaboly exhibited by *B. germanica* suggests that there are no dramatic transformations of the internal organs, as opposed to *D. melanogaster*, whose internal anatomy is heavily reconstructed during metamorphosis.

In striking contrast, BR-C proteins play complex morphogenetic roles in holometabolous species, leading to the formation of the pupal morphology. Pioneering genetic studies in *D. melanogaster* demonstrated that BR-C null mutants, in which not one of the isoforms is expressed, never molt to pupae [23,47,48]. Later, the use of a recombinant Sindbis virus expressing a BR-C antisense RNA fragment in *B. mori* reduced endogenous BR-C mRNA levels in infected tissues and the insect did not complete the larval–pupal transition [26]. More recently, experiments depleting BR-C mRNA levels with RNAi have been carried out in *T. castaneum* [11,27,28] and in the lacewing *Chrysopa perla* [28]. In all cases, RNAi treatments in larvae hampered larval–pupal transformation and produced individuals with larval, pupal and adult features, which indicates that BR-C isoforms promote the pupal developmental program while suppressing those of the larvae and the adult.

4.3. The isoforms

In *D. melanogaster*, BR-C encodes four zinc-finger protein isoforms (Z1, Z2, Z3 and Z4), which share most of the amino-terminal region called the BRcore, but they have a unique pair of zinc-fingers at their carboxy terminus [23,24]. Moreover, the common BRcore region contains a BR-C-Tramtrack-Bric-à-brac (BTB) domain involved in protein–protein interactions [24]. Mutants corresponding to isoform-specific regions form three complementing groups: *br* (broad), *rbp* (reduced bristle number on palpus) and *2Bc* [47,48]. Alleles belonging to the *npr1* (nonpupariating1) class of mutations do not complement mutations in each of the three complementing genetic functions. *npr1* mutations result in developmental arrest and lethality at pupariation; br^+ function is required for wing and leg imaginal disc development and for tanning the larval cuticle; rbp^+ and $2Bc^+$ functions are needed for larval tissues destruction and for gut morphogenesis; moreover, $2Bc^+$ is additionally required for complete closure of the thoracic epidermis, and all three functions must occur for central nervous system reorganization [47–50]. Mutant rescue experiments associated protein isoforms with genetic functions, and revealed that there were isoform-specific functions, although with some degree of redundancy [51]. Further studies in *D. melanogaster* have shown specific space–temporal distributions of different BR-C isoforms, thus suggesting distinct temporal function, especially in neural tissue morphogenesis [45,46,52]. Finally, the advent of RNAi allowed the functional study of individual BR-C isoforms (Z1 to Z5) in *T. castaneum* metamorphosis. As in *D. melanogaster*, results pointed to isoform-specific roles and partial redundancy [27,28].

Our functional studies on specific isoforms revealed that depletion of BR-C Z2 and BR-C Z6 gave discernible phenotypes, in both cases related to wing patterning. BR-C Z2 phenotype showed the CuP vein shorter and an associated notch at the wing edge (defect A), and the vein/intervein patterning disorganized in the anterior part (defect B), but it did not show the A-veins subdivided and incomplete (defect C). Moreover, the penetrance and severity of the defects were lower in comparison with the BR-C Core knockdowns, while wing size was practically unaffected. BR-C Z6 knockdowns phenotype showed t defects B and C, but not defect A, which is the most typical in the experiments depleting all isoforms simultaneously; wing size was unaffected. Phenotypes obtained in RNAi experiments on BR-C Z1, BR-C Z3, BR-C Z4 and BR-C Z5 were as in controls, including defect B, which seems qualitatively unspecific, although, importantly, its occurrence in BRCore or BR-C Z6 knockdowns is significantly higher than in controls. The relatively poor abundance of these isoforms in the pool of BR-C proteins, and the generally modest efficiency of these RNAi experiments in terms of transcript decrease, might explain the absence of differential phenotypes. However, given that phenotypes observed in BR-C Z2 and BR-C Z6 knockdowns encompass all the defects observed in BRCore knockdowns, we can presume that functions of the remaining isoforms might be redundant with those of BR-C Z2 and BR-C Z6.

4.4. Conclusion: the evolution of metamorphosis at the light of BR-C

The main function played by BR-C proteins in *B. germanica*, a phylogenetically basal, hemimetabolous species, during post-embryogenesis is to promote wing growth to reach the right size and form. Basically, this function has been conserved in the hemimetabolous species *O. fasciatus* and *P. apterus*, which are phylogenetically more distal than *B. germanica*. In these bugs, the wing buds are external, not embedded in a cuticular wing pad pocket, and the decrease in growth and attenuation of color pattern progression of BR-C knockdowns is externally visible [12,29]. In the beetle *T. castaneum*, RNAi of BR-C isoforms Z2 and Z3 results in pupae with shortened wings [27], which indicates that functions of BR-C related to wing size are conserved in basal holometabolous species. Functions of BR-C in determining wing size and form also appear to be present in the extremely modified, holometabolous species of fly *D. melanogaster*. Indeed, the name “broad” given to one of the complementation groups of BR-C derives from the oval, rather than from the elliptic form of the wings of these mutants, which were first described by Thomas H. Morgan and colleagues in 1925 [53]. Therefore, the functions of BR-C related to controlling wing size and form appear to be ancestral and conserved from cockroaches to flies. The same may hold true for the subtle functions related to vein patterning observed in *B. germanica*, as the classical *br* mutants of *D. melanogaster* also have defects on vein length [53]. Vein patterning in BR-C knockdowns of *O. fasciatus* and *P. apterus* was not reported [12,29].

Another interesting feature of BR-C in *B. germanica* is that its expression is enhanced by JH. Thus, both JH and BR-C mRNA levels are high in young nymphal instars and decrease in parallel during

the last nymphal instar, prior to metamorphosis. This contrasts with the situation found in holometabolous species, where JH inhibits BR-C expression in young larvae, and so high levels of JH correlate with low levels of BR-C expression [12].

A number of theories have been proposed to explain the evolutionary transition from hemimetaboly to holometaboly [4,54]. A classic theory originally argued by Lubbock [55] and formalized by Berlese [56], proposes that the larvae of holometabolous species arose by "de-embryonization", so that it was a sort of free living, often vermiform embryo. The "de-embryonization" theory was resuscitated and reinforced with modern endocrine data by Truman and Riddiford [54], who proposed that the holometabolous larva corresponds to the latest hemimetabolous embryonic stage that the latter authors called the pronymph, and that the origin of larval form would be explained by a shift in JH titers, from late embryonic stages in hemimetabolous species to earlier stages in holometabolous insect embryos. Thus, in postembryonic stages of holometabolous species, only when JH declines in the final larval instar does extensive morphogenesis resume and lead to differentiation of the pupa [57].

While this theory still is under debate [4], our present results show that, in a basal hemimetabolous species, JH enhances the expression of BR-C in young nymphs and that BR-C factors promote wing growth during nymphal life and refine wing patterning at metamorphosis, which contrasts with the endocrine regulation and complex functions observed in holometabolous species, as previously described in classical insect models. We propose that in the evolution from hemimetaboly to holometaboly, at least two key innovations appeared that affected BR-C and post-embryonic morphological development. The first was a shift of JH action, from stimulatory (as in present hemimetabolous species) to inhibitory (present holometabolous species) of BR-C expression, during young stages. Thus, BR-C expression had become inhibited during young larvae in holometabolous ancestors, with the resulting suppression of growth and development of BR-C-dependent tissues, for example the wing bud. The second great innovation was an expansion of functions, from one specialized in wing development and details of vein patterning, as in present hemimetabolous species, to a larger array of morphogenetic functions, which culminated with the pupal-specifier role that operates in present holometabolous species, from the basal *T. castaneum* to the distal *D. melanogaster* (Fig. 5). Paradoxically, the number of isoforms decreased from six to four in the evolution from cockroaches to flies, while their functions dramatically expanded.

The case of thysanopterans is very interesting because they show the so-called neometabolous development [5], with a life cycle including quiescent "propupal" and "pupal" stages that are reminiscent of the holometabolous pupae. The studies in *F. occidentalis* and *H. brevitubus* of Minakuchi et al. [44] have shown that BR-C mRNA levels are very low in young instars and show an acute peak around the formation of the propupae, as what occurred in holometabolous species, suggesting that BR-C expression in thysanopterans is hormonally regulated and specified as in holometabolous species. Reconstructions of insect phylogeny suggest that paraneopterans ((thysanopterans + hemipterans) + (psocopterans and phthirapterans)) are the sister group of holometabola [58]. If so, then the first steps towards the innovation of the pupal stage, including the endocrine determination, might have occurred in the ancestor of paraneoptera + holometabola. Interestingly, hemipterans also contain representatives of neometabolous development in the sternorrhyncha [4,5]. Our hypothesis contemplates that other hemipterans, and psocopterans and phthirapterans would have lost the ability to produce preimaginal quiescent stages in their life cycles. Whichever the case, although natural selection was able to make a first attempt to invent a sort of pupa in the ancestor of the paraneoptera + holometabola, the invention was fully perfected in the lineage of holometabola judging by the endless forms most beautiful that stand today in this insect superorder.

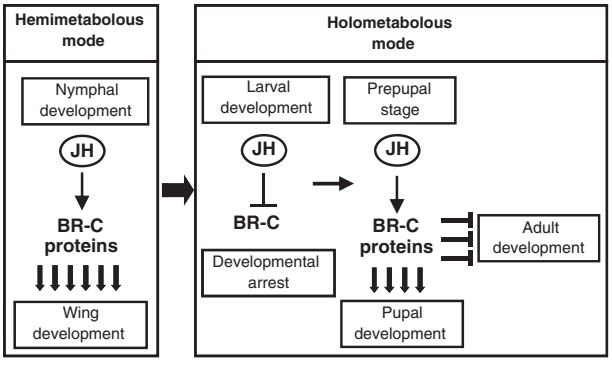

Fig. 5. BR-C and the evolution of insect metamorphosis. In the transition from hemimetaboly to holometaboly, the regulation and functions of BR-C would have experienced two main changes. First, a shift of JH action on BR-C expression, from stimulatory in hemimetabolous to inhibitory in holometabolous species. Second, an expansion of functions, from controlling wing pad growth by cell proliferation along nymphal instars, and regulating subtle features of vein development and patterning (hemimetabolous), to regulating pupal formation and inhibiting adult features at the end of the larval life (holometabolous).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2012.09.025.

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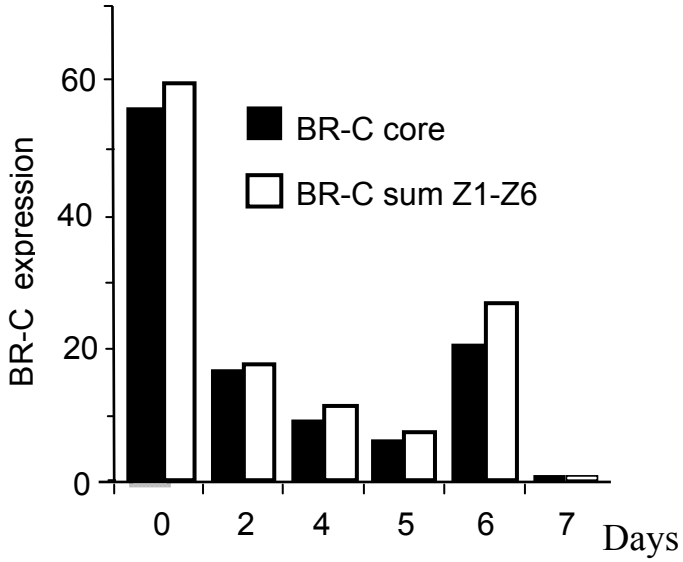
SUPPLEMENTARY DATA

Broad-complex functions in postembryonic development of the cockroach *Blattella germanica* shed new light on the evolution of insect metamorphosis

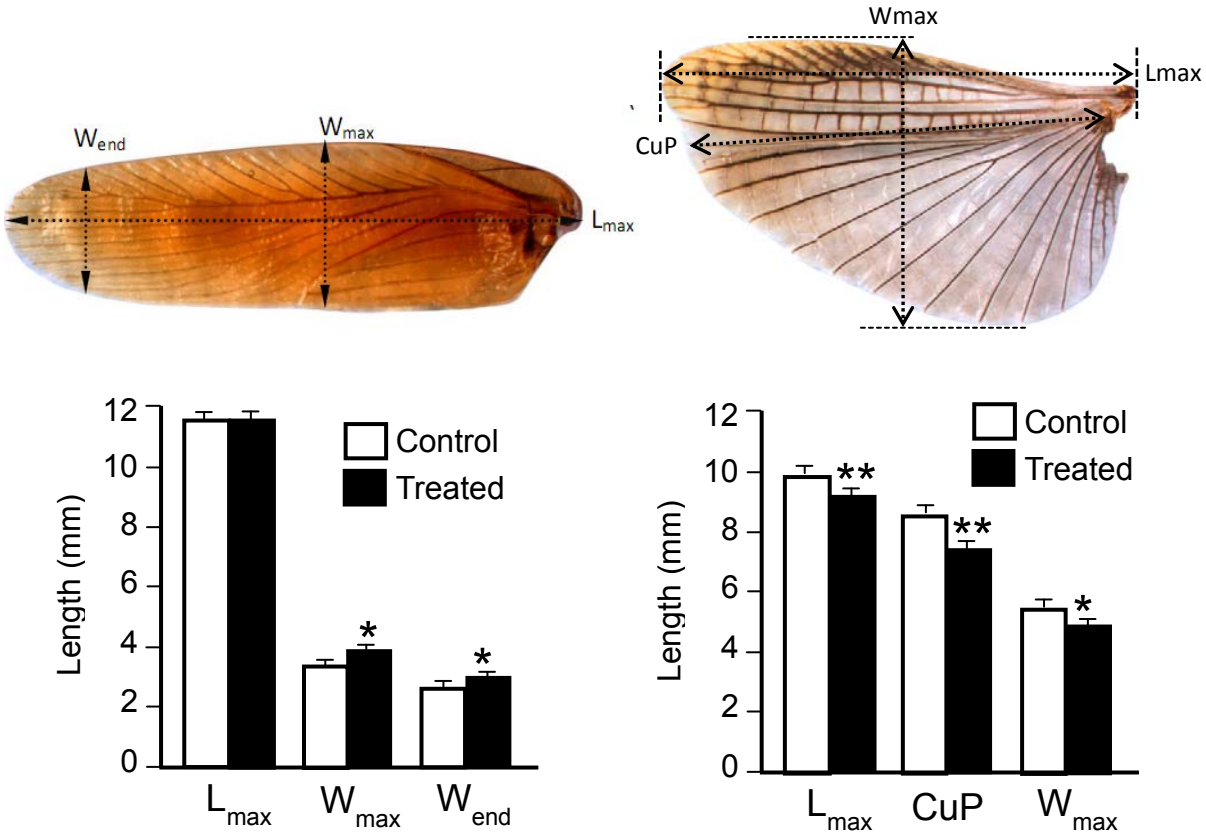
Jia-Hsin Huang, Jesus Lozano, Xavier Belles

CONTENTS

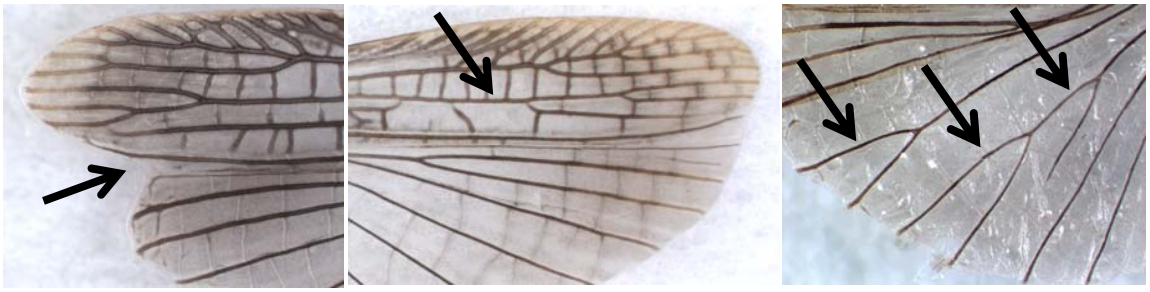
Supplementary Figure 1. Comparison of the joint expression of all BR-C isoforms, with the sum of the individual expression of the 6 isoforms, Z1 to Z6 represented in Figure 1C. Data are expressed as copies of BR-C mRNA per 1,000 copies of BgActin-5c.



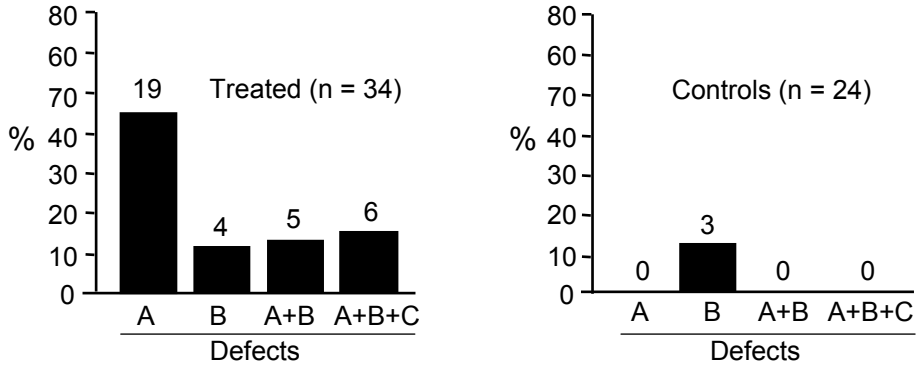
Supplementary Figure 2. Wings size of *Blattella germanica* resulting from a single dsBRCore treatment in N5 (treated) or from an equivalent treatment with dsMock (control). Top panel: Measurements carried out in the forewing (left) and hindwing (right). Bottom panel: data obtained; all values represent the mean ± SEM (n = 34 for treated, and n = 24 for controls); asterisks indicate statistically significant differences (* P < 0.01, ** P< 0.001, *t*-test).



Supplementary Figure 3. Venation defects in the hind wing of *Blattella germanica* resulting from a single dsBRCore treatment in N5. Top panel: A, B and C, the three main defects observed. Bottom: penetrance of each defect or sum of defects in specimens treated with dsBRCore (treated) and in those treated with dsMock (controls).



A: Short CuP (notch) B: Vein pattern disorganized C: A-veins broken



Supplementary Table 1. Primers used to detect the pool of all BR-C isoforms (BgBR-C) or to each isoform (BgBR-C Z1 to Z6). The accession numbers indicated in the "Encompassed region" column refer to the sequences of the different isoforms of *Blattella germanica* BR-C.

Primer set	Forward primer (5'-3')	Reverse primer (5'-3')	Encompassed region
BgBR-C	CGGGTCGAAGGGAAAGACA	CTTGGCGCCGAATGCTGCGAT	Nucleotide 699 to 774 of FN651774
BgBR-C Z1	CTTCAAGGGAGTACGGATGG	GGCGACGTAACCTCTGTAGC	Nucleotide 1298 to 1415 of FN651774
BgBR-C Z2	CTTCAAGGGAGTACGGATGG	ATGCTTGTCTGCAACGTGTC	Nucleotide 1298 to 1429 of FN651775
BgBR-C Z3	CTTCAAGGGAGTACGGATGG	TGGAGGAGGGATGCGATAAT	Nucleotide 1298 to 1372 of FN651776
BgBR-C Z4	CTTCAAGGGAGTACGGATGG	GAGAGGTAACTCGCCACTCG	Nucleotide 1298 to 1363 of FN651777
BgBR-C Z5	CTTCAAGGGAGTACGGATGG	GCAGTAAGGAGGTCCACTGC	Nucleotide 1298 to 1390 of FN651778
BgBR-C Z6	CTTCAAGGGAGTACGGATGG	CGCAGCTCATTTTGGATTTT	Nucleotide 1298 to 1398 of FN651779

Supplementary Table 2. Primers used to generate templates with PCR for transcription of dsRNAs designed to deplete all BR-C isoforms simultaneously (dsBrCore) or specific BR-C isoforms, BgBR-C Z1 to BgBR-C Z6 (dsBrZ1 to dsBrZ6). The accession numbers indicated in the "Encompassed region" column refer to the sequences of the different isoforms of *Blattella germanica* BR-C.

dsRNA	Length (bp)	Forward primer (5'-2')	Reverse primer (5'-3')	Encompassed region
dsBrCore	427	CATCAGAACAATCGCAGCATTC	TGTCCCTGAATACTGTCATGAAC	Nucleotide 744 to 1170 of FN651774
dsBrZ1	377	GCTCAGCAGGGACGTCAT	GATGAATTGAAACTTACTAACTCAAGG	Nucleotide 1317 to 1693 of FN651774
dsBrZ2	285	GCTCAGCAGGGACGTCAT	CACAGGTAACACCACCTTGGAAG	Nucleotide 1317 to 1601 of FN651775
dsBrZ3	411	GCTCAGCAGGGACGTCAT	TGTGTACATGAATGGATTTTTGG	Nucleotide 1317 to 1727 of FN651776
dsBrZ6	677	GCTCAGCAGGGACGTCAT	TCTTGCCATCATGATTAAATGAC	Nucleotide 1317 to 1993 of FN651777
dsBrZ4	612	GCTCAGCAGGGACGTCAT	AAATTTGATCATGGCCTTTG	Nucleotide 1317 to 1928 of FN651778
dsBrZ1	517	GCTCAGCAGGGACGTCAT	TTGCAACCAAATTCTTTAATAGG	Nucleotide 1317 to 1833 of FN651779

7. La funció repressiva de Krüppel homolog 1 és conservada en la metamorfosi dels insectes en espècies hemimetàboles i holometàboles

La funció repressiva de Krüppel homolog 1 és conservada en la metamorfosi dels insectes en espècies hemimetàboles i holometàboles

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Resum

La metamorfosi dels insectes està regulada per ecdisteroides, que indueixen les mudes, i per l'hormona juvenil (HJ), que inhibeix els canvis metamòrfics. L'acció molecular dels ecdisteroides s'ha estudiat a fons, però manca informació respecte a l'HJ, de la qual es coneixen dades només d'espècies holometàboles, com *Drosophila melanogaster* i *Tribolium castaneum*. Hem estudiat la funció de Krüppel homolog 1 (Kr-h1) a *Blattella germanica*, un model d'insecte hemimetàbol. Kr-h1 és un factor de transcripció amb dits de Zinc el qual s'ha caracteritzat recentment com a transductor de l'acció antimetamòrfica de l'HJ a *D. melanogaster* i *T. castaneum*. Els experiments amb RNA d'interferència mostrats en aquest treball van indicar que Kr-h1 transdueix l'acció antimetamòrfica de l'HJ també a *B. germanica*, suggerint que el seu paper és una condició ancestral que s'ha conservat en l'evolució dels insectes des d'espècies hemimetàboles a holometàboles .

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Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species

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Insect metamorphosis is regulated by ecdysteroids, which induce molts, and juvenile hormone (JH), which inhibits metamorphic changes. The molecular action of ecdysteroids has been thoroughly studied, but that of JH is poorly understood, with data currently only being available for holometabolous species, like *Drosophila melanogaster* and *Tribolium castaneum*. We studied the function of Krüppel homolog 1 (Kr-h1) in *Blattella germanica*, a hemimetabolous model. Kr-h1 is a Zn finger transcription factor whose function as transductor of the antimetamorphic action of JH has recently been demonstrated in *D. melanogaster* and *T. castaneum*. The RNAi experiments reported herein indicated that Kr-h1 transduces the antimetamorphic action of JH also in *B. germanica*, thereby suggesting that this role is an ancestral condition that has been conserved in insect evolution from hemimetabolous to holometabolous species.

Insect metamorphosis has fascinated mankind since the time of Aristotle, some two thousand years ago. Much later, Renaissance entomologists established that post-embryonic changes are most spectacular in insects like beetles, moths and flies, which undergo a dramatic morphological transformation from larva to pupa and adult, a phenomenon nowadays known as holometaboly, which is typical in endopterygote species. Exopterygote insects, such as locusts and cockroaches, also transform from last nymphal instar to adult, although the transformation is not as radical as the nymphs are relatively similar to the adult stage. However, they undergo qualitative metamorphic changes, such as formation of mature wings and external genitalia, amongst others, in a type of metamorphosis known as hemimetaboly[1–3]. Understanding insect metamorphosis at molecular level is still a challenging mystery because we still only have a few pieces of the puzzle. From an endocrine point of view, metamorphosis is regulated by two kinds of hormones, namely molting hormone, which induces molts, and juvenile hormone (JH), which modulates the quality of the molt: to an immature stage when it is present, and to the adult when it is absent; JH therefore plays a crucial repressive role in insect metamorphosis[1,4,5].

The effect of the commonest molting hormone, namely 20-hydroxyecdysone, is mediated by a cascade of transcription factors and starts upon its binding to the heterodimeric receptor composed by the ecdysone receptor and the ultraspiracle (or RXR), both of which belong to the nuclear receptor superfamily. This activates expression of a hierarchy of transcription factors, such as E75, E78, HR3, HR4 and FTZ-F1, which regulate the genes that underlie the cellular changes associated with molting and metamorphosis[6,7]. Most of the reported data on this cascade of transcription factors refer to *Drosophila melanogaster*, the fruit fly, a holometabolous insect that shows many highly derived characters, which has been the most thoroughly studied species from the point of view of molecular endocrinology[8,9]. From this point of view, the most widely studied hemimetabolous model is the German cockroach *Blattella germanica*, and results indicate that the transcription factors involved in 20-hydroxyecdysone signalling are generally conserved, although the functions of some of them and the precise epistatic relationships between them may differ with respect to *D. melanogaster*[10–14].

Conversely, the molecular mechanisms underlying the action of JH are poorly understood, and our current understanding relies completely on holometabolous models[4]. An important player is Methoprene tolerant (Met), a transcriptional regulator of the basic helix-loop-helix (bHLH) -Per-Arnt-Sim (PAS) domain family that was discovered in *D. melanogaster*[15] where it binds JH at physiological concentrations. This and other characteristics[4,16] suggest that Met plays the role of JH receptor, or is a component of a heterodimeric JH receptor. Key functional evidence that Met is required for the repressor action of JH on metamorphosis was not obtained in

D. melanogaster but in the beetle *Tribolium castaneum*, a holometabolous insect that shows fewer highly derived characters than *D. melanogaster*, where the RNAi of Met induced larvae to undergo precocious metamorphosis[17,18].

The transcription factor Krüppel homolog 1 (Kr-h1), whose antimetamorphic action has recently been demonstrated in *D. melanogaster*[19] and *T. castaneum*[20], is another important transducer of the JH signal. In *D. melanogaster*, the adult epidermis of head and thorax derive from imaginal discs[21], whereas that of the abdomen derives from larval histoblasts that start proliferating after puparium formation and give the pupal epidermal cells[22,23]. Ectopic application of JH prior to the prepupal stage prevents the normal differentiation of the abdominal epidermis, and the bristles that normally occur in the dorsal midline of the adult fly become shorter or simply do not form[24,25]. Moreover, ectopic expression of Kr-h1 in the abdominal epidermis during metamorphosis causes missing or short dorsal midline bristles, just as in the experiments with JH treatment, thereby suggesting that, in *D. melanogaster*, Kr-h1 mediates the antimetamorphic action of JH[19]. In *T. castaneum*, RNAi experiments have shown that Kr-h1 represses metamorphosis and that it works downstream of Met in the JH signalling pathway[20]. Kr-h1 therefore appears to be the more distal transcription factor in the JH signalling pathway whose role as mediator of the antimetamorphic action of JH has been conserved from beetles to flies, within the Endopterygota (=Holometabola) insect subclass.

As all functional data on the signalling pathway of JH have been obtained in holometabolous models, research in hemimetabolous species is needed if we aim at elucidating the evolution of insect metamorphosis. In light of this, we studied the function of Kr-h1 in *B. germanica*, a polyneopteran exopterigote that shows a gradual morphological transformation along the life cycle, which is representative of hemimetabolous metamorphosis[1,2]. Methodologically, our strategy was to knockdown Kr-h1 by RNAi, a technique that has been shown to be highly effective for silencing gene expression in *B. germanica*[26], and then to examine the phenotype obtained.

Results

The sequence of Krüppel-h1 is highly conserved in insects. Cloning of Kr-h1 cDNA in *B. germanica* was accomplished by a RT-PCR approach, combining the use of degenerate primers based on Kr-h1 conserved motifs to obtain a partial sequence, and 5'-RACE and 3'-RACE experiments to complete it. These amplifications rendered a cDNA of 2269 bp (GenBank accession number HE575250) where the putative start codon is preceded by in-frame stop codons, thus suggesting that a full-length open reading frame had been obtained. Database BLAST searches indicated that it encoded an ortholog of Kr-h1, which we called BgKr-h1. The conceptual translation rendered a 658 amino acid protein sequence containing the eight classical C_2H_2 zinc fingers towards the C-terminal region, and the "A" (LPLRKR) and "B" (RSRSVIHYA) motifs towards the 3'end in the N-terminal region, which are typical of Kr-h1 proteins[27].

The alignment of the Kr-h1 protein sequences (Supplementary Fig. 1 online) indicates that the most conserved region is the Zn finger domain, where the most apparent feature is an insertion-deletion of 25–47 amino acids located between the first and second Zn finger, which distinguishes the dipterans (that show the insertion) from the other insect orders. The percentage of identity of the BgKr-h1 Zn finger domain with respect to non-dipteran species is very high, ranging from 90% (with *Apis mellifera*), to 82% (with *Nasonia vitripennis*), whereas it is lower when compared with dipteran sequences, ranging from 68% (with *Aedes aegypti*) to 63% (with *D. melanogaster*). The percentage of identity in the Zn finger domain is also high with the homolog that we found in the crustacean *Daphnia pulex*, whose sequence presents only seven Zn fingers but having between 60 and 70% identity with the equivalent region in insects.

Krüppel-h1 expression decays suddenly in the last instar nymph and is up-regulated by juvenile hormone. To gain a first insight into the possible involvement of BgKr-h1 in cockroach metamorphosis, we studied its expression pattern in the whole body of females during the three last nymphal instars. The results (Fig. 1a) show that BgKr-h1 mRNA levels generally oscillate between 10 and 20 copies per 1000 copies of BgActin-5c but suddenly vanish one day after the molt to the sixth (last) nymphal instar. This down-regulation of BgKr-h1 expression coincides with decreasing levels of circulating JH (Fig. 1a), according to analytical data previously obtained in our laboratory[28]. Moreover, expression is not confined to a single or few tissue types, but is ubiquitously distributed amongst practically all tissues, especially the muscle, epidermis (pronotum, mesonotum and metanotum samples) and ovaries (Fig. 1b). Pattern coincidence suggests that BgKr-h1 expression is induced by JH, therefore to test this hypothesis we treated freshly emerged sixth instar female nymphs with JH III, and measured BgKr-h1 transcript levels 6 h, 2 days, 4 days and 6 days later. The results obtained indicate that JH up-regulates BgKr-h1 expression and that the stimulatory effect lasts practically the entire sixth instar nymph, although with decreasing intensity (Fig. 1c). A number of JH-treated specimens were left alive until the next molt, and they molted into adultoids with nymphal features (Supplementary Fig. 2 online), as expected.

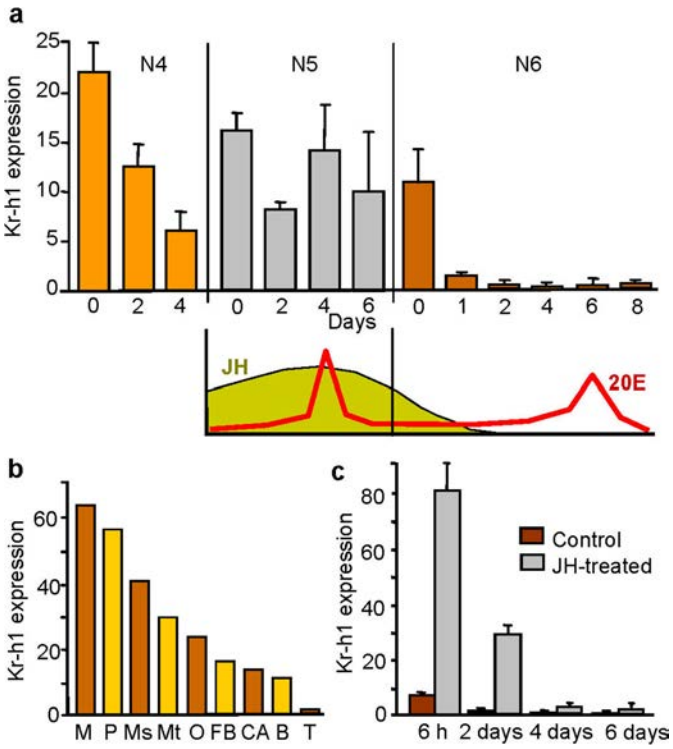

Figure 1 | Expression of BgKr-h1 mRNA in *Blattella germanica* determined by qRT-PCR. (a) Expression in female whole body in the three last nymphal instars: N4, N5 and N6. Relative titers of juvenile hormone III (JH) and 20-hydroxyecdysone (20E) in N5 and N6 are indicated below, according to Treiblmayr et al.[28] and Romaña et al.[39], respectively. (b) Expression in different tissues of females in day 0 of N6: muscle (M), pronotum (P), mesonotum (Ms), metanotum (Mt), ovaries (O), brain (B), fat body (FB), corpora allata (CA), and in testicles (T) from males of the same age. (c) Effect of the application of 20 μg of JH on freshly emerged N6 on BgKr-h1 mRNA levels. Data in (a) and (c) represent the mean ± SEM, and are indicated as copies of BgKr-h1 mRNA per 1000 copies of BgActin-5c; each point represents 4 biological replicates. Data in (b) represent a pool of 5 specimens. In (c), differences of JH-treated with respect to controls were statistically significant in all cases ($p < 0.05$), according to the REST software tool[40].

RNAi of Krüppel-h1 in fifth instar female nymphs results in precocious metamorphosis after the next molt. We approached the study of BgKr-h1 function in *B. germanica* by RNAi. In a first set of experiments, we injected a single 3-μg dose of dsRNA targeting BgKr-h1 (dsKr-h1) into the abdomen of freshly emerged fifth (penultimate) instar female nymphs. Controls received the same dose of unspecific dsRNA (dsMock). Transcript monitoring at 48 h intervals indicated that BgKr-h1 levels were significantly lower (52%) in dsKr-h1-treated specimens than in controls 6 days after the treatment (Fig. 2a). dsMock-treated (control) specimens (n = 40) molted to normal sixth instar nymphs ca. 6 days after the treatment. Females treated with dsKr-h1 (n = 41) required, on average, two or three days more than controls to perform the next molt (Fig. 2b), and this molt rendered individuals with adult features. About 71% of the specimens (Fig. 2c) had a general morphology and coloration intermediate between a sixth (last) instar nymph and an adult; of note, the structure of the latero-basal expansions of the mesonotum and metanotum (which correspond to the mesonotal and metanotal wing pads) was flexible and membranous, as in mature wings and tegmina (Fig. 2d). Most of these intermediates (86%) died between 6 and 10 days after the molt. In contrast, some of them (14%) were able to molt again (around day 9 of this sixth instar), although they were unable to properly shed the exuvia, and this resulted in mechanically deformed adults, with the wings well patterned but not well extended (Supplementary Fig. 3 online). The remaining 29% of the treated specimens (Fig. 2c) had the typical morphology and coloration of an adult, although they were smaller (having the size of a normal sixth instar female nymph) and their wings were membranous and well patterned, although not well extended (Fig. 2d). These precocious adults lived much longer than the nymph-adult intermediates (between 2 and 3 months, as average) and did not molt again.

In a second set of experiments we injected two doses of 3 μg each of dsKr-h, one into freshly emerged fifth instar female nymphs, and the

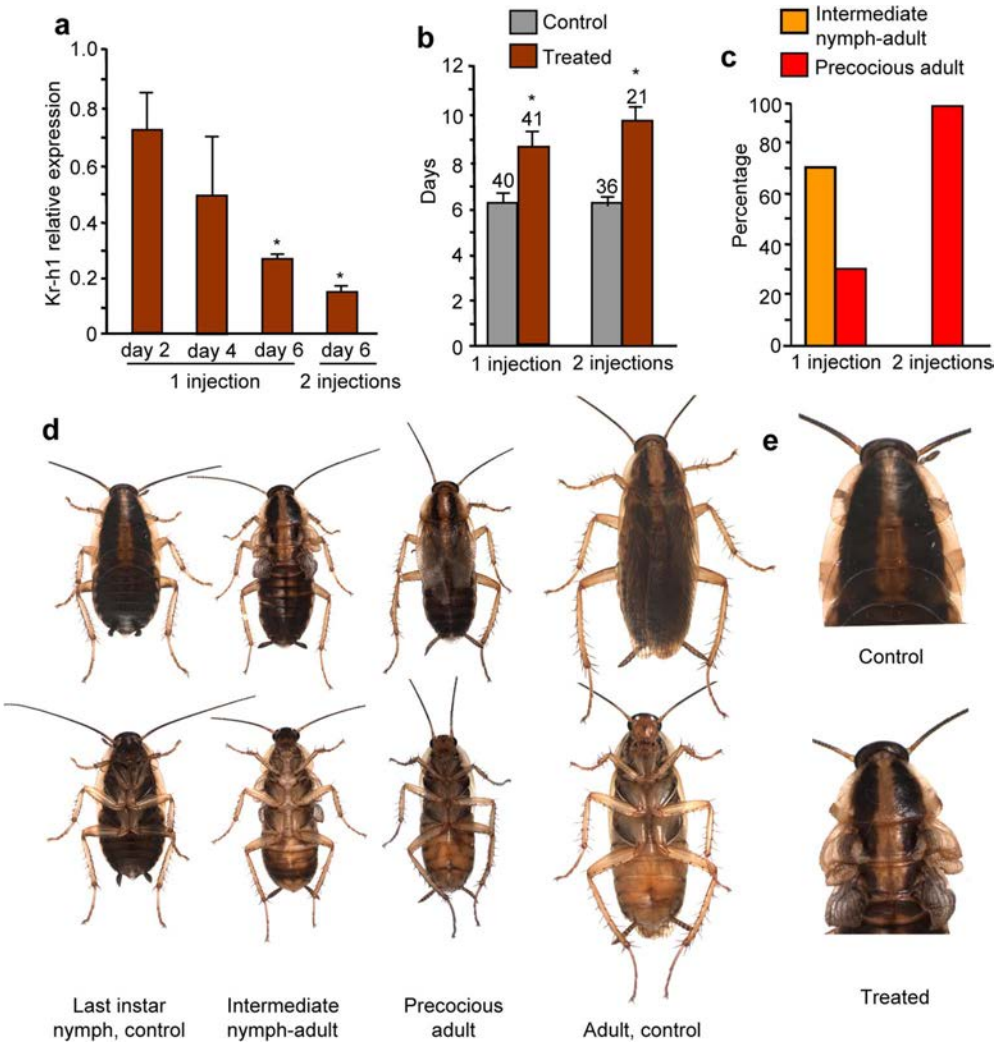

Figure 2 | Effects of BgKr-h1 depletion in fifth nymphal instar (N5) of *Blattella germanica* females. Females received 1 injection (3 μg-dose, on day 0 of N5), or 2 injections (3 μg each, on day 0 and day 3 of N5, respectively) of dsMock (control) or dsKr-h1 (treated). (a) Effects on BgKr-h1 mRNA levels measured by qRT-PCR on days 2, 4 and 6 of N5 in single-injection experiments, or on day 6 of N5 in two-injection experiments. (b) Length (days) of N5 in control and treated female specimens. (c) Percentage of specimens showing the intermediate nymph-adult phenotype or the precocious adult phenotype in the 1- or 2-injection experiments. (d) Dorsal and ventral view of phenotypes resulting from 1- or 2-injection experiments, compared with control females in last nymphal instar and with the adult stage. (e) Dorsal part of the thorax in the intermediate nymph-adult phenotype, showing the membranous structure of the wing pads. Data in (a) represent 4 biological replicates (mean ± SEM) and are normalized against the dsMock females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), according to the REST software tool[40]. Replicates in (b) are indicated at the top of each bar; the asterisk indicates that differences with the respective controls are statistically significant (student's *t*-test, $P<0.001$).

other one three days later. Controls were equivalently treated with dsMock. Six days after the treatment, BgKr-h1 mRNA levels were significantly lower (72%) in dsKr-h1-treated specimens than in controls (Fig. 2a). Females treated with dsKr-h1 (n = 21) took between three and four days more than controls (n = 36) to complete the fifth instar (Fig. 2b). In contrast with controls, which molted normally to the sixth nymphal instar, all dsKr-h1-treated specimens molted to precocious adults with imperfectly extended wings, in a similar manner to the 29% fraction of the single-injection experiments reported above (Fig. 2d). None of these precocious adults molted again.

RNAi in fourth instar female nymphs results in precocious metamorphosis after two molts. In order to test whether precocious metamorphosis could also be provoked in younger instars, we carried out experiments equivalent to those just described but using fourth instar female nymphs. Single-injection treatments were carried out on freshly emerged fourth (antepenultimate) instar female nymphs, and four days after the injection BgKr-h1 mRNA levels were already significantly lower (72%) in dsKr-h1-treated specimens than in controls (Fig. 3a). The length of the fourth instar was the same (ca. 5 days) in dsKr-h1-treated (n = 25) and in controls (n = 15), and all specimens molted normally to fifth instar. However, the length of the fifth instar in the dsKr-h1-treated specimens was almost twice that for the controls (Fig. 3b). After the next molt, control specimens became normal sixth instar nymphs, whereas six out of 25 specimens (24%) of the ds-Kr-h1-treated group (Fig. 3c) gave a phenotype intermediate between a sixth instar nymph and an adult, showing the typical membranous structure of the wing pads (Fig. 3d). These six intermediates died between 8 and 10 days after the molt. The remaining 19 dsKr-h1-treated specimens (76%) (Fig. 3c) molted into precocious adults with imperfectly extended wings (Fig. 3d), which did not molt again.

Two-injection experiments also induced a significant decrease (82%) of BgKr-h1 mRNA levels four days after the first injection (Fig. 3a), and the timing of the fourth and fifth nymphal instars was similar to that found in the single-injection experiments: no differences in the length of the fourth instar between dsKr-h1-treated (n = 20) and controls (n = 12), and a significant increase in the length of the fifth instar in the dsKr-h1-treated specimens (Fig. 3b). After the next molt, all dsKr-h1-treated specimens became precocious adults with imperfectly extended wings (Fig. 3d), which did not molt again. Controls molted to normal sixth instar nymphs.

Males are more sensitive to the silencing effects of Krüppel-h1 RNAi. A few male nymphs, all of which yielded precocious adults after two molts, were inadvertently included in the experiments involving injecting a single dose of dsKr-h1 into fourth instar female nymphs. This prompted us to test whether males could be more sensitive than females to the silencing effects of RNAi on BgKr-h1. First, we obtained data regarding BgKr-h1 mRNA levels on selected days of the penultimate and last nymphal instars in males. Our determinations indicated that the expression pattern (Fig. 4a) is similar to that of females (Fig. 1a), with a sudden decrease after the first day of the last nymphal instar.

We then used the design of single-injection RNAi experiments by administering a 3-μg dose of dsKr-h1 into freshly emerged fourth instar male nymphs (controls received the same dose of dsMock).

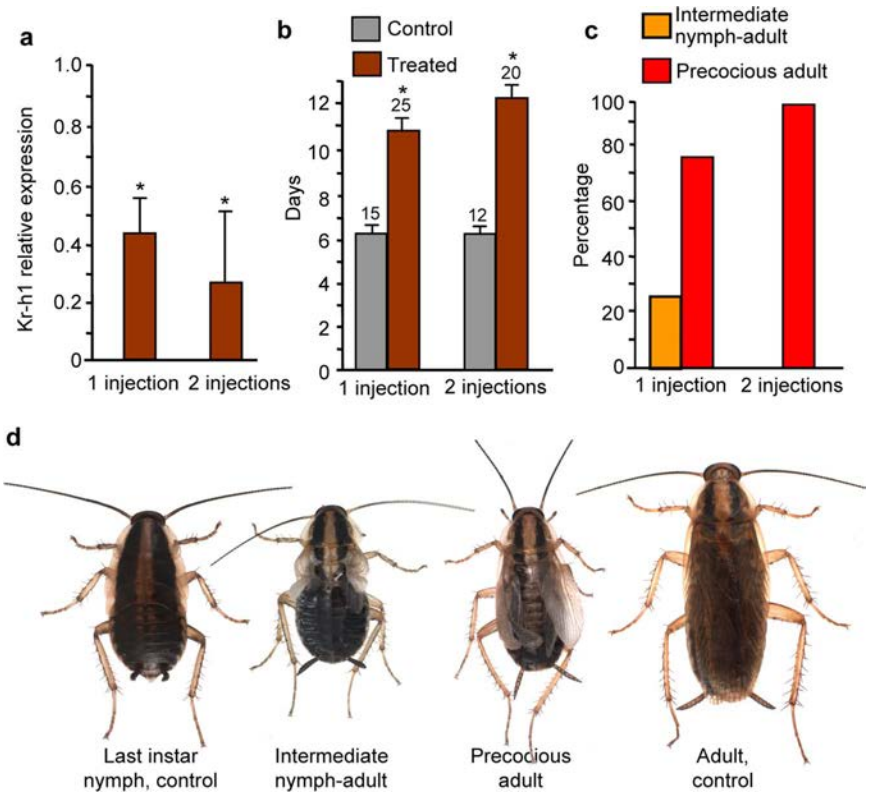

Figure 3 | Effects of BgKr-h1 depletion in fourth nymphal instar (N4) of *Blattella germanica* females. Females received 1 injection (3 μg-dose, on day 0 of N4), or 2 injections (3 μg each, on day 0 and day 3 of N4, respectively) of dsMock (control) or dsKr-h1 (treated). (a) Effects on BgKr-h1 mRNA levels measured by qRT-PCR on day 4 of N4 in 1- or 2-injection experiments. (b) Length (days) of N5 in control and treated specimens. (c) Percentage of specimens showing the intermediate nymph-adult phenotype or the precocious adult phenotype in the 1- or 2-injection experiments. (d) Dorsal view of phenotypes resulting from 1- or 2-injection experiments, compared with control females in last nymphal instar and with the adult stage Data in (a) represent 4 biological replicates (mean ± SEM) and are normalized against the dsMock females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), according to the REST software tool[40]. Replicates in (b) are indicated at the top of each bar; the asterisk indicates that differences with the respective controls are statistically significant (student's *t*-test, $P<0.001$).

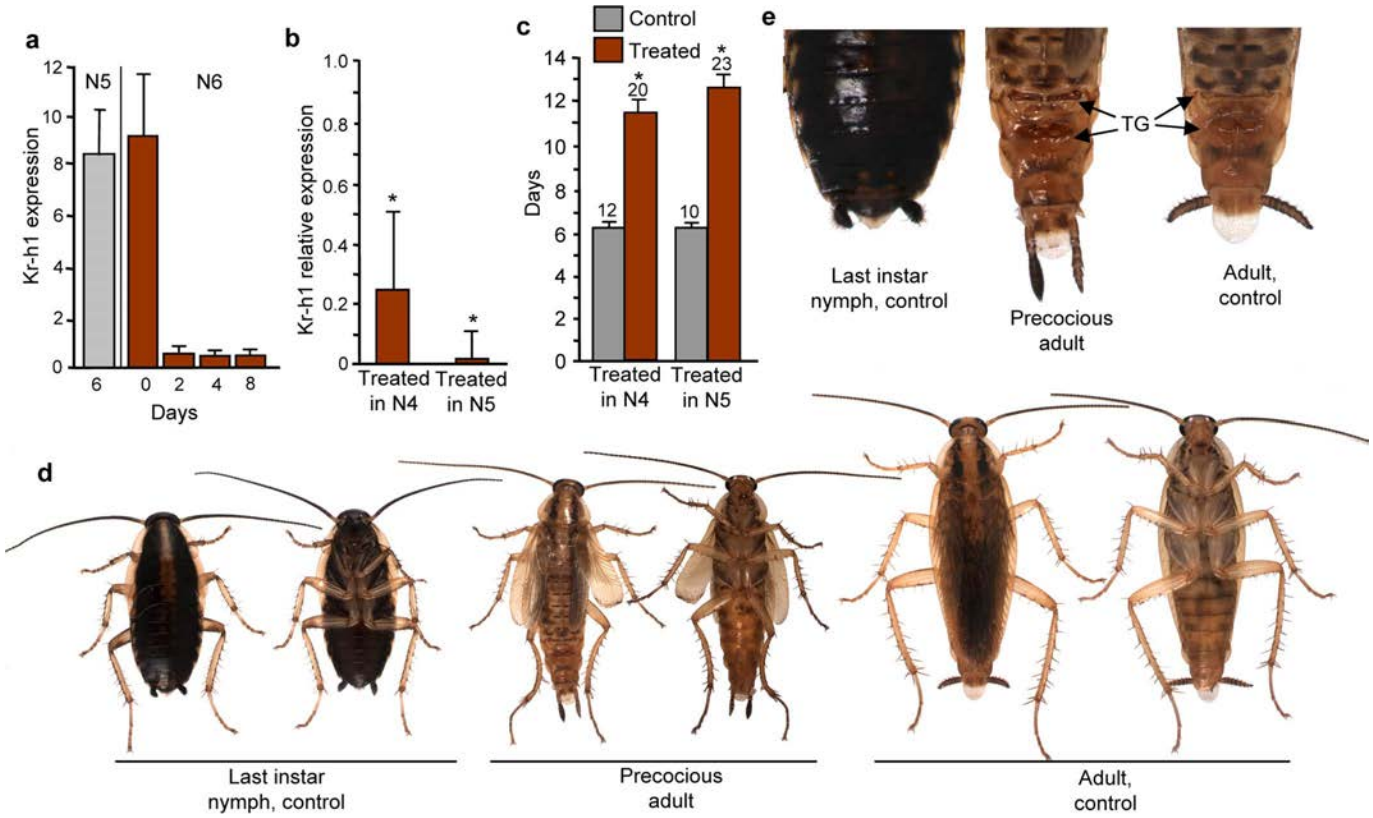

Figure 4 | Expression of BgKr-h1 and effects of its depletion in *Blattella germanica* males. Males received a single dose of 3 μg on day 0 of fourth (N4) or fifth (N5) nymphal instar of dsMock (control) or dsKr-h1 (treated). (a) Expression of BgKr-h1 in male whole body in selected days of N5 and N6. (b) Effects of dsKr-h1 on BgKr-h1 mRNA levels measured by qRT-PCR in specimens treated on N4 (transcript measured on day 4) or on N5 (transcript measured on day 6). (c) Length (days) of N5 in male specimens treated in N4 or N5. (d) Phenotype (dorsal and ventral view) resulting from the experiments carried out on N4 after two molts or in N5 after one molt. (e) Tip of the abdomen showing the area occupied by the tergal glands (TG) in the seventh and eighth tergites. Data in (a) represent the mean ± SEM, and are indicated as copies of BgKr-h1 mRNA per 1000 copies of BgActin-5c; each point represents 4 biological replicates. Data in (b) represent 4 biological replicates (mean ± SEM) and are normalized against the dsMock females (reference value = 1); the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), according to the REST software tool[40]. Replicates in (c) are indicated at the top of each bar; the asterisk indicates that differences with the respective controls are statistically significant (student's *t*-test, $P<0.001$).

This treatment induced a significant decrease (75%) of BgKr-h1 mRNA levels four days after the injection (Fig. 4b). Moreover, the fourth instar had the same length in dsKr-h1-treated and in control males, and all specimens molted normally to the fifth instar, whereas the length of the latter in dsKr-h1-treated males was practically twice that of the controls (Fig. 4c). All dsKr-h1-treated specimens (n = 20) then molted into precocious adults, with the same shape and coloration as a normal adult male, but with the size of a sixth nymphal instar, the membranous wings present but not well extended, and the tergal glands (which are absent in nymphs but present in adult males as paired pouches on the tergites 7 and 8) readily apparent (Fig. 4d). Of note, the posterior margin of tergite 7 was somewhat shorter and notched in these precocious adults, which allowed seeing directly the paired glands of the tergite 8, whereas they are partially hidden by the well developed tergite 7 in normal adults (Fig. 4e). Male precocious adults resulting from dsKr-h1 treatments did not molt again. Control specimens (n = 12) became normal sixth instar nymphs after the fifth molt.

Finally, we carried the same experiments but treating freshly emerged fifth instar male nymphs. In this case, the treatment induced a remarkable decrease (93%) of target transcript levels (Fig. 4b), much higher than that observed in females in equivalent experiments, and the length of the fifth instar was higher in treated than in controls (Fig. 4c), as in the former experiments. All dsKr-h1-treated specimens (n = 23) molted into precocious adults at the next molt, showing the same features as those described in the experiments carried out on males in the fourth nymphal instar (see Fig. 4d). Controls (n = 10) molted to normal sixth instar nymphs.

Discussion

The amino acid sequence of BgKr-h1, the Krüppel homolog 1 of the cockroach *B. germanica* (which undergoes a nymphal-adult transformation that is representative of hemimetabolous metamorphosis) is similar to those of ortholog sequences of other insects from holometabolous orders (i.e. those that undergo a full metamorphosis). Considering all sequences included in the alignment (Supplementary Fig. 1 online), the degree of conservation is high, even when comparing the sequence of *B. germanica* with that of the holometabolous insect *D. melanogaster*, which shows many highly derived characters. The similarity is particularly high in the Zn finger domain, where the most complex binding capacity of the molecule resides. Indeed, multiple-adjacent C_2H_2 zinc finger proteins bind 25–75% of the Zn fingers to DNA, whereas the remainder may bind to proteins and RNA, including dsRNA and DNA-RNA heterocomplexes[29]. The high degree of conservation found in the C_2H_2 zinc finger domain in all studied species suggests that the Kr-h1 function might have been generally conserved across the insect class. The discovery of a Kr-h1 homolog in the crustacean *D. pulex* opens the question of its function, which remains to be investigated.

Our mRNA determinations showed that BgKr-h1 is ubiquitously expressed in different tissues, whereas time-course studies indicated that expression vanishes after the first day of the last instar nymph. Similar decreases in Kr-h1 expression have been observed in the transition from the "propupa" to the "pupa" of thrips species (*Frankliniella occidentalis* and *Haplothrips brevitubus*)[30], which show a variant of hemimetaboly called neometaboly[1,31]. Similar decreases of Kr-h1 expression occur between the prepupal and pupal stages in holometabolous models, such as the fly *D. melanogaster*[19] and the beetle *T. castaneum*[20]. The coincidence of the patterns of BgKr-h1 and that of circulating JH (which decreases during the first days of the last nymphal instar[28]) in the cockroach *B. germanica* (Fig. 1a), suggested that BgKr-h1 expression is induced by JH. This hypothesis was assessed in experiments that showed that treatment of last instar nymphs with JH readily induced the re-expression of BgKr-h1. Early works on *D. melanogaster* reported that Kr-h1 is partially regulated by 20-hydroxyecdysone and, in turn, modulates ecdysteroid-dependent metamorphic processes[32,33]. However, more recent work revealed that Kr-h1 expression is induced by JH in the fly *D. melanogaster*[19], the beetle *T. castaneum*[20], and the thrips *F. occidentalis* and *H. brevitubus*[30], as is also the case in *B. germanica* (present work).

The results of RNAi experiments indicate that knockdown of BgKr-h1 in *B. germanica* in juvenile stages induces a precocious metamorphosis after the penultimate (fifth) nymphal instar. Of note, when the RNAi was carried out in the fourth nymphal instar, two molts were needed before the occurrence of precocious metamorphosis. Pioneering experiments have shown that metamorphosis can be precociously induced by dissecting out the corpora allata, i.e., the JH-producing glands. However, when such allatectomy is performed in very young larvae, one or two additional molts are currently needed before precocious adult features appear[34]. More recent studies have shown that depletion of JH through overexpression of JH esterase fails to cause premature pupation in larvae of the lepidopteran *Bombyx mori* if they are younger than third instar[35]. Similarly, metamorphic changes after RNAi treatment targeting Met in young larvae of *T. castaneum* typically require two or three molts before giving precocious adult features[17]. These observations, and those described herein in *B. germanica*, suggest that the immature insect must achieve a critical weight (thus a minimum time of postembryonic growth) in order to be able to metamorphose when JH vanishes, either in hemimetabolous as well as in holometabolous species. Connected with this issue is the observation showing that the stage previous to precocious metamorphosis in dsKr-h1-treated specimens, i.e., the fifth (normally the penultimate) nymphal instar is prolonged by 40–100% before molting, compared with the same instar in controls. Again, this delay could be necessary to reach a minimal critical weight or (perhaps more plausibly, given that there are no delays in the fourth nymphal instar) to unfold the developmental transition between the nymph and the adult.

RNAi was found to be more efficient in males than in females of *B. germanica*. Thus, the penetrance of the precocious adult phenotype in males using a single injection was 100%, irrespective of the stage of treatment, whereas in females it was between 29%, when females were treated in the fifth nymphal instar, and 76% when treated in the fourth nymphal instar (Supplementary Table 1 online). The experiments with males made clearer that there is a rough correlation between the percentage of transcript decrease after dsKr-h1 treatment and that of precocious adults obtained in the experiment. Moreover, the fact that target transcript decrease was higher in males than in females, especially in fifth nymphal instar (compare figures 2a and 4b), suggests that RNAi machinery[26] was more efficiently induced in males than in females after dsKr-h1 treatment, an aspect that may deserve further research.

According to the functional studies performed on *D. melanogaster*[19] and *T. castaneum*[20], Kr-h1 was considered the more distal transductor of the JH hierarchy whose antimetamorphic action had been conserved from beetles to flies, within the endopterygote suborder, whose species show holometabolous metamorphosis. Now, the results of the RNAi experiments reported herein indicate that BgKr-h1 plays the equivalent role in *B. germanica*, an exopterygote polyneopteran species showing an hemimetabolous mode of metamorphosis[1]. This suggests that the repressor role of Kr-h1 on metamorphosis is an ancestral condition that has been conserved from hemimetabolous to holometabolous species. Thus, the ancestral role of Kr-h1 can be a useful starting point to study the mechanisms underlying the evolutionary transition from hemimetaboly to holometaboly, which still remain a challenging enigma.

Methods

Insects. The specimens of *B. germanica* used in the experiments were obtained from a colony reared in the dark at 30 ±1°C and 60–70% RH. They were anaesthetized with carbon dioxide prior to injection treatments, dissections and tissue sampling.

Cloning of Kr-h1 cDNA. The *B. germanica* Kr-h1 homolog was obtained following a RT-PCR strategy using degenerate primers designed on the basis of conserved motifs from insect Kr-h1 sequences, and cDNA from one- to six-day-old fifth instar female nymphs of *B. germanica* as a template. The primers were: forward, 5'- GVCAYTACCGNACNCAYACBGGBGA -3'; reverse, 5'- TTBAGCACRTGRTTGTAGCCRAAG -3'. The sequence of the amplified fragment (419 bp) was highly similar to the equivalent region in known insect Kr-h1 sequences. Then, the sequence was completed by 5' and 3' RACE (5'- and 3'-RACE System Version 2.0; Invitrogen) using the same template. For 5'-RACE, reverse primer was 5'- CCTTGCCACAAATGACACAA -3' and the nested primer was 5'-AATGATTTGCTGCAATACTCGC -3. For 3'-RACE, forward primer was 5'-CTTGTCATACACATGCGCACTCATACAG-3' and the nested primer was 5'-GGAGAAACCCTATTCTTGTG -3'. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced.

RNA Extraction and retrotranscription to cDNA. All RNA extractions were performed using the miRNeasy Mini Kit (Qiagen). A 500-ng sample from each RNA extraction was treated with DNAse (Promega) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and random hexamers (Promega). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies).

Determination of mRNA levels by quantitative real-time PCR. Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems). A template-free control was included in all batches. The primers used to detect Kr-h1 mRNA were as follows: forward, 5'- GCGAGTATTGCAGCAAATCA -3' and reverse, 5'- GGGACGTTCTTTCGTATGGA -3'. The efficiency of this primer set was first validated by constructing a standard curve through four serial dilutions. mRNA levels were calculated relative to BgActin-5c (Accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA.

Treatments with juvenile hormone III in vivo. To study the effect of juvenile JH upon Kr-h1 expression, JH III (Sigma-Aldrich), which is the native JH of *B. germanica*[36], was applied topically to freshly emerged last instar nymphs at a dose of 20 µg per specimen in 1 µL of acetone. The commercial JH III is a mixture of isomers containing ca. 50% of the biologically active (10R)-JH III, thus the active dose applied was around 10 µg per specimen. Controls received 1 µL of acetone.

RNA interference. The detailed procedures for RNAi experiments were as described previously[37]. A dsRNA encompassing a 320-bp fragment located between nucleotides 611 and 930 (dsKr-h1), in the Zn finger domain, was designed. The primers used to generate the templates to prepare the dsKr-h1 were as follows: forward, 5'- GAATCTCAGTGTGCATAGGCG -3' and reverse, 5'- CCTTGCCACAAATGACACAA -3'. The fragments were amplified by PCR and cloned into the pSTBlueTM-1 vector. A 307-bp sequence from *Autographa californica* nucleopoyhedrovirus (Accession number K01149, from nucleotide 370 to 676) was used as control dsRNA (dsMock). The dsRNAs were prepared as reported previously[37]. A volume of 1 µL of dsRNA solution (3 µg/µL) was injected into the abdomen of specimens at chosen ages and stages. Control specimens were treated with the same dose and volume of dsMock.

Sequence comparisons. Insect sequences labelled as Krüppel homolog 1 were obtained from GenBank, and the list was enlarged by BLAST search using the *B. germanica* BgKr-h1 sequence as query. Finally, the protein sequences included in the analysis were the following (GenBank accession number and annotation details, if needed, in parenthesis). *Acromyrmex echinatior* (EGI66600.1), *Acyrtosiphon pisum* (XP_001946194, annotated as hypothetical protein), *Aedes aegypti* (EAT46451, annotated as Zinc finger protein), *Anopheles gambiae* (EAA13888.4, annotated as

Zinc finger protein, incomplete at the 3' end of the ORF), *Apis mellifera* (NP_001011566.1), *Blattella germanica* (HE575250), *Bombyx mori* (NP_001171332.1), *Bombus terrestris* (ACX50259.1), *Camponotus floridanus* (EFN62423.1), *Culex quinquefasciatus* (XP_001863529.1, annotated as Zinc finger protein), *Drosophila melanogaster* (CAA06543, Kr-h1 isoform B), *Frankliniella occidentalis* (BAJ41258.1, Kr-h1 isoform B), *Nasonia vitripennis* (XP_003425921.1), *Solenopsis invicta* (EFZ20948.1, annotated as Zn finger protein), *Spodoptera littoralis* (EZ981183.1, nucleotide sequence from transcriptome shotgun assembly), *Striacosta albicosta* (EZ585624.1, nucleotide sequence from transcriptome shotgun assembly), *Tribolium castanenum* (NP_001129235.1), *Pediculus humanus* (XP_002428656.1, annotated as Kr-18). In addition, we found a Kr-h1 orthologue of the Crustacean *Daphnia pulex* (EFX82007.1, annotated as Zn finger protein), which served as a reference for the sequences comparison. The sequences were aligned using the MAFFT program[38], with default parameters, and visualized using Geneious Software.

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Author contributions

X.B. initially conceived and designed the research. J.L. carried out the experiments and the sequences comparison. X.B. wrote the manuscript. Both authors discussed the results, decided about subsequent experiments and commented on the manuscript.

Additional information

Accession code: The new sequence BgKr-h1 has been deposited to GenBank under accession code HE575250.

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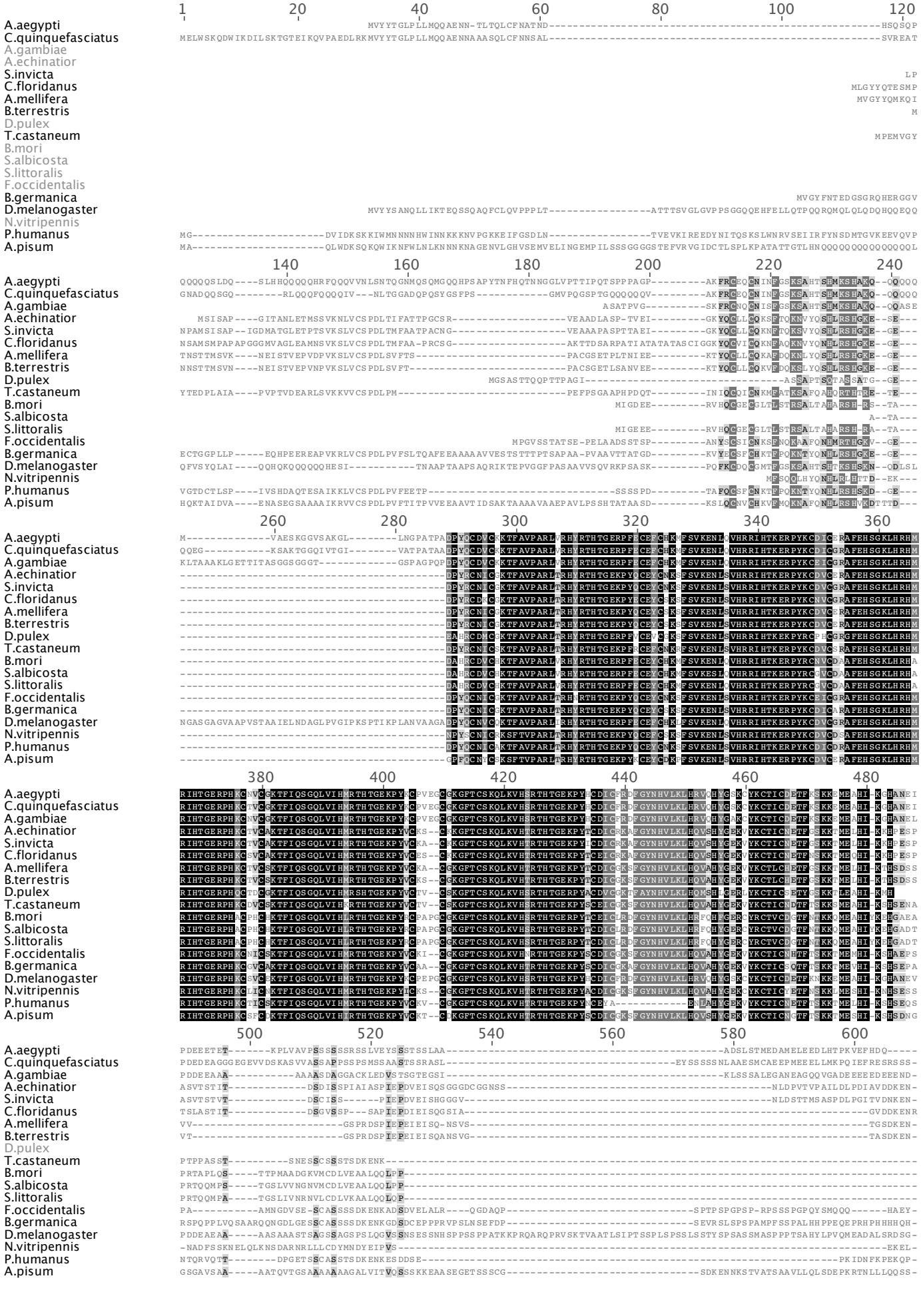
Supplementary information

Conserved repressive function of Krüppel homologue 1 on insect metamorphosis in hemimetabolous and holometabolous species

Jesus Lozano & Xavier Belles

3 Supplementary Figures
1 Supplementary Table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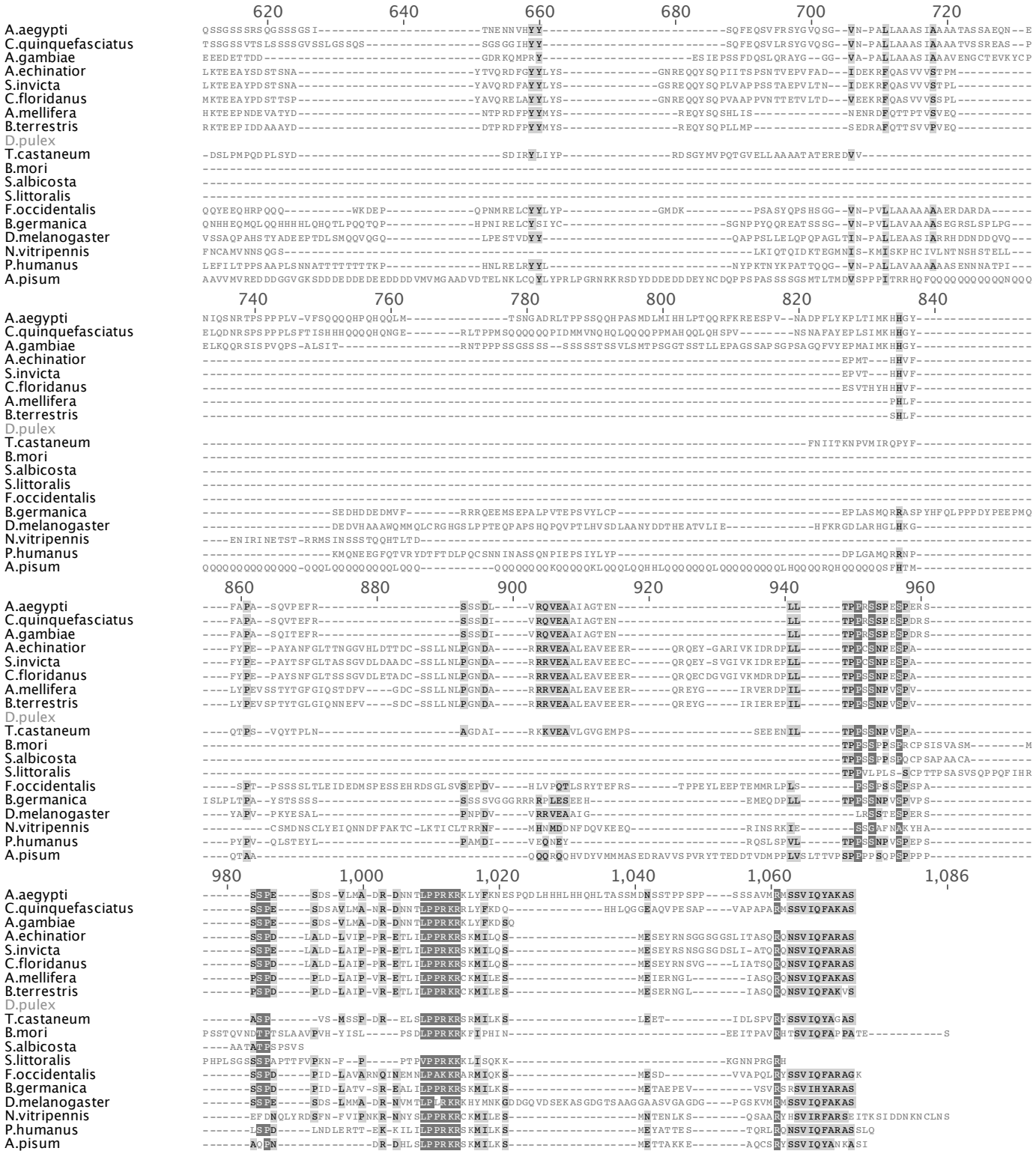

Supplementary Figure S1. Alignment of the Krüppel homolog 1 protein sequences from insects and from the Crustacean *Daphnia pulex*. The alignment was carried out using MAFFT program and visualiced in Geneiolus Software. Positions with 100% of identity are indicated in black, 80 to 99% in dark grey, 60 to 79% in bright grey and in white with less than 59%.

Supplementary Figure 2. Dorsal and ventral view of a supernumerary nymph of *Blattella germanica*. They were obtained after treating freshly ecdysed sith (last) instar female nymphs with 20 µg of JH III. The wings were partially extended, but the general shape and dark coloration, in particular in the abdomen, correspond to a nymph.

Supplementary Figure 3. Incomplete imaginal ecdysis of nymph-adult intermediates of *Blattella germanica*. Nymph-adult intermediates were obtained instead the normal sixth (last) nymphal instar after treating freshly ecdysed fifth instar nymphs with dsKr-h1. Most of them (86%) died between 6 and 10 days after the molt, but 14% were able to molt again, although did not complete the ecdysis. The image shows one of these specimens, still with part of the exuvia covering the tip of the abdomen. The wings are unextended, but the general shape and yellowish coloration, in particular in the abdomen, correspond to an adult.

Supplementary Table

Supplementary Table 1. Summary of the RNAi experiments to knockdown BgKr-h1 in *Blattella germanica*. Experiments were carried out on males (M) or females (F), with a single dose of 3 µg of dsKr-h1 injected on day 0 of the corresponding nymphal instar, or with two doses of 3 µg of dsKr-h1 each, injected on day 0 and day 3. The phenotype refers to the external morphology of the specimens.

Sex	**Nymphal instar**	**dsKr-h1 day 0**	**dsKr-h1 day 3**	**n**	**Phenotype**
F	4th	x		25	6 (24%) intermediate nymph-adult* 19 (76%) precocious adult*
F	4th	x	x	20	20 (100%) precocious adult*
F	5th	x		41	29 (71%) intermediate nymph-adult** 12 (29%) precocious adult**
F	5th	x	x	21	21 (100%) precocious adult**
M	4th	x		20	20 (100%) precocious adult*
M	5th	x		23	23 (100%) precocious adult**

*Phenotype obtained after two molts

** Phenotype obtained after one molt

8. MiR-2 regula la metamorfosi dels insectes mitjançant el control de la via de senyalització de l'hormona juvenil

MiR-2 regula la metamorfosi dels insectes mitjançant el control de la via de senyalització de l'hormona juvenil

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Resum

Els miRNAs juguen un paper clau en la metamorfosi hemimetàbola, però el mecanisme exacte que controla el procés encara és desconegut. Sabem que si inhibim l'enzim que catalitza l'últim pas de la biosíntesi dels miRNA (Dicer-1) la metamorfosi en *Blattella germanica* no es produeix. Hem observat que aquest fenotip s'associa a un augment en els nivells d'mRNA de Krüppel homolog 1 (Kr-h1), un factor de transcripció dependent d'hormona juvenil que reprimeix la metamorfosi, i que la metamorfosi pot ser restaurada mitjançant RNAi de Kr-h1 en els individus interferits per Dicer-1. Hem predit que a la regió 3'UTR de l'mRNA de Kr-h1 hi ha un lloc d'unió a miRNAs, i l'hem validat com a funcional per a miR-2. Totes aquestes dades suggereixen que la inhibició de la metamorfosi causada pel bloqueig de la síntesi de miRNAs va ser resultat d'una desregulació de l'expressió Kr-h1 i que aquesta desregulació podria ser deguda a una deficiència de miR-2. Això va ser corroborat pel tractament de l'últim estadi nimfal de *B. germanica* amb un inhibidor de miR-2, la qual cosa impedia la metamorfosi, i pel tractament amb un mimètic de miR-2 realitzat en exemplars interferits per a Dicer-1, la qual cosa va restaurar la metamorfosi. D'aquesta manera, miR-2 es mostra com un dels elements clau que participen en el control dels nivells de transcripció Kr-h1, per tant crucial per al desenvolupament correcte de la metamorfosi.

MiR-2 regulates insect metamorphosis by controlling the juvenile hormone signaling pathway

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Abstract

It has been reported that depletion of dicer-1, the enzyme that catalyzes the final step of miRNA biosynthesis, prevents metamorphosis in *Blattella germanica*. However, the precise regulatory roles of miRNAs in the process have remained unknown. We have observed that dicer-1 depletion results in an increase of mRNA levels of Krüppel homolog 1 (Kr-h1), a juvenile hormone-dependent transcription factor that represses metamorphosis, and that depletion of Kr-h1 expression in dicer-1 knockdowns rescues metamorphosis. We have also found that the 3'UTR of Kr-h1 mRNA contains a functional binding site for miR-2. These data suggest that metamorphosis impairment caused by dicer-1 and miRNA depletion is due to a deregulation of Kr-h1 expression and that this deregulation derives from a miR-2 deficiency. We corroborated this by treating the last nymphal instar of *B. germanica* with a miR-2 inhibitor, impairing metamorphosis, and treating dicer- 1-depleted specimens with a miR-2 mimic, which restored metamorphosis. Taken together, the data indicate that miR-2 scavenges Kr-h1 transcripts when the transition from nymph to adult should be taking place, thus crucially contributing to the correct development of metamorphosis.

Significance

MicroRNAs are short, single-stranded RNAs that bind target mRNAs and block their translation. Five years ago it was observed in the cockroach *Blattella germanica* that general depletion of microRNAs prevents metamorphosis. This observation led to two key questions: which microRNAs are involved in this action, and which target do they act on. The results reported herein show that the microRNA is miR-2, and the target is the transcription factor Krüppel homolog 1, a master repressor of insect metamorphosis. The data presented indicate that miR-2 rapidly clears Krüppel homolog 1 transcripts in the last nymphal instar, a process that is crucial for proper metamorphosis. This reveals the elegant mechanism of a single miRNA leading metamorphosis to its correct conclusion.

Introduction

MicroRNAs (miRNAs) are endogenous, *ca*. 22-nucleotide, single-strand, non-coding RNAs that regulate gene expression by acting post-transcriptionally through basepairing between the so-called "seed" sequence of the miRNA (nucleotides 2–8 at its 5'end) and the complementary seed match sequence in the target mRNA (1). Since miRNAs were first discovered in *Caenorhabditis elegans* in the 1990s (2) and subsequently detected in other models (3), a remarkable diversity of them has been reported in a variety of organisms, including insects, plants, viruses and vertebrates (4). Nowadays, we know that miRNAs pervade gene regulatory networks, driving cell differentiation, conferring robustness and channeling the development of multicellular organisms (5-8). Furthermore, the escalation in complexity in early bilaterian evolution has been correlated with a strong increase in the number of miRNAs (9-11). In insects, miRNAs have been shown to be involved in fine-tuning a number of biological processes, like cell proliferation, apoptosis and growth, oogenesis and development, as well as in response to biological stress (12).

In 2009 we first looked whether miRNAs might have a relevant role in the regulation of insect metamorphosis. Only a few published studies had considered the contribution of miRNAs to this process, but these focused on specific aspects, such as wing formation and neuromuscular development (13-16). They also used the fly *Drosophila melanogaster* as model, a species that follows the holometabolan mode of metamorphosis, in which the juvenile stages are extremely divergent from the adult body plan and thus requires a dramatic transformation for adult morphogenesis (17-20). To answer the above question we used the cockroach *Blattella germanica* as model, as it displays the more primitive hemimetabolan mode of metamorphosis, where the juvenile stages already have the adult body plan, thus making the transition from nymph to adult much less dramatic than in holometabolan species. In both, hemimetabolan and holometabolan modes, the basic regulation of metamorphosis is ensured by two hormones, the molting hormone that triggers non-metamorphic and metamorphic molts, and the juvenile hormone that represses the metamorphic character of the molts (21). Our approach involved silencing the expression of dicer-1, the ribonuclease that produces mature miRNAs from miRNA precursors (22), following a method that has been used successfully in zebrafish (23). The approach was also effective for *B. germanica*, as depletion of dicer-1 mRNA levels resulted in depleted levels of mature

miRNAs, and, importantly, inhibited metamorphosis, as last instar nymphs molted to supernumerary nymphs or to nymph-adult intermediates instead of normal adults (24).

The results obtained for *B. germanica* indicated that miRNAs played a crucial role in regulating metamorphosis, at least in this hemimetabolan model. Our next goal was to unveil which miRNAs and targets were involved, and we started by obtaining a complete catalogue of the miRNAs present in the transition from nymph to adult (25) and then identifying which miRNAs were differentially expressed in this transition; these were let-7, miR-125 and miR-100 (26). A functional study of these miRNAs, however, revealed that they play minor roles in metamorphosis, and are only related to wing vein patterning, as depleting them resulted in disorganized vein/intervein patterns and anomalous bifurcations of the A-veins (27). Given that none of these miRNAs explained the total inhibition of metamorphosis observed after depleting dicer-1 (24), we shifted to a miRNA-targeted approach, choosing Krüppel-homolog 1 (Kr-h1) as a candidate.

Kr-h1 is a JH-dependent transcription factor that represses metamorphosis in both hemimetabolan (28, 29) and holometabolan insects (30, 31), and is a crucial element of the JH signaling pathway comprising Methoprene tolerant (Met), Kr-h1 and E93, the MEKRE93 pathway (32), which switches adult morphogenesis on and off in all insects. As in other species, Kr-h1 mRNA is expressed in juvenile instars of *B. germanica* but its expression decreases in the last nymphal instar, when JH titer in the haemolymph also decreases, which is followed by adult morphogenesis (29). RNAi experiments have shown that depleting Kr-h1 in the pre-last nymphal instar leads to precocious metamorphosis (29), thus demonstrating the repressor role of this transcription factor in adult morphogenesis. An interesting feature of Kr-h1 in *B. germanica* is that the decrease of transcript levels in freshly-emerged last nymphal instars is very abrupt (29), a fact which suggested to us the hypothesis that miRNAs could contribute towards modulating it. The present work is addressed to test this hypothesis.

Results and Discussion

Depletion of dicer-1 increases Kr-h1 expression and depletion of Kr-h1 in dicer-1 knockdowns rescues metamorphosis. To gain a first insight into whether Kr-h1 might be a promising candidate, we tested whether depletion of dicer-1, impairing mature miRNA formation, would affect Kr-h1 mRNA levels. We proceed as in our previous work (24) and treated penultimate instar female nymphs (N5) of *B. germanica* with double-stranded RNA targeting dicer-1 (dsDcr1). As expected, dicer-1 mRNA levels became depleted and mature miRNAs were also depleted (Fig. S1). The insects (n = 10) molted to the next instar nymph (N6) and subsequently to either a perfect supernumerary nymph (N7) (80%) or to nymphoids with membrane-like lateral expansions in the metathorax (20%) (Fig. S1). Two out of the eight N7 insects molted to second supernumerary nymphs (N8) and then to adults, which were bigger than normal and had a blackish abdomen similar in color to that of a nymph (Fig. S2). Control (dsMock-treated) specimens showed normal levels of dicer-1 mRNA and let-7, and all of them (n = 10) molted to N6 and then normal adults (Fig. S1). Interestingly, mRNA levels of Kr-h1 measured at the beginning of N6 were very low in dsMock-treated animals, as expected, although they were higher in dsDcr1-treated specimens (Fig. S1). Double dsDcr1 treatment experiments, one on N5D0 and the other on N5D3, were more efficient in reducing dicer-1 mRNA levels, and Kr-h1 expression increased more dramatically than in the single dsDcr1 treatment experiments; in all cases (n=15) these treatments resulted in perfect supernumerary N7 nymphs that died after molting (Fig. S3).

We then wondered whether Kr-h1 was the main, if not the only factor affected by miRNA depletion in the context of metamorphosis. Thus, we designed a phenotype rescue experiment by depleting Kr-h1 mRNA levels in dicer-1 knockdowns. Freshly emerged N5 were treated with dsDcr1 and, just after molting to N6, the same specimens were treated with dsKrh1. Controls were equivalently treated with dsMock. All specimens treated with dsDcr1 and dsKrh1 (n=18) molted to N6 and then to adults, just as the dsMock-treated controls did (n=11) (Fig. 1). The only difference was observed in the membranous hindwings of the specimens treated with dsDcr1+dsKrh1, which were somewhat smaller, wrinkled and presented defects in the vein/intervein pattern (Fig. 1), reminiscent of the phenotype obtained after depleting let-7, miR-100 and miR-125 (27). As expected, mRNA levels of Kr-h1 were depleted in the specimens treated with

dsDcr1+dsKrh1 (Fig. 1). We also measured the expression of the other component of the MEKRE93 pathway, Met and E93, as well as Broad Complex (BR-C), a family of JH-dependent transcription factors that in *B. germanica* are involved in wing formation (33). Results showed that transcript levels of Met, which is upstream of Kr-h1, were not affected, those of BR-C were higher than controls in dsDcr1-treated specimens, and returned to normal levels in dsDcr1+dsKrh1-treated specimens. Conversely, E93 transcript levels were reduced in dsDcr1-treated specimens, and tended to recover normal levels in the dsDcr1+dsKrh1-treated group (Fig. S4). These results are consistent with the epistatic relationships of these factors in the JH signaling pathway and in the MEKRE93 axis (32). Interestingly, the intermediate levels of E93 expression in dsDcr1+dsKrh1-treated specimens (Fig. S4), associated to low levels of Kr-h1 (Fig. 1) and of BR-C (Fig. S4) expression, are sufficient to result in a 100% adult morphogenesis.

Kr-h1 mRNA contains a functional binding site for miR-2. The above results suggest that inhibition of metamorphosis caused by the absence of mature miRNAs is mainly due to impairing the Kr-h1 mRNA decrease that occurs at the beginning of N6 in *B. germanica*, which would appear to be regulated by miRNAs. To test this hypothesis we started by predicting miRNA binding sites in the 3'UTR of Kr-h1 mRNA using RNAhybrid, miRanda and PITA algorithms. As queries, we used the miRNAs identified by high throughput sequencing in *B. germanica* N6 (25). All three algorithms coincided in predicting a top-scored site for miR-2 located at the beginning (nucleotide 67) of the 3'UTR of the Kr-h1 mRNA (Fig. S5A, Table S1). In *B. germanica* N6, miR-2 coexists with miR-13a and miR-13b, two structurally close miRNAs, which are less abundant and only differ from each other in 1 to 3 nucleotides outside the seed region (Fig. S5B) (25). miR-13a and miR-13b are predicted to bind at the same miR-2 site on the Kr-h1 mRNA (Table S1). miR-13a and miR-13b have been grouped into the so-called miR-2 family (34), and they appear functionally redundant as they have an identical seed region and are only differentiated by a few nucleotides. Interestingly, a top-scored site for miR-2 is also predicted in the 3'UTR of Kr-h1 mRNA of other hemimetabolan insects, like the true bug *Pyrrhocoris apterus*, the kissing bug *Rhodnius prolixus* and the aphid *Acyrthosiphon pisum* (Table S1), spanning an evolutionary distance of more then 300 myr (35). Conversely, these same algorithms did not predict a miR-2 site in the

3'UTR of Kr-h1 mRNA in holometabolan species, like the fly *Drosophila melanogaster*, the beetle *Tribolium castaneum* or the silkmoth *Bombyx mori*.

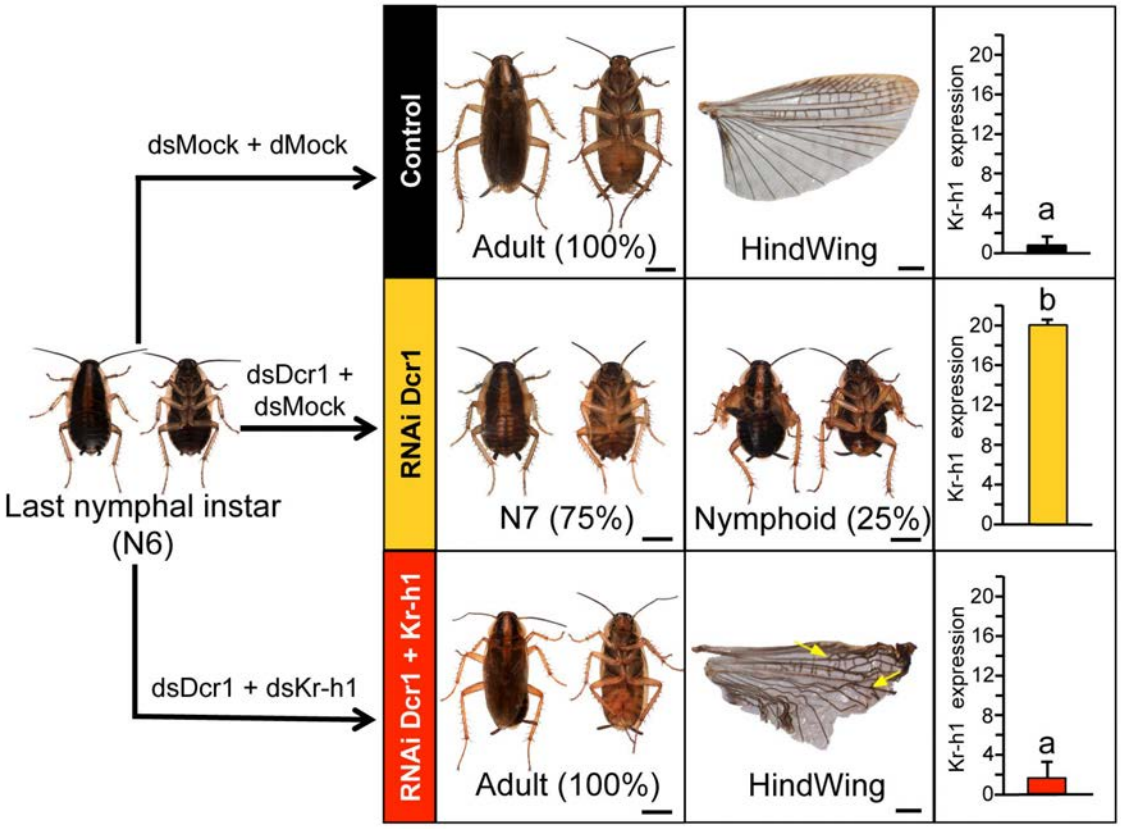

Fig. 1. Effects of dicer-1 mRNA depletion on Kr-h1 expression and of rescue experiments with RNAi of dicer-1 plus Kr-h1 in *Blattella germanica*. Females freshly ecdysed to the 5th nymphal instar (N5D0) received an injection (3 µg) of dsMock or dsDcr-1. The same specimens received a second injection (3 µg) of either dsMock (control) or dsKr-h1 on N6D0. Experimental specimens molted to normal N6 in all cases. Subsequently, dsMock+dsMock treated specimens (n=10) molted from N6 to normal adults, with perfect hindwings; Kr-h1 mRNA levels in N6D4 were low. Specimens treated with dsDcr+dsMock (n=14) molted from N6 to perfect supernumerary nymphs (N7) (75%) or nymphoids with membrane-like lateral expansions in the metathorax (25%); Kr-h1 mRNA levels in N6D4 were high. Insects treated with dsDcr+dsKrh1 (n=18) molted from N6 to normal adults, with imperfect hindwings (smaller, wrinkled and with defects in the vein/intervein pattern); Kr-h1 mRNA levels in N6D4 were low. Data on Kr-h1 expression represent the mean ± SEM (n=4), Kr-h1 mRNA levels in N6D4 were high. Insects treated with dsDcr+dsKrh1 (n=18) molted from N6 to normal adults, with imperfect hindwings (smaller, wrinkled and with defects in the vein/intervein pattern); Kr-h1 mRNA levels in N6D4 were low. Data on Kr-h1 expression represent the mean ± SEM (n=4), and are indicated as copies of Kr-h1 mRNA per 1000 copies of Actin-5c; the different letters at the top of the columns indicate statistically significant differences when treatments are compared ($p \leq 0.05$), according to the REST software tool. Scale bars in hindwing = 1 mm, in whole body= 3 mm.

The expression pattern of miR-2, miR-13a and miR-13b in females of the last two nymphal instars, N5 and N6, and the beginning of the adult stage is similar, fluctuating around 1 copy per copy of U6, although miR-2 is generally the most abundant (Fig. 2A). Intriguingly, these miRNA expression patterns do not correlate with those of Kr-h1 (Fig. 2A). A similar fluctuating pattern of miR-2 expression has been observed in the bug *Nilaparvata lugens* (36).

In order to functionally validate the predicted miR-2 binding site, we linked the entire Kr-h1 3'UTR containing the miR-2 site to a luciferase reporter gene, and expressed this wild type (WT) construct in Drosophila S2 cells. As a negative control, we used the equivalent construct, but linked a Kr-h1 3'UTR version where the miR-2 site had been removed (Mut construct). The first measurements revealed that luciferase activity was significantly higher in the Mut construct than in the WT (Fig. 2B), due to the high levels of endogenous mir-2 reported for S2 cells (3, 37). We then determined that miR-2 levels in our S2 cells could be lowered using a miR-2 LNA (locked nucleic acid) inhibitor (miR-2 LNA-i) (Fig. S5C). When S2 cells expressing the WT construct were incubated with miR-2 LNA-i, the translation of the construct was de-repressed as indicated by the significant increase in the luciferase signal, as expected. Conversely, the same treatment with miR-2 LNA-i did not modify the luciferase signal in S2 cells expressing the Mut construct (Fig. 2C). This suggests that the miR-2 site predicted in the 3'UTR of Kr-h1 mRNA is functional.

We then assessed the functionality of this site by treating *B. germanica in vivo* with miR-2 LNA-i on N6D0 and N6D1, and measuring miRNA levels on N6D2. The results showed that miR-2 levels were significantly depleted (as were those of miR-13a and miR-13b), whereas those of let-7, used as a negative control, were not (Fig. 2D). Kr-h1 mRNA levels were significantly higher than in the controls (Fig. 2E), suggesting that miR-2 miRNAs control Kr-h1 transcript levels. Interestingly, one of the ten specimens treated with miR-2 LNA-i that was still alive attempted to molt, completed the apolysis but arrested and died without ecdysing. It presented superimposed double cuticular structures, reminiscent of the molting-defective phenotype obtained after depleting the ecdysone receptor (EcR) (38). Observed by transparency, the new exosqueleton had the general morphology of a supernumerary nymph (N7), and mechanical removal of the exuvia of the mesonotum and metanotum revealed that there were neither tegmina nor wings developed (Fig. S6).

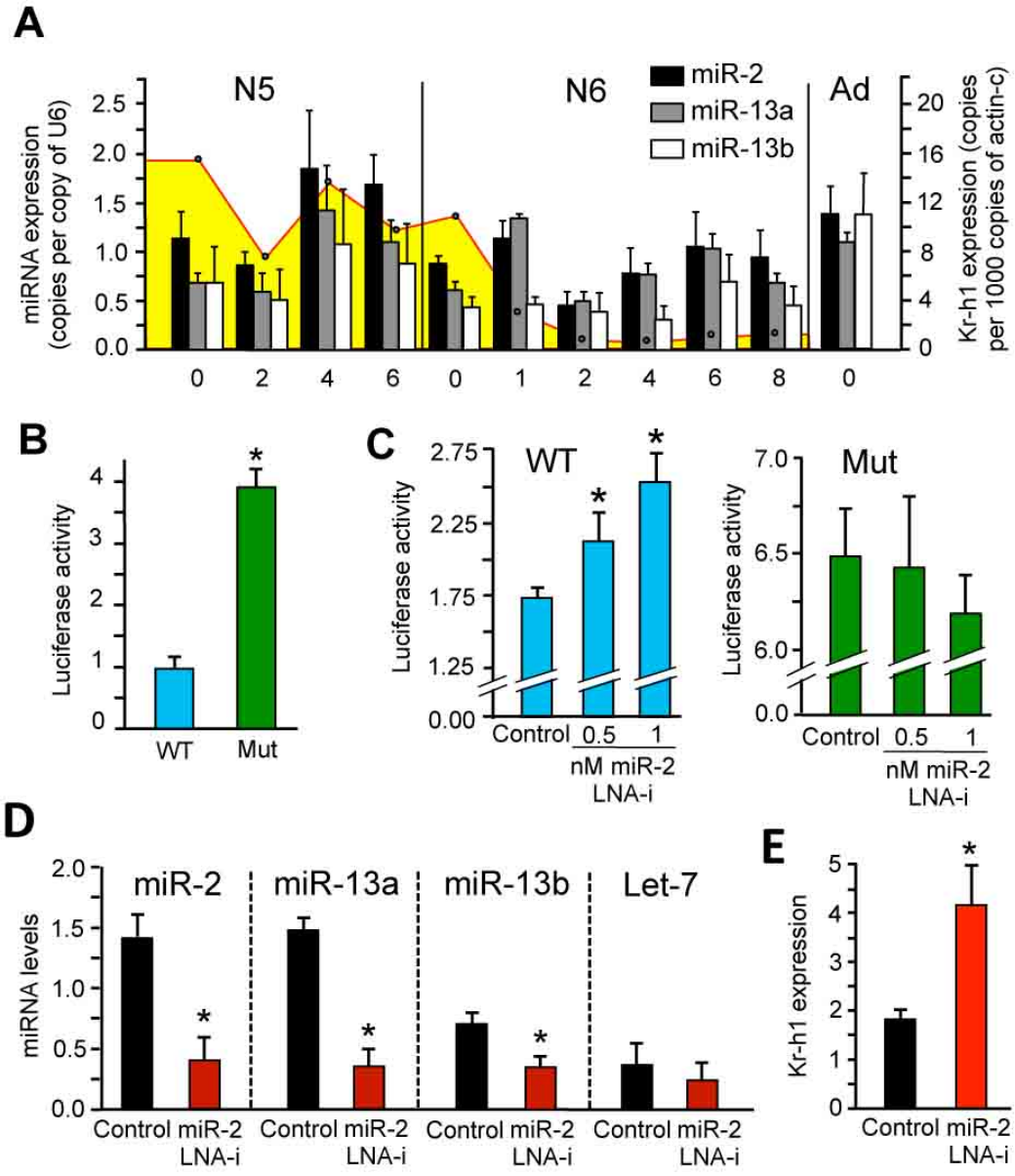

Fig. 2. Expression of miR-2 miRNAs and functional validation of the miR-2 site in Kr-h1 mRNA of *Blattella germanica*. (*A*) Expression pattern of miR-2, miR-13a and miR-13b during N5 and N6, as well as in freshly emerged female adults (Ad); expression of Kr-h1 mRNA levels according to Lozano and Belles (2011) is also shown in yellow. (*B*) Luciferase activity measured in Drosophila S2 cells transfected with the construct containing the 3'UTR of Kr-h1 mRNA, and hence containing the miR-2 binding site (WT), and transfected with an equivalent construct lacking the miR-2 binding site (Mut). (*C*) Luciferase activity measured in Drosophila S2 cells transfected with either the WT or Mut construct, incubated for 48 h with miR-2 LNA inhibitor (miR-2 LNA-i) or Scramble LNA (control) at a concentration of 0.5 µM. (*D-E*) Effect *in vivo* of miR-2 LNA-i treatment on miR-2, miR-13a, miR-13b and let-7 levels (*D*) and Kr-h1 expression (*E*); *B. germanica* female nymphs were treated with 25 nM of miR-2 LNA-i on N6D0 and N6D1, and miRNAs or Kr-h1 mRNA levels were quantified on N6D2. In all cases, the data represent the mean ± SEM (n = 3-5); miRNA levels are indicated as copies per copy of U6 and Kr-h1 expression is indicated as copies of mRNA per 1000 copies of Actin-5c; the asterisks at the top of the columns indicate statistically significant differences in comparison with the respective control ($p \leq 0.05$) according to the REST software tool, or *t*-test in *C*.

Treatment with a miR-2 inhibitor impairs metamorphosis and treatment of dicer-1-depleted specimens with a miR-2 mimic restores it. The supernumerary nymph obtained with the miR-2 LNA-i treatment encouraged us to carry out new experiments *in vivo* following the same approach in order to study the phenotype. To achieve greater efficiency in the miR-2 depletion, we used a more potent antagonist, namely mir-2 LNA Power Inhibitor (miR-2 LNA-pi), administered in two doses, on N6D0 and N6D1. Measuring miRNA levels on N6D2 showed that the treatment efficiently depleted miR-2 (as well as miR-13a and miR-13b), whereas let-7 was unaffected (Fig. 3A). The same samples showed that Kr-h1 mRNA levels were higher in specimens treated with miR-2 LNA-pi than in the controls (Fig. 3B), thus indicating that Kr-h1 transcripts had not been properly down-regulated in the absence of miR-2 miRNAs.

At phenotypic level, 6 out of 34 miR-2 LNA-pi-treated specimens died shortly after the second treatment. Of the 28 survivors, 6 (21%) molted to supernumerary nymphs (N7) and 22 (79%) molted to normal adults (Fig. 3C). These results further suggest that miR-2 miRNAs remove Kr-h1 transcripts at the beginning of the last nymphal instar (N6) of *B. germanica*, thus contributing towards triggering correct metamorphosis. The relatively modest 21% of specimens that did not metamorphose when treated with miR-2 LNA-pi is possibly due to the fact that only this percentage of specimens maintained high enough Kr-h1 levels to prevent metamorphosis.

Another way to demonstrate that the absence of miR-2 miRNAs in dicer-1 depleted specimens is the main explanation for metamorphosis inhibition (24), is through a rescue experiment using a miR-2 mimic. Thus, freshly emerged N5 nymphs were treated with dsDcr1 and some of these specimens were then treated with the miR-2 mimic or received an equivalent treatment with miRNA Scramble (controls). Transcript measurements showed that Kr-h1 mRNA levels in dsDcr1-treated specimens were higher than in the controls, as expected, whereas in those treated with both dsDcr1 and miR-2 mimic the levels were similar to those in the controls (Fig. 3D). At the phenotypic level, the specimens treated with both dsMock and miRNA Scramble (n = 10) molted to N6 and normal adults, and those treated with dsDcr1 and miRNA Scramble (n = 10) molted to N6 and then to N7. In the group of specimens treated with both dsDcr1 and miR-2 mimic (n=33), 6 (18%) molted to N6 and then to N7, whereas the remaining 21 specimens molted to N6 and then to adults (Fig. 3E). These adults presented a normal morphology except for the occurrence of small defects in the

vein/intervein pattern in the membranous hindwings (Fig. S7). These defects are reminiscent of those observed in the dsDcr1+dsKrh1 treatments described above (Fig. 1) and those reported by Rubio and Belles (27) in specimens that were depleted for let-7, miR-100 and miR-125 in N6.

As expected, miR-2 levels increased dramatically in the specimens treated with dsDcr1 plus miR-2 mimic, whereas those of let-7, used as negative control, were unaffected (Fig. S8). Transcript levels of Met were not modified by the treatments, those of BR-C were higher than controls in dsDcr1-treated specimens, and returned to normal levels in dsDcr1+ miR-2 mimic-treated specimens. Conversely, E93 transcript levels were reduced in dsDcr1-treated specimens, and tended to recover normal levels in dsDcr1+ miR-2 mimic-treated specimens (Fig. S8). Interestingly, these results parallel those obtained with the treatments of dsDcr1+ dsKr-h1 (Fig. S4) that rescued 100% normal metamorphosis. In this case, however, the rescue was not complete (82% of specimens molted to the adult stage), which is possibly associated to the still low average levels of recovery of E93 expression after miR-2 mimic treatment.

A miRNA that leads metamorphosis to a correct conclusion. In 2009 we demonstrated that in *B. germanica* depletion of dicer-1 and consequent reduction of miRNA levels prevented metamorphosis (24). The results presented herein show that depletion of dicer-1 results in increased levels of Kr-h1 mRNA, and that RNAi of Kr-h1 in dicer-1 knockdowns rescues metamorphosis. Both results suggest that the inhibition of metamorphosis caused by miRNAs depletion (24) is due to a deregulation of Kr-h1 expression. Moreover, the 3'UTR of Kr-h1 mRNA contains a functional binding site for miR-2 miRNAs, suggesting that the deregulation of Kr-h1 might be due to a deficiency of miR-2 (and the redundant miR-13a and miR-13b) in dicer-1-depleted specimens. This was corroborated by treating N6 of *B. germanica* with a miR-2 inhibitor, impairing metamorphosis, and by treating dicer-1-depleted specimens with a miR-2 mimic, which restored metamorphosis. Taken together, the data indicate that miR-2 modulates Kr-h1 mRNA levels and that this is crucial for metamorphosis.

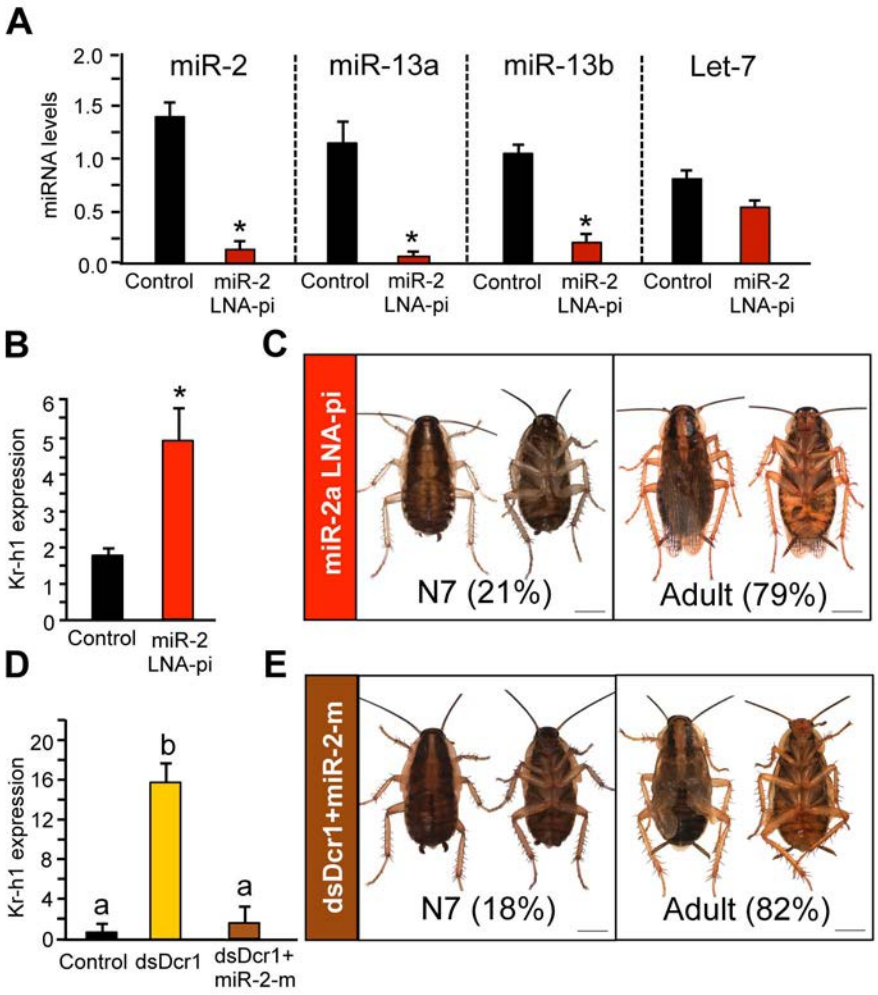

Fig. 3. Treatment of *Blattella germanica* N6 with a miR-2 inhibitor impairs metamorphosis and treatment of dicer-1-depleted specimens with a miR-2 mimic restores it. (*A*) Effect of miR-2 LNA Power Inhibitor (miR-2 LNA-pi) on miRNA levels; specimens were treated with two doses of 25 nM of miR-2 LNA-pi or of miRNA Inhibitor Negative Control (control), one on N6D0 and the other on N6D1, and miRNAs were measured in N6D2. (*B*) Kr-h1 mRNA levels measured in the same samples. (*C*) Representative N7 and adult specimens obtained from miR-2 LNA-pi treatments. (*D*) Effects of dsDcr1 and miR-2 mimic (miR-2-m) treatments on Kr-h1 transcript levels; dsDcr1 and dsMock treatments to deplete dicer-1 mRNA were carried out on N5D0 as described in figure 1; the group further treated with miR-2-m, received three doses of 25 nM each, on N5D4, N6D0 and N6D1, respectively; the controls of these experiments received an equivalent treatment with miRNA mimic Scramble (controls); Kr-h1 mRNA levels were measured on N6D4. (*E*) Representative N7 and adult specimens obtained from these dsDcr1 plus miR-2 mimic treatments. Data in *A*, *B* and *D* represent dsDcr1+miR-2 mimic treatments. Data in *A*, *B* and *D* represent the mean $\pm$ SEM (n = 4), the asterisk in *A* and *B* and the different letters in *D* indicate significant differences with respect to the corresponding control or reference treatment ($p \leq 0.05$), according to the REST software tool. Scale bars = 3 mm.

Recent studies have shown that the transcription factor Met is the JH receptor, and that downstream of Met, the JH signal is transduced by Kr-h1 (39). In pre-final stages, Kr-h1 exerts its antimetamorphic action by repressing E93 (32), a transcription factor that triggers adult morphogenesis (40). Thus the Met-Kr-h1-E93 axis, or the MEKRE93 pathway (32), switches adult morphogenesis on and off, as observed, among other models, in *B. germanica* (29, 32, 40, 41). Kr-h1 is expressed in juvenile instars, but its expression abruptly decreases at the beginning of N6 (29), coinciding with the gradual decrease of JH levels in the hemolymph (42). Treatment with JH at this pre-adult stage re-induces Kr-h1 expression and inhibits adult morphogenesis, indicating that the decrease in Kr-h1 expression at the beginning of N6 is crucial for metamorphosis and that it is primarily triggered by the decrease of JH levels (29). However, the decrease of Kr-h1 mRNA levels is very abrupt, which suggests that there are additional mechanisms regulating it, one of these being the repression exerted by E93 on Kr-h1 expression (32, 40). This led us to wonder whether this repressive action is mediated by miR-2, and to test whether E93 stimulates the expression of miR-2 using E93-depleted specimens as in previous experiments (32). MiR-2 levels in these specimens were very similar to those of respective controls (Fig. S9), which suggests that the antagonistic actions of E93 and miR-2 over Kr-h1 mRNAs are independent. Therefore, miR-2 appears to play a role of Kr-h1 transcript scavenger in a precise developmental stage that is of paramount importance for correct metamorphosis. This would suggest that the respective expression patterns should inversely correlate. However, this is not the case (Fig. 2A), possibly because miR-2 regulate multiple targets, as recorded in other species (43), as well as the fact that Kr-h1 expression is constantly enhanced while JH is present, and it is only at the beginning of N6, when JH vanishes, that there is full, efficient depletion of the Kr-h1 transcripts.

The presence of a miR-2 binding site in the 3'UTR of Kr-h1 mRNA of hemipterans (Table S1), and the fact that the expression pattern of Kr-h1 in hemipterans and other paraneopterans also decreases at the beginning of the pre-adult stage (*P. apterus*: (28); Thrips species: (44), suggest that the clearing of Kr-h1 transcripts by miR-2, allowing correct metamorphosis, is a common mechanism in hemimetabolan species. We were not able to identify miR-2 sites in the Kr-h1 mRNA of holometabolan species, indicating that the above inhibitory mechanism of miR-2 upon Kr-h1has been lost in the evolutionary transition from hemimetaboly to holometaboly. Indeed, RNAi of dicer-1 in

T. castaneum only results in occasional wing expansion defects (45), suggesting that in holometabolan species miRNAs only play refining roles in particular morphogenetic processes, such as wing formation. A number of reports support this hypothesis; for example, during adult morphogenesis of *D. melanogaster*, let-7 is required for neuromusculature remodeling (16), whereas let-7 and miR-124 are needed for proper timing in wing cell cycle and for the maturation of neuromuscular junctions (14). Also in *D. melanogaster*, iab-4 attenuates Ultrabithorax expression and causes a transformation of halteres to wings (15), whereas miR-9a contributes to wing development by modulating the expression of the transcription factor dLMO, which represses Apterous, a factor required for the proper dorsal identity of the wings (13). Specific miRNA functions in wing development have also been described in hemimetabolan species like *B. germanica*, where depletion of let-7 and associated miRNAs affects vein patterning (27). Thus, we propose that the contribution of miRNAs to wing development is an ancestral function in insects, which perhaps originated with the first pterygotes, some 350 million years ago (46, 47).

Taken as a whole, the data presented herein indicate that miR-2 miRNAs rapidly clear Kr-h1 transcripts at the beginning of the last nymphal instar (N6) of *B. germanica*, a process that appears crucial for proper adult morphogenesis. IIt is a miRNA, therefore, that leads metamorphosis to its correct conclusion, reminiscent of the role of a single miRNA, the cell's Nepenthe, that removes maternal transcripts during the maternal-to-zygotic transition (48) to allow a new life to start. In this case the miRNA clears away a single transcript that is preventing transition to the adult stage. An stage that also, in some ways, represent a new life with respect to that of the nymph.

Materials and Methods

Insects. Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 ºC and 60–70% relative humidity. Freshly ecdysed female nymphs were selected and used at the appropriate ages. They were anaesthetized with carbon dioxide prior to injection treatments, dissections and tissue sampling.

RNA Extraction and retrotranscription to cDNA. We performed total RNA extraction from the whole body (excluding the head and the digestive tube to avoid ocular pigments and intestine parasites), using the miRNeasy extraction kit (QIAGEN). For mRNA quantification a 500-ng sample from each RNA extraction was treated with DNAse (Promega) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and random hexamers (Promega). RNA quantity and quality was estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies). For miRNA quantification an amount of 500 ng of total RNA was reverse transcribed with the NCodeTM miRNA first-strand synthesis and qRT- PCR kit (Invitrogen), following the manufacturer protocol. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced.

Quantification of miRNAs and mRNAs by quantitative real-time PCR. Quantitative real time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real- Time PCR Detection System (Bio-Rad Laboratories), using SYBR®Green (Power SYBR® Green PCR Master Mix; Applied Biosystems). A template-free control was included in all batches. Primer efficiency was first validated by constructing a standard curve through four serial dilutions and were assesed , using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). mRNA levels were calculated relative to BgActin-5c (Accession number AJ862721) expression Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA. Primers are described in Table S2. The U6 from *B. germanica* (accession number FR823379) was used as a reference gene for miRNA quantification and all procedures were made as previously reported (*18*). Results are given as copies of RNA per copies of U6. Primers are described in Table S2.

RNA interference. The detailed procedures for RNAi have been described previously (24). Primers to prepare the dsRNAs are described in Table S2. The sequences were amplified by PCR and cloned into the pSTBlue-1 vector. A 307 bp sequence from *Autographa californica* nucleopolyhedrosis virus (Accession number K01149, from nucleotide 370 to 676) was used as control dsRNA (dsMock). A volume of 1 µl of the respective dsRNA solution (3 µg/µl) was injected into the abdomen of specimens at chosen stages.

Prediction of miRNA binding sites. To predict binding sites in the 3'UTR of Kr-h1 with the highest and comparable reliability, we used the following three algorithms and parameter sets. RNAhybrid (http:// bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (49) , with a distribution probability of parameter ξ = 1.98 and θ = 1.16; miRanda (http://www.microrna.org) (50), with a score threshold of 134; and PITA (http://genie. weizmann.ac.il/index.html) (51), with the seed limitation between 5 and 8 and wit Firefly luciferase as a 5' upstream.

Luciferase assay to assess the effect of miR-2 on the mRNA of Kr-h1. Two reporter constructs were generated by cloning downstream of Firefly luciferase coding region, under the control of a Ac5 promoter, a wild type (WT) or a mutated (Mut) 3'-UTR of BgKr-h1. Both 3'-UTRs were generated by PCR amplification from a pSTBlue-1 vector (Novagen) with the BgKr-h1 transcript, using the primers described in Table S2, for the entire 3'UTR (WT) or for a truncated version of the 3'UTR with 87 nucleotides deleted in the 5`region, where the miR-2 site is placed (Mut). We modified the plasmid pAc5.1 and inserted a Firefly luciferase coding region in KpnI site and NotI and under the control of the Ac5 promoter. Then, we added a new multicloning site from pStBlue. BgKr-h1 3'-UTR was finally cloned downstream of the Firefly luciferase coding region of plasmid pAc5+Luciferase. All PCR fragments were cloned in the pAc5 vector and confirmed by sequencing. To perform the luciferase essay, S2 cells were transfected in 24-well plates with Insect GeneJuice (Novagen) following the manufacturer's instructions. Transfection was performed in triplicate with the following mixture per well: 500 ng of Firefly luciferase reporter plasmid, WT or Mut, 500 ng of the *Renilla* luciferase, as a transfection control plasmid. In the experiments with miR-2 antagonists, miR-2 LNA-i or Scramble LNA were added at a concentration of 0.5 and 1 nM. After 48 h of transfection, Dual-Glo luciferase assays (Promega) were performed following the manufacturer's instructions and measure in an Orion II microplate luminometer (Titertek-Berthold). Results from three independent replicates were averaged and normalized to the value of the Scramble LNA control. Statistical significance was assessed using the t test. Results for transiently expressed BgKr-h1 3'UTRs reporters, coinjected with antisense miR-2a into S2 cells shown moderate effect in strength but very reproducible (Figure 2C). Columns represent ratio of renilla/firefly luciferase

activity, averaged over 3–5 independent replicates and normalized to the value of the scramble control ± SEM; *p < 0.05, *t*-test.

Experiments with miRNA inhibitors and miRNA mimics . To deplete miR-2 in the experiments for validating miRNA binding site, miR-2 miRCURY LNA™ microRNA Inhibitor (miR-2 LNA-i) (Exiqon) was used. In the experiments to inhibit metamorphosis, miR-2a miRCURY LNA™ microRNA Power Inhibitor (miR-2 LNA-pi) (Exiqon), which has more powerful action and resistance against DNases, was used. miRCURY LNA™ microRNA Inhibitor Negative Control A (Exiqon) was used as control. In all experiments, specimens were treated with two doses of 25 nM each of inhibitor or negative control, one on N6D0 and the other on N6D1, and effects were determined on N6D2. To restore metamorphosis in dicer-1-depleted specimens, GMR-miR microRNA double-stranded mimics from Genepharma (52) (miR-2a mimic and miRNA Scramble as negative control) were used. Specimens that had been treated with dsDcr1 in N5D0, were treated with three doses of 25 nM each of the miR-2 mimic o the corresponding control in N5D4, N6D0 and N6D1, and results in terms of transcript measurements were studied on N6D4.

Statistical analysis. In all experiments, significance of the differences between treated and control samples was determined with the REST software tool (53).

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Supporting information

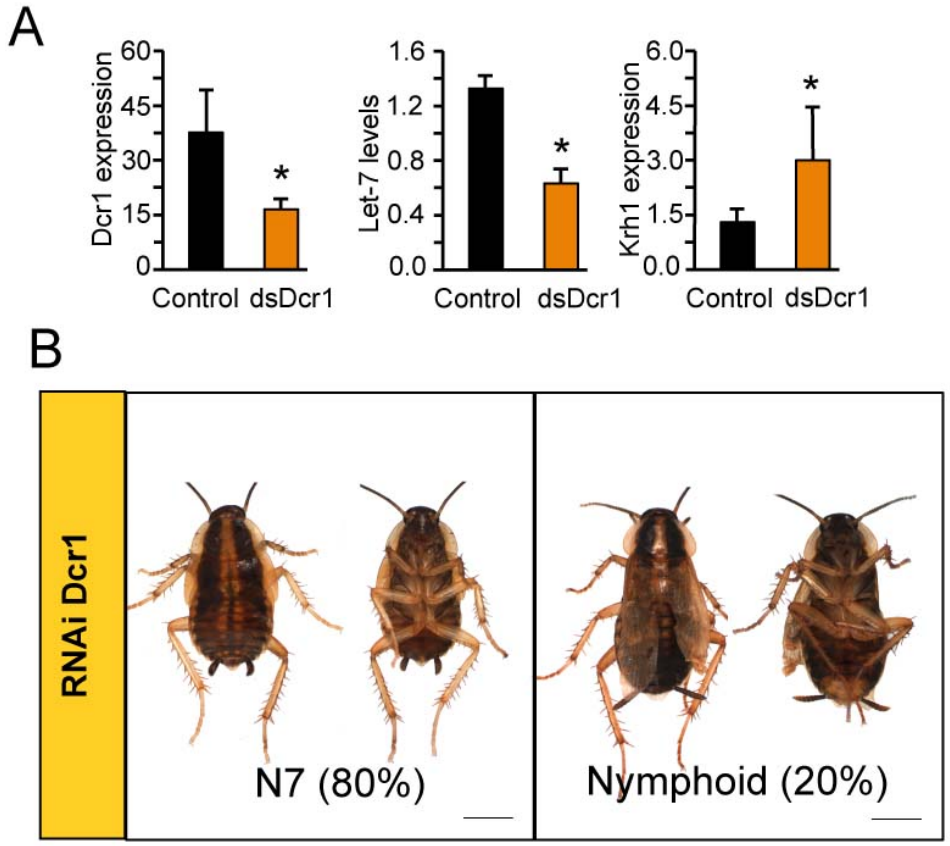

Fig. S1. Effects of dicer-1 depletion in *Blattella germanica*. Females freshly ecdysed to the 5th nymphal instar (N5D0) received an injection (3 µg) of dsMock or dsDcr-1. (*A*) The levels of dicer 1 mRNA and let-7 on N6D2 were significantly lower than in the controls, whereas Kr-h1 mRNA levels were higher; data represent the mean ± SEM (n = 4), dicer-1 and Kr-h1 expression is indicated as copies of the respective mRNA per 1000 copies of Actin-5c, let-7 levels are indicated as copies of let-7 per copy of U6; the asterisks at the top of the columns indicate statistically significant differences in comparison with the respective control ($p \leq 0.05$) according to the REST software tool. *(B)* Experimental specimens molted to normal N6 in all cases; subsequently, dsMock-treated specimens (n=10) molted from N6 to normal adults, whereas dsDcr-treated specimens (n=10) molted from N6 to perfect supernumerary nymphs (N7) (80%) or to nymphoids with membrane-like lateral expansions in the metathorax (20%).

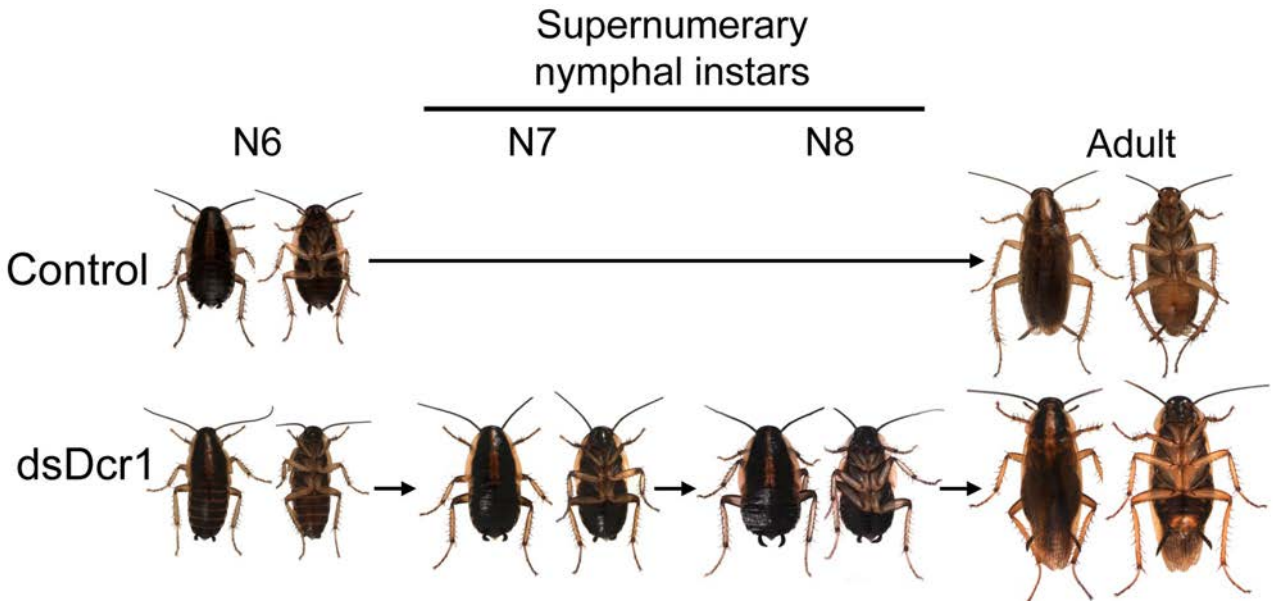

Fig. S2. Second supernumerary nymphal instar in dsDcr1-treated specimens of *Blattella germanica*. Most (80%) of the specimens (n = 10) treated with 3 µg of dsDcr-1 in the 5th nymphal instar (N5D0) molted to normal N6 and then to perfect supernumerary nymphs (N7). Two out of eight of the perfect N7 moulted to a second morphologically perfect supernumerary nymph (N8), and then to adults that were bigger than normal and presented a blackish abdomen, similar in colour to that of a nymph.

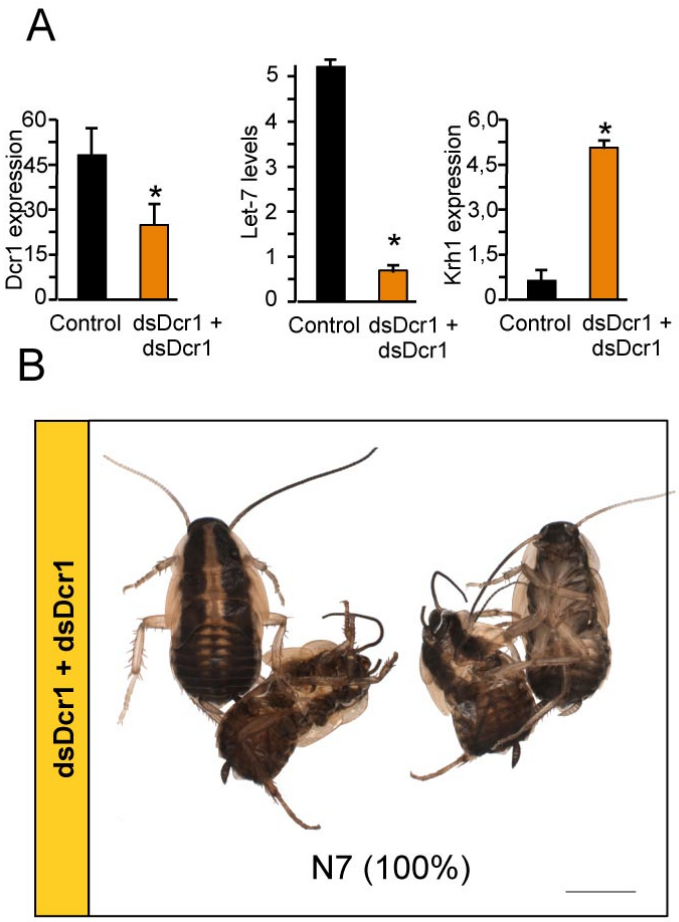

Fig. S3. Effects of double treatment with dsDcr1 in *Blattella germanica*. Females received two 3 µg injections of dsMock or dsDcr1, one when freshly ecdysed to the 5th nymphal instar (N5D0) and the other on N5D3. *(A)* Levels of dicer-1 mRNA and let-7 on N6D2 were significantly lower than in the controls, whereas Kr- h1 mRNA levels were dramatically higher; data represent the mean ± SEM (n = 4), dicer-1 and Kr-h1 expression is indicated as copies of the respective mRNA per 1000 copies of Actin-5c, let-7 levels are indicated as copies of let-7 per copy of U6; the asterisks at the top of the columns indicate statistically significant differences in comparison with the respective control ($p \leq 0.05$) according to the REST software tool. (*B*) In each case, all experimental specimens moulted to normal N6, and then to N7 (100%); however, they died during ecdysis, being unable to detach from the exuvia.

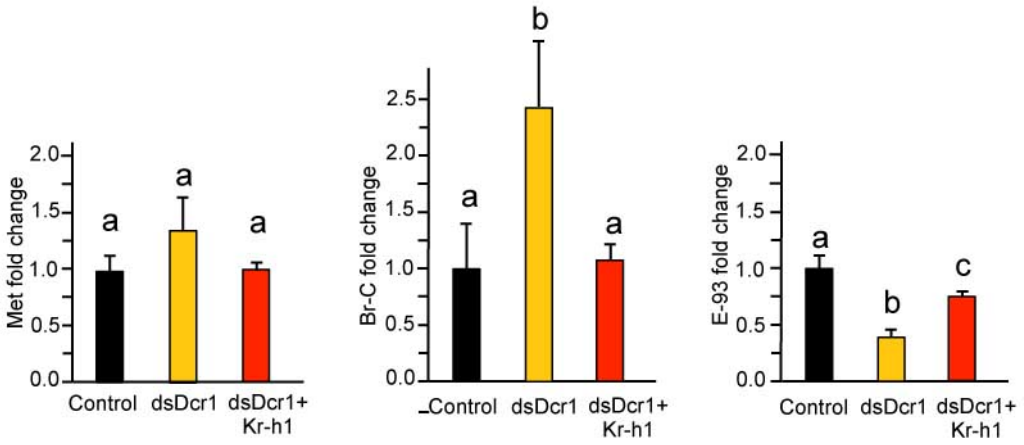

Fig. S4. Effects of dicer-1 mRNA depletion on Kr-h1 expression and of rescue experiments with double RNAi, of dicer-1 and Kr-h1 in *Blattella germanica* on the expression of transcription factors Methoprene tolerant (Met), Broad-complex (BR-C) and E93. Females freshly ecdysed to 5th nymphal instar (N5D0) received an injection (3 μg) of dsMock or dsDcr-1. The same specimens received a second injection (3 μg) either of dsMock (Control) or of dsKr-h1 on N6D0. Data represent the mean ± SEM (n = 4), and are normalized against the dsMock-treated samples (reference value=1); the different letters at the top of the columns indicate statistically significant differences comparing treatments ($p \leq 0.05$), according to the REST software tool.

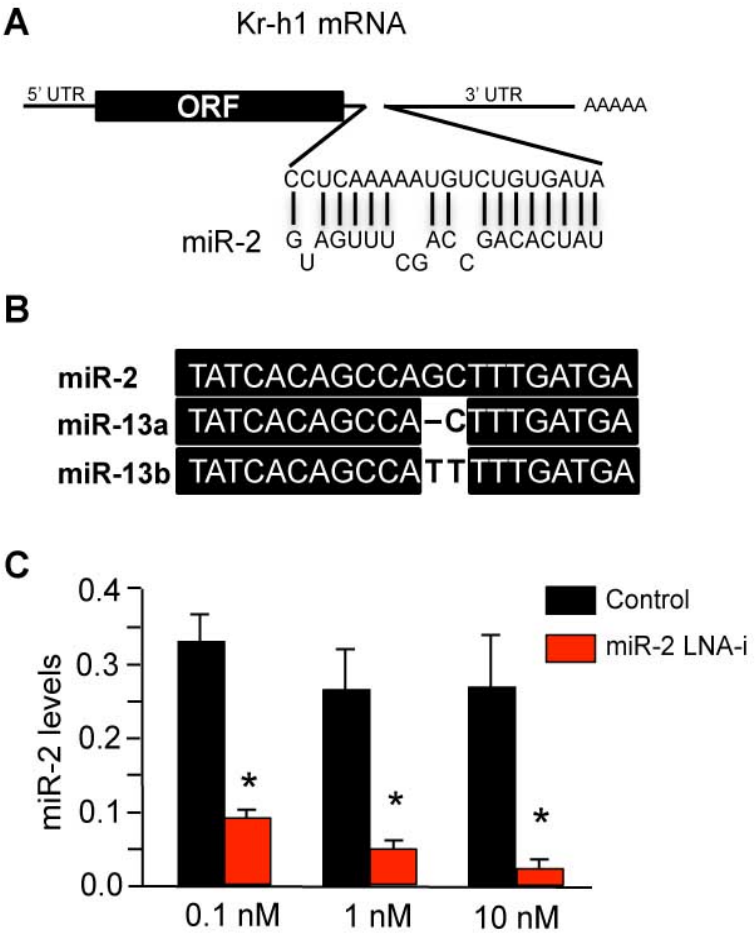

Fig. S5. miR-2a binding site and effects of miR-2 LNA inhibitor in vitro in *Blattella germanica*. (*A*) Schematic representation of Kr-h1 mRNA showing the predicted miR-2 binding site at nucleotide 67 on the 3'UTR. (*B*) Alignment of the three members of the miR-2 family found in the last nymphal instar (N6) of *B. germanica*. (*C*) Effect of a miR-2 LNA inhibitor (miR-2 LNA-i) on levels of miR-2, miR-13a and miR13b in Drosophila S2 cells incubated in vitro. Control cells were treated with scrambled LNA. miRNA measurements were carried out after 48 h of incubation; data represent the mean ± SEM (n = 3-5); miRNA levels are indicated as copies per copy of U6; the asterisk indicates statistically significant differences compared with the respective control ($p \leq 0.05$), according to the REST software tool.

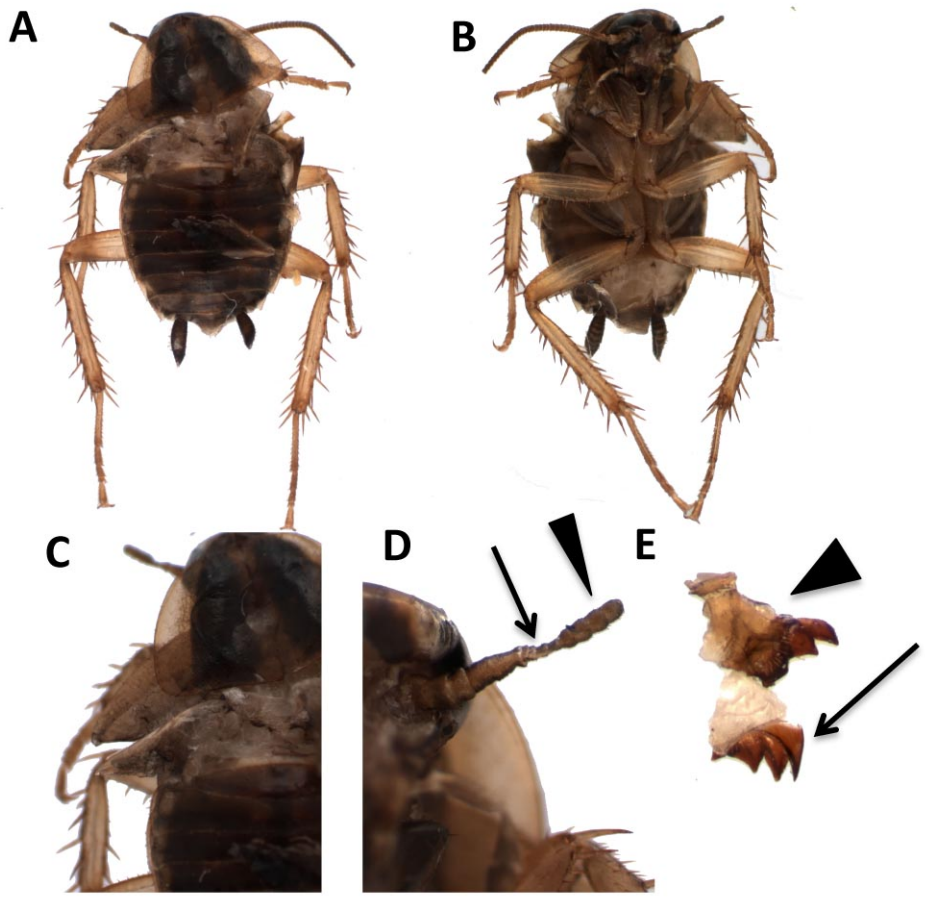

Fig. S6 .Supernumerary nymph (N7) obtained after miR- 2a LNA-i treatment in N6. *B. germanica* female nymphs were treated with 25 nM of miR-2 LNA-i on N6D0 and N6D1. Only one out of ten specimens was able to complete the apolysis, did not undertake the ecdysis, and showed morphological features of a N7. Dorsal (*A*) and ventral (*B*) view of this specimen. (*C*) Detail of the dorsal region of the thorax, with the exuvia partially removed, showing that the specimens did not form and extend tegmina or wings. (*D*) Detail of the antennae showing double, superimposed cuticle. (*E*) Mandibles presenting double cuticular structures corresponding to the old (arrow) and new (arrowhead) cuticle.

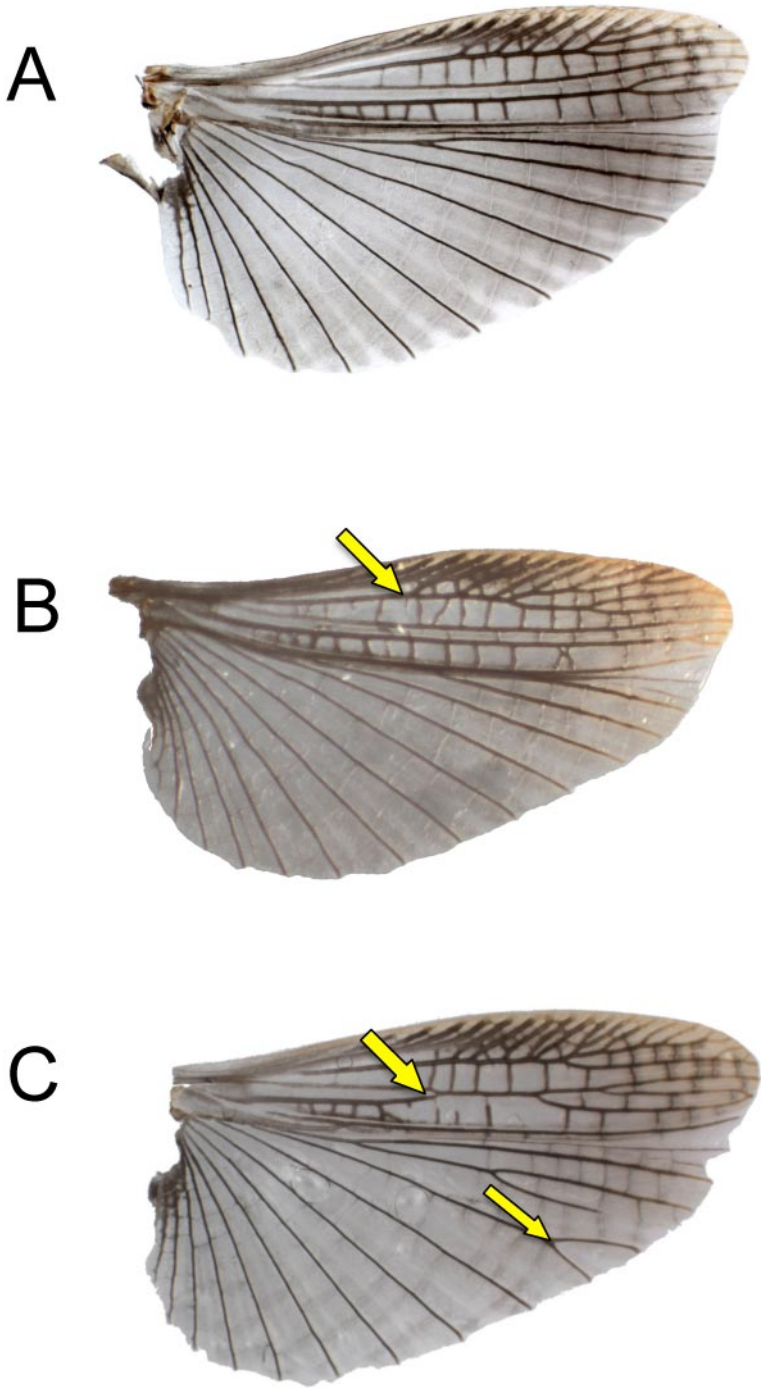

Fig. S7. Hindwings from adult female *Blattella germanica*. (*A*) From a control specimen treated with dsMock in N5D0 and scrambled miRNA on N5D4, N6D0 and N6D1. (*B*) From a specimen treated with dsDcr1 on N5D0 and with miR-2 mimic on N5D4, N6D0 and N6D1; note the disorganized vein/intervein pattern in the anterior section (arrow). (*C*) From a specimen treated with let-7 LNA inhibitor (let-7 LNA-i) on N6D0; data and image are from Rubio and Belles (2013); note that the wing show defects in the vein/intervein patterning in the anterior section, as well as anomalous vein bifurcation in the posterior section (arrows).

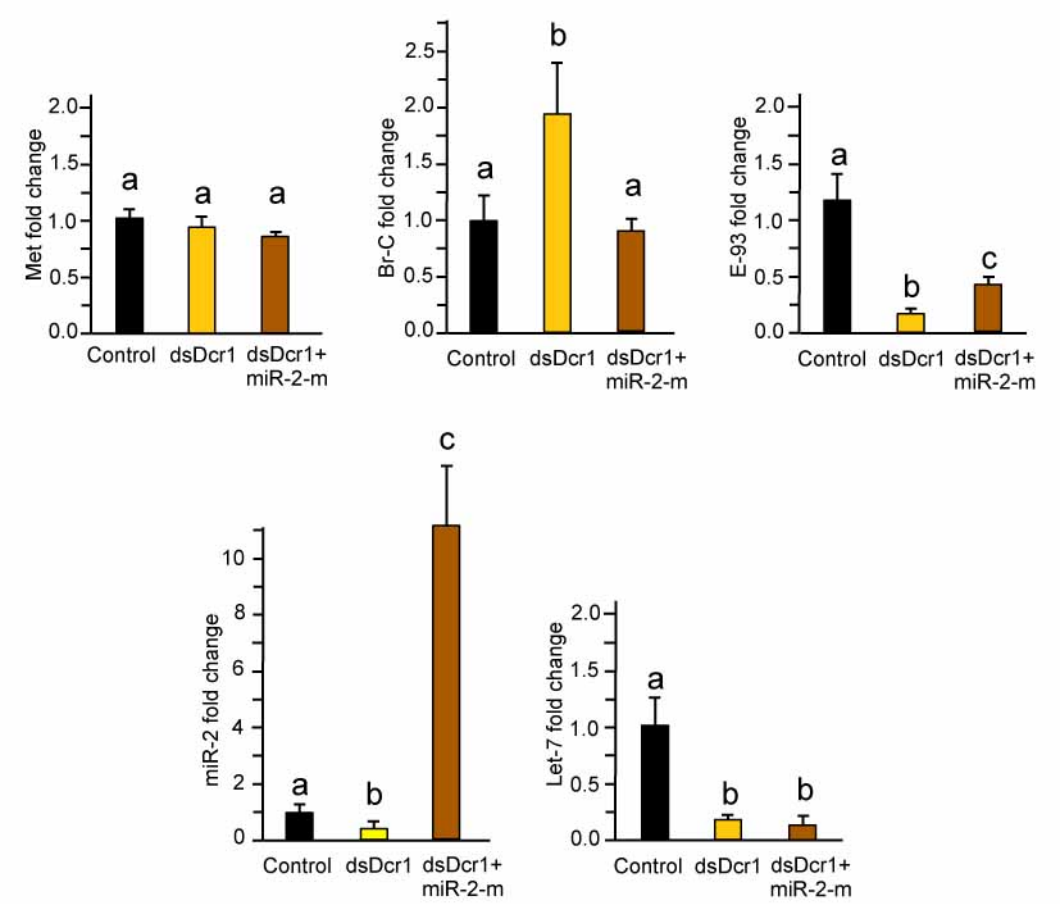

Fig S8. Effects of miR-2 mimic (miR-2-m) treatment on dicer-1 depleted specimens of *B. germanica*. Females freshly ecdysed to 5th nymphal instar (N5D0) received an injection (3 µg) of dsMock or dsDcr-1; the group further received three doses of 25 nM each of miR-2-m, on N5D4, N6D0 and N6D1; controls received an equivalent treatment with miRNA mimic Scramble (Controls); mRNA levels of Methoprene tolerant (Met), Broad-complex (BR-C) and E93, as well as levels of miR-2 and let-7, were measured on N6D4. Data represent the mean ± SEM (n = 4), and are normalized against the dsMock-treated samples (reference value=1); the different letters at the top of the columns indicate statistically significant differences comparing treatments ($p \leq 0.05$), according to the REST software tool.

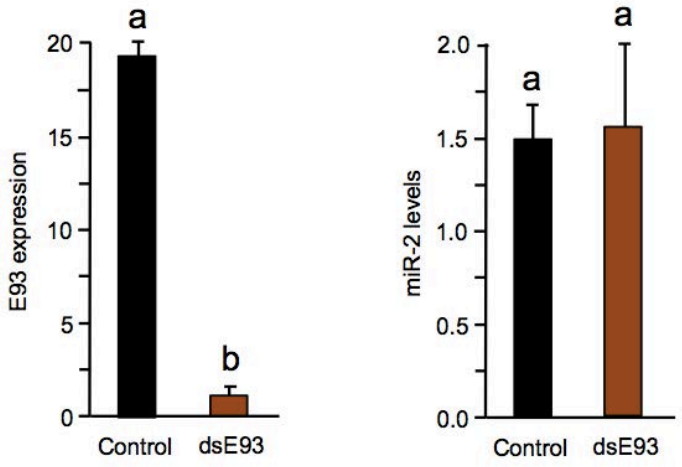

Fig S9. Effects of E93 depletion on miR-2 expression levels in *B. germanica*. RNA extracts were from E93-depleted specimens, and the respective controls (dsMock-treated), obtained by Belles and Santos (2014). Males freshly ecdysed to 5th nymphal instar (N5D0) received an injection (2 µg) of dsMock or dsE93, and were dissected on N6D4. Data represents the mean ± SEM (n = 4). E93 expression is indicated as copies of mRNA per 1000 copies of Actin-5c, and levels of miR-2 are indicated as copies per copy of U6; different letters at the top of the columns indicate statistically significant differences in comparison with the respective control ($p \leq 0.05$) according to the REST software tool.

Table S1. Prediction of miRNA binding sites for miR-2 and miR-2 variants (miR-13a and miR13b) in the 3'-UTR of *Blattella germanica* Kr-h1 mRNA. The sites were independently predicted by the following three algorithms: RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), used with a distribution probability of parameter ξ=1.98 and Θ=1.16; miRanda (http://www.microrna.org), with a score threshold of 100; and PITA (http://genie.weizmann.ac.il/index.html), with the seed limitation between 5 and 8. 3'UTR site indicates the first nucleotide were the miRNA seed region would binds, according the mRNA sequence available in GenBank. In order to have a comparable quality estimator value among methods, the different scores are obtained by the normalization to 1 with reference to the best prediction. "Rel. energy" indicates the averaged relative value of energy, "Rel. score" the relative value of scores, and "Rel. total" is the product Rel. energy x Rel. score.

Species	miRNA	3'UTR site	rel_en	rel_sec	rel_total
Blattella germanica	miR-2	67	0.8	0.8	0.6
Blattella germanica	miR-13a	67	0.7	0.7	0.5
Blattella germanica	miR-13b	67	1.0	0.9	0.9
Pyrrhocoris apterus	miR-2	73	0.7	0.8	0.6
Rhodnius prolixus	miR-2	71	0.8	0.8	0.6
Acyrtosiphon pisum	miR-2	300	0.8	0.8	0.8

Table S2. Forward (Fw) and reverse (Rv) primers used to measure mRNA and miRNA levels by qRT-PCR, to syntesize dsRNAs for RNAi, and to amplify the 3'UTR region of Kr-h1 mRNA, wild type (WT) or without the miR-2 site (Mut) for luciferase assays.

Primers qRT-PCR	5'-3'
Kr-h1 Forward	GCGAGTATTGCAGCAAATCA
Kr-h1 Reverse	GGGACGTTCTTTCGTATGGA
Met Forward	CTGTTGGGACATCAGCAGAA
Met Reverse	GGCAGGTGATGGAGTGAAGT
BR-C Forward	CGGGTCGAAGGGAAAGACA
BR-C Reverse	CTTGGCGCCGAATGCTGCGAT
E93 Forward	TCCAATGTTTGATCCTGCAA
E93 Reverse	TTTGGGATGCAAAGAAATCC
Dcr1 Forward	GCACTTGGGCACGAAAATCT
Dcr1 Reverse	ATCCAAAGGTGCCCATTCAA
Firefly luciferase Forward	CCGCCGTTGTTGTTTTG
Firefly luciferase Reverse	CTCCGCGCAACTTTTTC
Actin Forward	AGCTTCCTGATGGTCAGGTGA
Actin Reverse	TGTCGGCAATTCCAGGGTACATGG
miR-2	TCACAGCCAGCTTTGATGA
miR-13a	TATCACAGCCACTTTGATGA
miR-13b	TATCACAGCCATTTTTGACGA
Let-7	TGAGGTAGTAGGTTGTATAGT
U6	CGATACAGAGAAGATTAGCATGG

Primers dsRNA	5'-3'
dsDcr1 Forward	GGTGAGTCGGTTTGGTGAAT
dsDcr1 Reverse	CATGGCATGGCTAGGAATCT
dsKr-h1 Forward	GAATCTCAGTGTGCATAGGCG
dsKr-h1 Reverse	CCTTGCCACAAATGACACAA
dsMock (Polyhedrin) Forward	ATCCTTTCCTGGGACCCGGCA
dsMock (Polyhedrin) Reverse	ATGAAGGCTCGACGATCCTA

Primers luciferase	5'-3'
Kr-h1 3'UTR WT Forward	GAGGAAGATTGATCTCACAG
Kr-h1 3'UTR WT Reverse	TGAGTCATTTAATTTTTATT
Kr-h1 3'UTR Mut Forward	ATGTGGTATTCTGGCCTATA
Kr-h1 3'UTR Mut Reverse	TGAGTCATTTAATTTTTATT

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9. Discussió general

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Durant els diferents capítols d'aquesta tesi hem descrit com alguns factors de transcripció i reguladors d'aquests, com els microRNAs (miRNAs), regulen la transducció de l'hormona juvenil (HJ) en el context de la metamorfosi de la panerola *Blattella germanica*. Concretament, hem estudiat el receptor de l'HJ, format per Methoprene-tolerant (Met) i per Taiman (Tai), com es transdueix el senyal hormonal mitjançant els factors de transcripció BR-C i Krüppel homolog 1 (Kr-h1) i, finalment, quin és el paper de miR-2 sobre Kr-h1 i sobre el resultat de la metamorfosi, en general.

Receptor de l'hormona juvenil

En el primer capítol d'aquesta tesi hem descrit, a la panerola *Blattella germanica*, el paper del receptor de l'HJ, el factor de transcripció Methoprene-tolerant (Met). Els nostres estudis amb RNAi de Met en fases nimfals demostren que Met és necessari per la transducció del senyal hormonal, ja que la seva supressió provoca una metamorfosi precoç. L'RNAi de Met realitzat a l'avant-penúltima fase nimfal fa que l'insecte mudi primer a penúltima fase i després a adult precoç, mentre que en el mateix experiment realitzat en penúltima fase nimfal fa que l'insecte mudi directament a adult precoç (Lozano and Belles 2014). Aquests resultats suggereixen que l'insecte ha d'assolir una determinada mida crítica per tal que es pugui desencadenar la metamorfosi. La supressió de Met també va portar a altes taxes de mortalitat, suggerint un possible paper en altres funcions addicionals de caràcter vital. Tot i ser un gen induïble per l'HJ, l'expressió de Met és present a la darrera fase nimfal, quan no hi ha producció d'HJ ni aquesta es troba circulant a l'hemolimfa. Això suggereix altres funcions de Met independents d'aquesta hormona. L'eliminació de Met en la darrera fase ninfal va provocar retardament de la metamorfosi, defectes en el creixement de l'ala i problemes d'ècdisi. Aquests indicis van portar a estudiar l'afectació de factors dependents d'ecdisona, per la qual cosa vam quantificar l'mRNA de factors participants en aquesta via, com EcR o el factor de transcripció E-75. Els resultats obtinguts suggerien que Met està implicat en la senyalització de la 20E, resultats que ens remeten als problemes d'ècdisi trobats en lepidòpters interferits per Met abans de la fase pupal

publicats anteriorment (Guo *et al*. 2012). Els problemes de creixement trobats en els individus tractats també ens feu especular sobre una possible afectació de la via de la insulina. En els individus tractats amb RNAi de Met a l'inici de la darrera fase nimfal, vam mesurar els nivells d'mRNA d'una d'aquestes hormones peptídiques, l'ILP-1, i vam comprovar que efectivament s'expressava menys que en els individus control. Aquesta relació entre Met i la via de la insulina mereixeria nous estudis adreçats específicament a dilucidar els mecanismes subjacents. Met transdueix el senyal de l'HJ, i per tant, contribueix a reprimir la metamorfosi a la panerola *B. germanica*, un insecte hemimetàbol basal representatiu dels polineòpters. Els nostres resultats estenen, doncs, aquesta funció als insectes polineòpters, ja que abans tan sols era coneguda en holometàbols (Konopova and Jindra 2008, Parthasarathy *et al*. 2008a) i paraneòpters (Konopova *et al*. 2011),, tot cobrint el clade dels neòpters, establint la primera regularitat en la via de senyalització de l'HJ en els insectes metamòrfics (excepte en els paleòpters, en els que encara no s'ha estudiat Met). La proteïna de Met de *B. germanica* és molt similar a la d'insectes ametàbols, com *Thermobia domestica* (Konopova *et al*. 2011), això suggereix que Met de *B. germanica* pot ser un bon model per insectes amb metamorfosi propera al mode metamòrfic ancestral. Donat que *T. domestica* és una espècie ametàbola, la funció de Met en la mateixa no pot estar relacionada amb metamorfosi. Possiblement, la seva funció estaria relacionada amb la transducció de l'HJ per a l'expressió del gen de la vitel·logenina, com s'ha comprovat en l'heteròpter *Pyrrhocoris apterus* (Smykal *et al*. 2014). A falta d'estudis funcionals amb insectes ametàbols, els estudis recentment realitzats en crustacis, on s'ha trobat que Met hi és present i pot transduïr el senyal d'anàlegs de l'HJ, suggereix que la seva funció com a receptor hormonal es propia del clade Pancrustacea i precediria, per tant, l'emergència dels insectes, dels insectes alats i de la metamorfosi (Miyakawa *et al*. 2013).

Taiman (Tai) s'havia convertit en el millor candidat a actuar com a heterodímer de Met en la recepció de l'HJ i en la repressió de la metamorfosi, tot i que cap experiment *in vivo* havia pogut demostrar aquesta funció (Bitra *et al*. 2009, Smykal *et al*. 2014). Seguint una aproximació *in vivo*, el nostre treball mostra com a més de Met, Tai també està implicat en transduir el senyal de l'HJ en la repressió de la metamorfosi. Hem descobert que Tai de *B. germanica* posseeix quatre isoformes resultants de la combinació de dues insercions/delecions (indels) a la regió carboxi-terminal de la proteïna, i posteriorment hem examinat les funcions respectives de diferents grups

d'isoformes. La presència d'un homoleg de la primera inserció (in-1) a les seqüencies de Tai d'altres insectes, com *T. castaneum o D. melanogaster*, suggereix que la presència d'isoformes podria ser comuna a tots els insectes, i que aquesta inserció té alguna funció biològica. A *B. germanica*, Tai s'expressa al llarg de les dues darreres fases nimfals, amb patrons d'expressió similars als de Met. L'RNAi de Tai realitzat a quarta o cinquena fase nimfals de *B. germanica* va reduir l'expressió de totes les isoformes i provocà la mort de tots els individus tractats en la mateixa fase nimfal en la que s'havia realitzat el tractament, tal i com va succeir també en els tractaments a nimfes de *P. apterus* i larves de *T. castaneum* (Bitra *et al*. 2009, Smykal *et al*. 2014). No obstant, vam poder determinar una caiguda dels nivells d'expressió de gens dependents d'HJ com són Kr-h1 i BR-C, indicant possibles funcions de Tai relacionades amb la metamorfosi. Va ser quan vam eliminar específicament les isoformes que contenien la inserció-1 de Tai, que vam provocar una metamorfosi precoç en els insectes tractats, a l'igual que una caiguda de l'expressió dels gens de la via de senyalització de l'HJ: Met, Kr-h1 i BR-C (Lozano *et al*. 2014). També vam mesurar els efectes sobre la expressió de gens pertanyents a la via de l'ecdisona, i vam observar qu es reduia l'expressió d'EcR, RXR i E75A. També vam observar caiguda de l'expressió d'un Insulin-Like-Peptide (ILP) de *B. germanica*, l'ILP-1, en els individus tractats. La relació de Met amb factors pertanyents a la via de senyalització de l'ecdisona ja havia estat descrita a *D. melanogaster* i a *Aedes aegypti* (Bai *et al*. 2000, Zhu *et al*. 2006), però la relació de Met amb la via de la insulina obre uns camins nous d'estudi, i tot plegat reforça la idea de que en la regulació de la metamorfosi, no hi ha vies senyalitzadores independents, sinó més aviat una xarxa d'interacció entre vies. La reducció de l'expressió de les isoformes de Tai que contenen la segona de les insercions a *B. germanica* no va generar cap fenotip relacionat amb la metamorfosi. No descartem que aquest resultat podria ser conseqüència de la mida reduïda de la dsRNA utilitzada per suprimir aquesta inserció. Reduir l'expressió d'isoformes selectivament suggereix que només alguna d'elles, com a mínim les isoformes que contenent la inserció-1, presenten una funció relacionada amb la metamorfosi, i d'altres tenen funcions vitals. Val a dir, però, que la moderada reducció de transcrit obtinguda en alguns dels experiments d'RNAi impedeix sentar conclusions definitives sobre especificitat de funcions de les diferents isoformes de Tai de *B. germanica*. Amb les dades obtingudes en els experiments amb RNAi, sumat a l'informació disponible d'estudis *in vitro* que demostren la interacció entre Met i Tai, i la funcionalitat d'aquest heterodímer com a

receptor de l'HJ (Zhang *et al*. 2011, Kayukawa *et al*. 2012, Kayukawa *et al*. 2013, Charles *et al*. 2011, Miyakawa *et al*. 2013), proposem que una o més isoformes de Tai actuen com a heterodímers de Met en la recepció de l'HJ durant la repressió de la metamorfosi a *B. germanica.*

Aprofitant que Tai de *T. castaneum* també presenta una inserció-1 homòloga a la de *B. germanica* vam dur a terme assajos específics per avaluar la transducció d'HJ tot fent servir les eines disponibles en aquest insecte (Kayukawa *et al*. 2012, Kayukawa *et al*. 2013). Vam quantificar l'activitat transcripcional de cèl·lules a les quals se'ls introdueix un constructe amb Taiman fusionat al gen de luciferasa i sota el control d'un determinat promotor. Els assajos van indicar que tant la isoforma de Tai de *T. castaneum* que conté la inserció-1, en conjunció amb Met i HJ, com la isoforma que no present aquesta inserció presenten la capacitat d'activar l'element de resposta a HJ del promotor del gen Kr-h1 (kJHRE) (Lozano *et al*. 2014). Les diferencies de transducció entre ambdós isoformes són menors i els resultats suggereixen que les dues isoformes poden participar en la transducció del senyal antimetamòrfic de l'HJ a Kr-h1. Tanmateix, no hem de descartar que les diferents isoformes de Tai actuïn de manera diversa tot dimeritzant amb altres proteïnes (p. ex. altres proteïnes bHLH o receptors nuclears com βFtz-F1 (Li *et. al* 2011)), d'aquesta manera contribuint a modular l'expressió d'altres gens. De fet, els nostres resultats indiquen que Tai de *B. germanica* presenta alguna funció relacionada amb la senyalització d'ecdisona, funció prèviament caracteritzada a *D. melanogaster* (Bai *et. al.* 2000) i *A. aegipti* (Zhu *et al*. 2006). El conjunt de dades disponibles suggereix que les proteïnes Tai poden realitzar les funcions més variades mitjançant la unió a d'altres proteïnes, i postulem que les isoformes de Tai poden ser rellevants a l'hora de determinar amb quines altres proteïnes dimeritzen i quin paper juguen. Tai també presenta proteïnes homòlogues a vertebrats i que han estat estudiades a mamífers, on s'anomenen SRC, de les quals s'ha pogut comprovar que exerceixen les seves funcions a través d'isoformes (Hayashi *et al*. 1997, Meiher *et al*. 2005, Needham *et al*. 2000). La proteïna SRC1 de mamífer, per exemple, posseeix com a mínim 5 isoformes que difereixen en l'extrem carboxi-terminal i que afecten la interacció amb receptors nuclears en diferents vies de senyalització hormonal (Kamey *et al*. 1996). Sembla raonable postular que es produeixin fenòmens similars en les Tai d'insectes.

Factors de transcripció controlats per l'hormona juvenil

Broad complex

Un factor de transcripció que participa en la senyalització de l'HJ i que també és dependent d'ecdisona és Broad-Complex (BR-C) (DiBello *et al*. 1991). Els nostres estudis a *B. germanica* revelen funcions ancestrals de BR-C relacionades amb divisió cel·lular i creixement de l'ala, i amb la formació d'un patró de venes correcte, alhora que aporten noves pistes que ajuden a entendre l'evolució de la metamorfosi dels insectes. A *B. germanica,* BR-C presenta un total de 6 isoformes que divergeixen entre elles en els dits de zinc, a l'extrem carboxi-terminal de la proteïna. L'expressió de BR-C és més intensa als teixits metamòrfics, com els primordis alars, on els patrons d'expressió semblen determinats per l'ambient hormonal: els nivells màxims de BR-C coincideixen amb pics de producció de 20E en presència de nivells alts d'HJ. La coincidència de pics d'expressió de BR-C i aquells de 20E reflecteixen les relacions causa-efecte, atès que l'expressió de BR-C és dependent de la 20E (Karim *et al*. 1993). Conseqüentment, els nivells d'mRNA de BR-C davallen a la darrera fase nimfal (N6), en paral·lel amb la desaparició d'HJ, només amb petits rebrots d'expressió encara observables en dia 6 coincidents amb els pics d'ecdisona. Les dades aportades al nostre treball indiquen també una clara regulació per HJ (experiments d'inducció per HJ i experiments amb RNAi de Kr-h1 on BR-C redueix l'expressió), de manera que la caiguda d'expressió i el declini observat a la sisena (i darrera) fase nimfal pot ser explicada, si més no en part, per la desaparició d'HJ. Els experiments amb RNAi de BR-C en nimfes de panerola han resultat en una disminució de la intensa proliferació cel·lular que experimenten els primordis alars en condicions normals a la darrera fase nimfal (Huang *et al*. 2013). La funció principal de les proteïnes BR-C a *B. germanica*, una espècie hemimetàbola filogenèticament basal, durant el desenvolupament postembrionari és, doncs, promoure el creixement del teixit alar fins arribar a assolir una mida i una forma correctes. Com aquesta funció es dóna també a *D. melanogaster* (Bayer *et al*. 1996), les funcions de BR-C relacionades amb controlar la mida i forma de l'ala semblen ser ancestrals i conservades des de dictiòpters a dípters. El mateix podria ser cert per les funcions subtils relacionades amb el patró de venació observats a *B. germanica*, ja que els clàssics mutants de BR-C de *D. melanogaster* també presenten defectes en el forma i mida de les venes alars (Morgan *et al*. 1925).

La nostra descripció dels factors BR-C com agents de canvi durant els diferents passos evolutius de la metamorfosi ha estat recolzada per diferents evidencies, i en aqust sentit, els patrons d'expressió de BR-C observats en els tisanòpters *Frankliniella occidentalis* i *Haplothrips brevitubus* són d'especial interès (Minakuchi *et al*. 2011). Els tisanòpters segueixen un desenvolupament essencialment hemimetàbol, en el sentit que les nimfes són morfològicament similars als adults, però el cicle de vida inclou d'una a tres fases pràcticament quiescents que recorden l'estadi pupal dels holometàbols, i que s'anomenen propupa i pupa i on els primordis alars es desenvolupen considerablement. Aquest cicle particular ha estat definit com a desenvolupament neometàbol (Sehnal *et al*. 1996). *F. occidentalis* passa per dos estadis nimfals seguits d'una fase de propupa i una de pupa, mentre que *H. brevitubus* posseeix també dues fases nimfals seguides d'una fase propupal i dos estadis pupals. A ambdues espècies, l'expressió de BR-C és baixa en les primeres fase nimfals, fa un pic cap al final de la segona fase nimfals i davalla a la fase de propupa. A més a més, el tractament de la fase de propupa amb un anàleg d'HJ indueix la re-expressió de BR-C a la pupa (Minakuchi *et al*. 2011). El patró d'expressió de BR-C en aquests tisanòpters mostra un pic just abans de la transició de nimfa a propupa, i l'efecte estimulant de l'HJ sobre l'expressió de BR-C a la propupa són reminiscents del determinisme endocrí de l'estadi pupal de les espècies holometàboles. Estudis en estrepsípters també suggereixen la importància de BR-C com a especificador pupal, i remarca el paper clau d'aquest gen (Erezyilmaz *et al*. 2014). Alguns membres derivats de l'ordre endoparasit dels estrepsípters han adquirit una forma extrema de dimorfisme sexual pel quals els mascles experimenten una metamorfosi i existeixen com a adults de vida lliure, mentre que les femelles romanen durant tot el cicle vital larviformes, aconseguint la maduresa sexual dins dels seus hostes. En aquests insectes BR-C presenta una expressió alta durant els períodes del desenvolupament pupal dels mascles, mentre que en les femelles, sense fase de pupa, s'expressa constitutivament a l'igual que en insectes hemimetàbols (Erezyilmaz *et al*. 2014). Aquestes dades suggereixen que el canvi de funció de BR-C en el context evolutiu, de promotor del creixement de l'ala a especificador de l'estadi pupal, s'hagi donat abans de la separació entre polineòpters (paneroles, saltamartins) i el clade Eumetabola, constituït per paraneòpters (hemípters, tisanòpters) i endopterigots (escarabats, mosques, papallones).

Krüppel homolog 1

Un altre element important que participa en la transducció de l'HJ en relació amb la metamorfosi és el factor de transcripció Krüppel-homolog 1 (Kr-h1). El nostre estudi va determinar que Kr-h1 es troba present en tots els estadis nimfals de la panerola i que la seva expressió desapareix sobtadament després del primer dia de la darrera fase nimfal (Lozano and Belles 2011). En la transició de "propupa" a "pupa" en les espècies de hemimetàboles de tisanòpters (*F. occidentalis* i *H. brevitubus*) han estat observades caigudes similars de l'expressió de Kr-h1 (Minakuchi *et al*. 2011). També s'ha observat reducció de l'expressió de Kr-h1 entre els estadis prepupals i pupals dels models holometàbols, com la mosca *D. melanogaster* i l'escarabat *T. castaneum* (Minakuchi *et al*. 2008, Minakuchi *et al*. 2009). La coincidència de patrons de Kr-h1 i els d'HJ circulant (que davalla també durant els primers dia de la darrera fase nimfal) (Treiblmayr *et al*. 2006) a la panerola *B. germanica*, suggereix que l'expressió de Kr-h1 és induïda per l'HJ. Aquesta hipòtesi fou corroborada amb experiments on tractàvem la panerola amb HJ exògena a la darrera fase nimfal, en els què vam observar que Kr-h1 es re-expressava. Els resultats dels experiments amb RNAi de Kr-h1 indiquen que la interferència d'aquest gen a *B. germanica* en quart i cinquè estadi juvenil indueix una metamorfosi precoç després del penúltim (cinquè) estadi nimfal (Lozano and Belles 2011). La supressió de Kr-h1 mitjançant RNAi en nimfes o larves joves porta a una metamorfosi precoç, i aquesta caiguda és condició necessària per desencadenar la metamorfosi. Cal fer notar, però, que quan l'RNAi fou realitzada en quarta fase nimfal, varen caldre dues mudes abans de que es donés la metamorfosi precoç. Estudis pioners ja havien demostrat que la metamorfosi pot ser induïda precoçment tot extirpant els *corpora allata*. No obstant, quan aquesta operació era feta en larves molt joves, calien una o dues mudes addicionals abans que es formessin precoçment els caràcters adults (Wigglesworth 1954). Estudis en larves del lepidòpter *Bombyx mori* han mostrat que la disminució de nivells d'HJ a través de manipulacions moleculars, com la sobre-expressió d'una esterasa de l'HJ, no donen lloc a una pupa prematura fins després del tercer estadi larvari (Tan *et al*. 2005). Estudis més recents també efectuats a *Bombyx mori* obtenen resultats similars: utilitzant mutants de *mod*, els quals no no poden sintetitzar HJ expoxidasa, es demostra com malgrat presentar nivells residuals d'mRNA de Kr-h1 durant els primers estadis larvals les papallones no entren en fase de pupa fins la penúltima fase nimfal (Smykal *et al.* 2014). De manera similar, els canvis

metamòrfics després d'un tractament amb RNAi de Met en larves joves de *T. castaneum* o nimfes joves de *P. apterus* requereixen típicament dues o tres mudes abans de donar caràcters d'adults precoços (Konopova and Jindra 2007, Smykal *et al.* 2014). Aquestes observacions, i les nostres descrites a *B. germanica*, suggereixen que els insectes immadurs semblen insensibles als efectes de l'HJ en els estadis juvenils més primerencs i necessiten arribar a una mida crítica (per tant un temps mínim de creixement postembrionari) per ser capaços de fer la metamorfosi quan desapareix l'HJ, tant en espècies hemimetàboles com holometàboles.

Un altre descobriment interessant va ser que l'RNAi de Kr-h1 era més eficient en mascles que en femelles de *B. germanica*. L'RNAi de Kr-h1 realitzat en mascles amb una sola dosi de dsRNA presentava una penetrància del fenotip adult precoç d'un 100%, independentment de l'estadi del tractament, mentre que en femelles va resultar entre el 29%, quan les femelles van ser tractades a la cinquena fase nimfal, i 76%, quan va ser tractades en la quarta fase nimfal (Lozano and Belles 2011). Els experiments amb mascles van aclarir que existeix una sòlida correlació entre els percentatges de caiguda d'RNA missatger després del tractament amb RNAi de Kr-h1 i els adults precoços obtinguts en l'experiment. A més, el fet que la caiguda de transcrit fos més acusada en mascles que en femelles suggereix que la maquinària d'RNAi és més eficient, o va ser més eficientment induïda, en mascles que en femelles, un aspecte que mereixeria futures recerques.

Els resultats dels experiments d'RNAi indiquen que Kr-h1 desenvolupa el paper de repressor mestre de la metamorfosi a *B. germanica*, un exopterigot polineòpter que mostra un tipus de metamorfosi hemimetàbola. Això suggereix que el paper de repressor de Kr-h1 en la metamorfosi és una condició ancestral que s'ha conservat en espècies hemimetàboles i holometàboles. El nostres resultats van ser publicat en paral.lel als trobats en els hemípters *P. apterus* i *R, prolixus* (Konopova *et al*. 2011), la qual cosa dona més suport als nostres resultats obtingut en *B. germanica*, i els generalitza. Treballs posteriors han mostrat que la regió promotora del gen Kr-h1 conté elements de resposta a l'HJ, als quals s'uneix el complex Met-Taiman en presència de l'HJ, la qual cosa activa la transcripció de Kr-h1 (Kayukawa *et al*. 2012, Kayukawa *et al*. 2013). Els nous reptes són descobrir els factors que especifiquen l'estadi adult i són reprimits per Kr-h1, com el recentment descobert factor E-93 (Ureña *et al*. 2014, Belles and Santos 2014). A més, el descobriment d'un homòleg de Kr-h1 en *Daphnia pulex*

obre la qüestió de la seva funció en crustacis (Miyakawa *et al*. 2013, Miyakawa *et al*. 2014), la qual caldria ser investigada, de la mateixa manera que caldria investigar la seva funció en insectes ametàbols.

Regulació de Kr-h1 mitjançant l'acció de microRNAs

Els microRNAs (miRNAs) són una classe d'RNAs petits no codificants que regulen l'expressió de gens a nivell transcripcional mitjançant la regulació de l'mRNA (Bartel 2004, Bentwich *et al*. 2005, Berezikov *et al*. 2005). Per tal de desvetllar la funció dels miRNAs durant el desenvolupament de *B. germanica*, es van dur a terme experiments suprimint l'expressió de Dicer-1, enzim que participa en la biosíntesi dels miRNAs. Els experiments de RNAi de Dicer-1 realitzats a la penúltima fase nimfal de *B. germanica* van provocar una reducció dels nivells de miRNAs i una inhibició de la metamorfosis (Gomez-Orte and Belles 2009). Els resultats presentats en aquesta tesi mostren com als individus interferits de Dicer-1 els nivells d'mRNA de Kr-h1 augmenten, i que si es realitza un RNAi de Kr-h1 sobre individus interferits per Dicer-1, hom aconsegueix restablir la metamorfosi. Hem predit que a la regió 3'UTR de l'mRNA de Kr-h1 hi ha un lloc d'unió a miRNAs que hem validat com a funcional per a miR-2 Els experiments inhibint l'expressió de miR-2 a la fase premetamòrfica també van resultar en una inhibició de la metamorfosi i el tractament amb mimètic de miR-2 en individus interferits per a Dicer-1 va restablir la metamorfosi.

La transducció de l'HJ s'inicia quan l'hormona s'uneix al seu receptor, Met, i aquest transdueix l'acció antimetamòrfica a través de Kr-h1. Recentment s'ha demostrat que Kr-h1 exerceix la seva funció tot reprimint el factor de transcripció E93 (Belles and Santos 2014). Kr-h1 cau abruptament al l'inici de la darrera fase nifmal, i aquesta reducció és propiciada en part per l'acció repressiva del factor E93 (Belles and Santos 2014, Ureña *et al*. 2014). El nostre treball ha permés explicar que miR-2 contribueix simultàniament a l'eliminació de l'expressió de Kr-h1 durant l'inici de la darrera fase nimfal. L'acció de miR-2 possiblement és clau en aquesta fase premetamòrfica, ja que és en aquest moment quan Kr-h1 deixa de ser induit per HJ, E93 reprimeix l'expressió de Kr-h1, mentre que miR-2 s'encarrega d'eliminar ràpidament les còpies d'mRNA de Kr-h1 romanents, tot desencadenant-se la metamorfosi. La presència de llocs d'unió de miR-2 predits per a les regions 3'UTR de Kr-h1 de diversos hemípters, sumat al fet que

Kr-h1 davalla a l'inici de la darrera fase nimfal d'aquests insectes (Konopova *et al*. 2011, Minakuchi *et al*. 2011) suggereix que la regulació de Kr-h1 per miR-2 és un tret comú als insectes hemimetàbols. Per contra, els programes de predicció no detecten llocs d'unió per a miR-2 a l'mRNA de Kr-h1 d'insectes holometàbols, per la qual cosa postulem que aquest mecanisme de control post-transcripcional de Kr-h1 mitjançant miR-2 s'hagi perdut durant la transició evolutiva a insectes holometàbols. El fet que les funcions dels miRNAs descrites en processos morfogenètics estiguin relacionades amb el desenvolupament d'ala a *D. melanogaster* (Ronshaugen *et al*. 2005, Biryukova *et al*. 2009) i a *B. germanica* (Rubio and Belles 2013), suggereix que la funció ancestral dels miRNAs en la metamorfosi dels insectes està relacionada amb la morfogènesi de l'ala.

10. Conclusions

10. Conclusions

1. La reducció de nivells d'expressió de Met a fases nimfals de *Blattella germanica* provoca una metamorfosi precoç. Això suggereix que Met participa a la cascada de senyalització de l'HJ en la inhibició de la metamorfosi, actuant possiblement com al receptor de l'HJ, tal i com s'ha comprovat prèviament en altres insectes.

2. La reducció de l'expressió de les isoformes que contenen la inserció-1 de Tai a *B. germanica* provoca una metamorfosi precoç. Això suggereix que aquestes isoformes de Tai participen a la cascada de senyalització de l'HJ en la inhibició de la metamorfosi, actuant possiblement com a co-receptores de l'HJ, tal i com prèviament s'ha comprovat en experiments *in vitro*. La presència d'aquesta inserció en isoformes de Tai d'altres espècies suggereix que el mecanisme de transducció de l'activitat antimetamòrfica de l'HJ mitjançant aquestes isoformes és un fenomen conservat en insectes.

3. La reducció de nivells d'expressió dels factors BR-C a *B. germanica* provoca una disminució de la divisió cel·lular i creixement d'ala, i una organització incorrecta del patró de venació de les ales. Això suggereix que la funció ancestral d'aquest complex de proteïnes en aquesta espècie està relacionada amb el control de mida i forma de l'ala.

4. La reducció de nivells d'expressió de Kr-h1 a fases nimfals a *B. germanica* indueix una metamorfosi precoç. Això suggereix que Kr-h1 participa a la cascada de senyalització de l'HJ en la inhibició de la metamorfosi, actuant possiblement com diana de Met-Tai, atès que la reducció de l'expressió d'aquests factors redueix també la de Kr-h1.

5. La dismuinució de nivells de miR-2, incrementa els nivells de transcrit de Kr-h1 i inhibeix la metamorfosi. Aquest resultats i d'altres associats, suggereixen que miR-2 regula la davallada de l'expressió del transcrit de Kr- h1 a la darrera fase nimfal de *B. germanica*, la qual cosa propicia que la metamorfosi es desenvolupi correctament.

11. Bibliografia

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