nature COMMUNICATIONS

ARTICLE

Received 31 Oct 2012 | Accepted 15 Apr 2013 | Published 14 May 2013

DOI: 10.1038/ncomms2868

A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans

Hitoshi Miyakawa[1], Kenji Toyota[1,2], Ikumi Hirakawa[1,3], Yukiko Ogino[1,2], Shinichi Miyagawa[1,2], Shigeto Oda[1], Norihisa Tatarazako[4], Toru Miura[5], John K. Colbourne[6] & Taisen Iguchi[1,2]

Juvenile hormone is an essential regulator of major developmental and life history events in arthropods. Most of the insects use juvenile hormone III as the innate juvenile hormone ligand. By contrast, crustaceans use methyl farnesoate. Despite this difference that is tied to their deep evolutionary divergence, the process of this ligand transition is unknown. Here we show that a single amino-acid substitution in the receptor Methoprene-tolerant has an important role during evolution of the arthropod juvenile hormone pathway. Microcrustacea *Daphnia pulex* and *D. magna* share a juvenile hormone signal transduction pathway with insects, involving Methoprene-tolerant and steroid receptor coactivator proteins that form a heterodimer in response to various juvenoids. Juvenile hormone-binding pockets of the orthologous genes differ by only two amino acids, yet a single substitution within *Daphnia* Met enhances the receptor's responsiveness to juvenile hormone III. These results indicate that this mutation within an ancestral insect lineage contributed to the evolution of a juvenile hormone III receptor system.

[1] Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Sciences, Higashiyama, Myodaiji, Okazaki 444-8787, Japan. [2] Department of Basic Biology, School of Life Science, Graduate University for Advanced Studies (Sokendai), Nishigonaka, Myodaiji, Okazaki 444-8585, Japan. [3] Ecogenomics, Inc., 1-1 Hyakunenkouen, Kurume, Fukuoka 839-0864, Japan. [4] Environmental Quality Measurement Section, Research Center for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan. [5] Laboratory of Ecological Genetics, Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810, Japan. [6] School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK. Correspondence and requests for materials should be addressed to T.I. (email: taisen@nibb.ac.jp).

Juvenile hormones (JHs) are acyclic sesquiterpenoid hormones that regulate important physiological and developmental processes among arthropods, including metamorphosis, moulting, growth, reproduction, sex and caste determination[1–7]. As exposure of JHs to animals can easily deflect developmental pathways, JHs are well studied in crustaceans for aquaculture[8–10] and in insects for agricultural pest control[11,12]. Crustaceans mainly use methyl farnesoate (MF) as the innate JH ligand[13] whereas insects use an epoxide form of MF called JH III (JH III), which is catalysed by the MF epoxidase, CYP15A1 (ref. 14). Therefore, MF is the precursor form of active JH in insects[15–17]. However, little is known about their receptor systems and how they evolved.

Freshwater microcrustaceans *Daphnia pulex* and *D. magna* show striking JH-mediated polyphenisms such as environmental sex determination, induction of haemoglobin in response to reduced oxygen availability in aquatic habitats and inducible morphological defenses against predators signalled by kairomones[8–10,18–21]. In particular, the relationship between environmental sex determination and JH has been well studied over the last decade[8–10,22]. Most *Daphnia* species usually produce female offspring by parthenogenesis; however, once environmental conditions become barren, such as starvation or crowding, they produce male offspring and sexual reproduction occurs[23]. Male production is also induced by topical application of JH and JH analogues independent of environmental condition[8–10], suggesting that JH is a key endocrine factor for sex determination working downstream of environmental stimuli in *Daphnia*. To date, several chemicals, including MF, JH III and four artificially synthesized juvenoids used in this study (that is, fenoxycarb, pyriproxyfen, methoprene and epofenonane), have been shown to be able to induce male daphniids with various concentration ranges[10,22] (Table 1). Although receptors of JH are still unknown in any of the crustacean species, basic information obtained from these ecotoxicological studies is helpful in understanding crustacean JH pathway. There is every possibility that the difference in the capacity to induce male, shown by these chemicals, reflects ligand selectivity of JH receptor systems of *Daphnia* directly.

Methoprene-tolerant (Met) and steroid receptor coactivator (SRC) are proteins that belong to the basic helix–loop–helix (bHLH)–Per–Arnt–Sim (PAS) family of transcription factors. Both have important roles in the JH reception and downstream transcriptional activation[24–26]. Met is regarded as a leading candidate for the JH receptor in insect studies[27–29]. SRC—whose name is from the mammalian homologue SRC-1 with orthologs named FISC and Taiman in *Aedes* and *Drosophila* species—is also involved in the reception and signal transduction of JH[24–26]. Although Met exists as a homodimer (or heterodimer with the *Drosophila*-specific paralog, germ cell-expressed (GCE)) in the absence of JH[30], the homodimer is remodelled to form a heterodimer with SRC followed by transcriptional activation of downstream elements upon JH binding to the carboxy-terminal PAS domain (PAS-B) of Met[24–26].

To understand the molecular underpinnings of the JH reception system in daphniids and ligand transition that likely occurred when hexapods evolved from their pancrustacean ancestor[31], we tested the JH sensitivity of Met and SRC in two crustacean species, *D. pulex* and *D. magna*. As a result, *Daphnia* Met and SRC showed capability to respond to various juvenoid in different dose ranges, respectively. Moreover, we demonstrate that a single amino-acid substitution within *Daphnia* Met enhances the receptor's responsiveness to JH III, suggesting that this mutation contributed to the evolution of a JH III receptor system.

Results

Cloning and characterization of *Daphnia Met* and *SRC*. We cloned and characterized the full-length sequences of *Met* and *SRC* genes from two *Daphnia* species, designated as *Dappu-Met* and *Dappu-SRC* for *D. pulex*, and *Dapma-Met* and *Dapma-SRC* for *D. magna* (accession numbers: AB698067–AB698070). Pairwise sequence identities of bHLH, PAS-A and PAS-B domains between *Daphnia* and selected insect species range from 15% to 78% (Fig. 1a–c). Six of eight polymorphic amino-acid residues within PAS-B domain forming JH-binding pockets are conserved in Met of both *Daphnia* species[25] (Fig. 1d). At the other two sites, valine in insects is substituted for threonine in *Daphnia*, and threonine in insects is substituted by serine (Fig. 1d). We hypothesize that one or both amino-acid substitutions cause differences in ligand specificity of Met between crustaceans and insects. Both genes are required for development; transcriptional knockdown of *Dapma-Met* or *Dapma-SRC* result in embryonic death (Fig. 2 and Table 2).

JH-dependent heterodimerization of *Daphnia* Met and SRC. We tested juvenoid-dependent Met–SRC protein interactions in *Daphnia* by two-hybrid luciferase assay[25,32] (see Methods). MF (JH in crustaceans), JH III (JH in insects) and four artificially synthesized juvenoids (fenoxycarb, pyriproxyfen, methoprene and epofenonane) were used for this study. As described above, all these chemicals induce male offspring development in *Daphnia*[10,22] (Table 1). We detected concentration-dependent luciferase activation from the heterodimerization of Met with SRC by all juvenoid treatments, except for epofenonane (Fig. 3a,b). Although the effective dose responses to the juvenoids vary for both species, parallel responses are observed

Table 1 | EC_{50} of transcriptional activation via heterodimerization between SRC and Met (mutant) used in this study.

	D. pulex				*D. magna*				
	wt	T292V	S323T	T292V, S323T	wt	T296V	S327T	T296V, S327T	*D. magna* male induction*
Methyl farnesoate	4.906E−07	4.349E−08	3.631E−07	3.308E−08	4.764E−07	2.670E−08	2.064E−07	2.372E−08	2.777E−07
JH III	8.519E−06	6.056E−07	6.096E−06	7.681E−07	5.707E−06	3.869E−07	5.207E−06	4.900E−07	4.009E−06
Fenoxycarb	1.412E−08				1.068E−08				3.053E−09
Pyriproxyfen	8.880E−07				5.183E107				4.387E−10
Methoprene	NA†	1.021E−05	NA†	4.937E−06	NA†	5.889E−06	NA†	4.296E−06	NA†
Epofenonane	NA†				NA†				2.108E−07

NA, not applicable; wt, wild type.
*EC_{50} based on the data of previous studies (Tatarazako *et al.*[10], and Oda *et al.*[22]).
†EC_{50} could not be determined because of low concentration response.

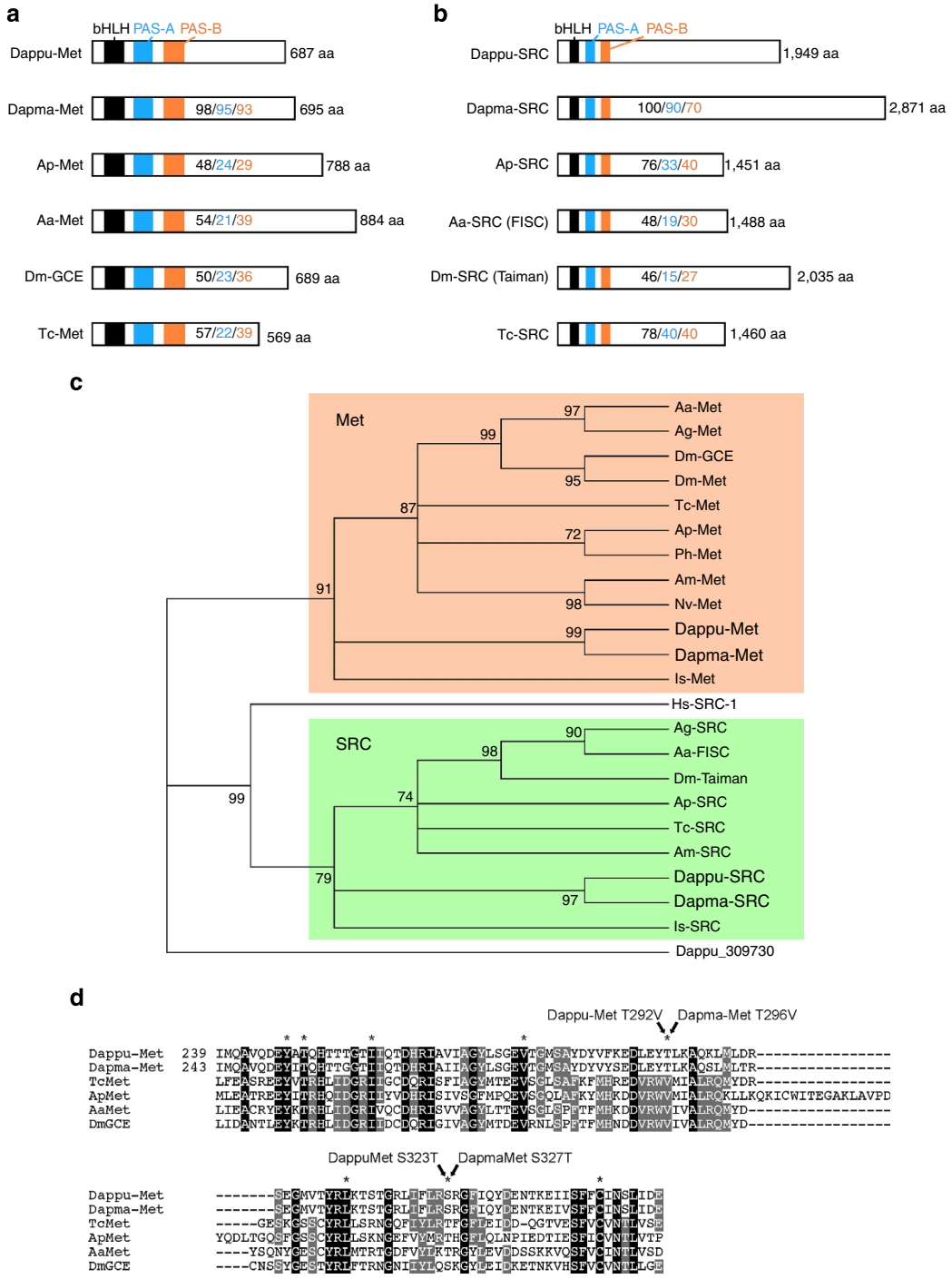

Figure 1 | Met and SRC in two *Daphnia* species. (**a**,**b**) Met and SRC in two *Daphnia* species, *D. pulex* (Dappu) and *D. magna* (Dapma), and homologues in insect species. Both *Daphnia* Met (**a**) and SRC (**b**) contain bHLH domain and PAS (PAS-A and PAS-B) domain in their sequences. Values in each sequence indicate percentage identity of amino-acid sequences of bHLH (black), PAS-A (blue) and PAS-B (orange) domain with those of *D. pulex*. Characteristic SRC family motifs LXXLL and glutamine-rich (Q-rich) regions were identified within the central and C-terminal regions of both Dappu-SRC and Dapma-SRC[39]. (**c**) Maximum Likelihood tree based on the amino-acid sequences of Met and SRC suggested that *Daphnia* Met and SRC were orthologs of insect Met and SRC, respectively. Dappu_309730 is another protein with bHLH–PAS domain found in the genome database of *D. pulex*. Strongly supported nodes (>70%) are labelled with the percentage of bootstrap 1,000 replications. (**d**) Multiple alignment of PAS-B domain in *Daphnia* and insect Met. Asterisks indicate eight amino acids involved with JH III binding in *Tribolium* Met[24]. In this study, two amino acids indicated by arrows were mutated to the corresponding amino acids used in insects. Species abbreviations and accession codes are as follows: *Aedes aegypti* (Aa) Met (XP_001660262.1) and SRC (FISC) (ABE99837.2); *Anopheles gambiae* (Ag) Met (XP_316059.4) and SRC (XP_001689140.1); *Apis mellifera* (Am) Met (XP_395005.4) and SRC (XP_394114.4); *Acyrthocyphon pisum* (Ap) Met (XP_003246905.1) and SRC (XP_001944363.2); *D. magna* (Dapma) Met (AB698069) and SRC (AB698070); *D. pulex* (Dappu) Met (AB698067) and SRC (AB698068); *D. melanogaster* (Dm) Met (NP_511126.2), GCE (NP_511160.2) and SRC (Taiman) (AAG16637.1); *Homo sapiens* (Hs) SRC-1 (AAB50242.1); *Ixodes scapularis* (Is) Met (XP_002410323.1) and SRC (XP_002402360.1); *Nasonia vitripennis* (Nv) Met (XP_001606775.2); *Pediculus humanus* (Ph) Met (XP_002430841.1); *Tribolium castaneum* (Tc) Met (EFA00995.1) and SRC (EFA04027.1). aa, amino acid.

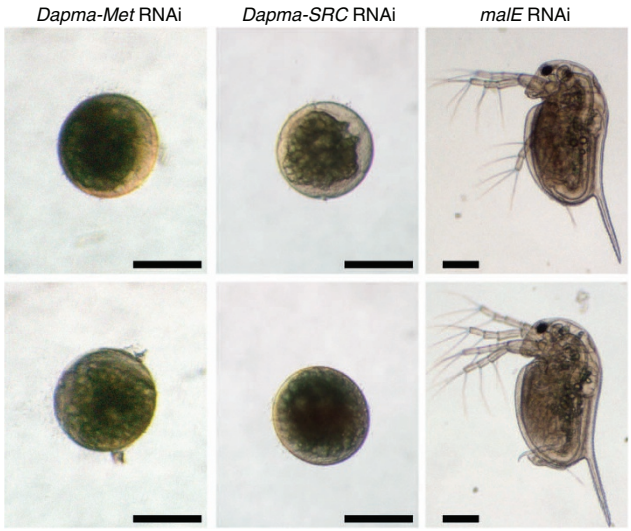

Figure 2 | Phenotypes of RNA interference (RNAi) targeting of the *Dapma-Met* and *Dapma-SRC*. Development of *Dapma-Met* or *Dapma-SRC* dsRNA-injected embryos was aborted at 20–30 h after oviposition. *E. coli malE* dsRNA-injected embryos grew into the first instar juveniles at 80 h after oviposition. Scale bars, 200 μm.

Table 2 | Summary of the result of RNAi transcriptional knockdown experiments.

	Dapma-Met	*Dapma-SRC*	*malE*
Number of aborted eggs/injected eggs	11/13	11/11	0/6
Percentage of phenotypes	84.6	100	0

RNAi, RNA interference.

suggesting that the Met–SRC complex functions as a JH receptor in *Daphnia* species, as in insects[24–26]. In addition, the effect of MF on heterodimerization is nearly tenfold greater than that of JH III, which is consistent with the knowledge that crustaceans use MF as an active form of JH (Fig. 3a,b and Table 1), not JH III. Dappu-Met and Dapma-Met were also swapped in combination with Dapma-SRC and Dappu-SRC for each of the treatments. These swapped pairs also exhibited juvenoid-dependent dimerization (Fig. 5), yet combinations of both Met–Met and SRC–SRC did not show juvenoid-dependent responses (Fig. 5), thus further confirming that the Met and SRC protein complex functions as a JH receptor in daphniids.

Transition of JH ligand sensitivity. Finally, we tested our hypothesis that the threonine → valine and/or serine → threonine amino-acid substitutions in Met cause different ligand specificities of the receptor between crustaceans and insects, by measuring ligand-dependent heterodimerization using mutant *Daphnia* Met (Fig. 1d) that have amino acids T292V in Dappu-Met and T296V in Dapma-Met exchanged with Val-297 of *Tribolium* Met, and have amino acids S323T in Dappu-Met and S327T in Dapma-Met exchanged with Thr-330, also of the *Tribolium* gene (Fig. 1d). MF, JH III and methoprene treatments were used in this last experiment. For both *Daphnia* Met genes, the Val-297 mutation significantly increases the receptor sensitivity to JH III (Fig. 3c and Table 1). By contrast, mutations S323T and S327T did not alter ligand sensitivity (Fig. 3c and Table 1).

Discussion

Although there are a lot of reports about the effect of juvenoids on environmental sex determination in daphniids[8–10,22], their JH receptor molecules have been unclear to date. Here, we cloned *Met* and *SRC* from two *Daphnia* species, and demonstrated that Met and SRC proteins form heterodimer only in the presence of juvenoids, suggesting that these molecules act as a JH receptor in crustaceans as in insects[24–26]. Moreover, ligand selectivity that was estimated from the half maximal effective concentration (EC_{50}) values for heterodimerization was generally in accord with previous data for inducing male offspring development when testing six juvenoids (EC_{50} values: fenoxycarb < MF < JH III < methoprene; Table 1 and Fig. 4)[10,22]. Although EC_{50} values of pyriproxyfen and epofenonane were not consistent between these two different experiments, the observed difference between the effective ranges for male offspring induction in the two species is likely caused by ligand selectivity of Met (or the Met–SRC complex) for juvenoids. Epofenonane is the only tested compound that does not induce heterodimerization, despite its effect at inducing male offspring development (Fig. 3a,b and Table 1). For most juvenoids, male induction is also associated with a great reduction of the number of offspring. In contrast, epofenonane can also induce male production with only a slight change in brood size[10,22]. It is therefore likely that epofenonane induces male production by a different mechanism from the other juvenoids and their synthetic derivatives, which might not involve Met and SRC. On the other hand, in several insects, juvenoids lead to dissociation of Met–Met homodimer[25,30], yet combinations of two Met plasmids in daphniids indicated neither homodimerization nor juvenoid-dependent responses (Fig. 5), thus further confirming that the Met and SRC protein complex functions as a JH receptor in daphniids.

For both *Daphnia* Met genes, the Val-297 mutation significantly increases the receptor sensitivity to JH III (Fig. 3c and Table 1), suggesting that amino-acid substitution to valine at this site had contributed to the transition of JH ligand during insect evolution. Furthermore, this amino-acid substitution to valine is thought to be involved in reception of methoprene, to which daphniids show lower sensitivity than insects, consistent with a previous report demonstrating that a single amino-acid mutation at Val-297 in *Tribolium* Met inhibited binding of methoprene to Met[24]. This mutation, however, also increases sensitivity to MF (Fig. 3c and Table 1). Several studies report detectable amounts of MF in insect haemolymph, together with other juvenoids[7,33,34]. Further studies are required to determine whether both JH III and MF target the same insect receptor. By contrast, mutations S323T and S327T did not alter ligand sensitivity (Fig. 3c and Table 1), possibly because both serine and threonine have similar biochemical characteristics[35]. Given that a similar substitution to threonine can also be found within the *Drosophila melanogaster* GCE (Fig. 1d), it is thought to be a less functional polymorphism during the evolution of JH receptor.

In summary, present study strongly suggests that the Met–SRC complex operates as a JH receptor in daphniids as well as insects, but their ligand sensitivities are somewhat different from each other. It can be a reason why each juvenoid shows so varied effects and toxicity depending on animal species and taxa. Moreover, a single amino-acid substitution is revealed important during evolution of the arthropod JH pathway, by comparative functional genetic studies using a branchiopod crustacean model species. This knowledge, coupled with the identification of a JH-synthesizing organ and isolation of JH-responsive elements

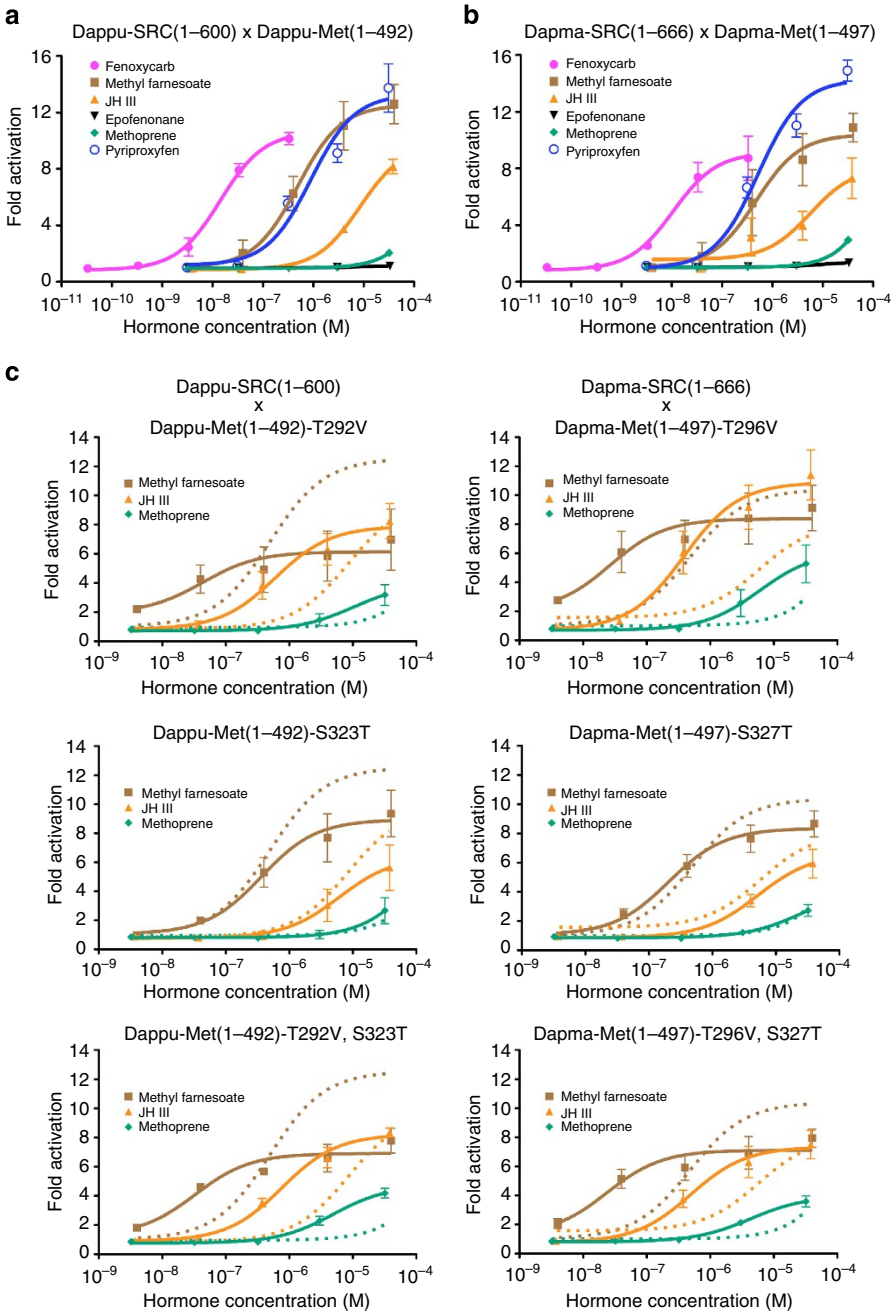

Figure 3 | Transcriptional activation via heterodimerization between *Daphnia* Met and SRC exposed to various juvenoids. (**a**) Concentration–response profile for SRC(1–600) and wild-type Met(1–492) of *D. pulex*. (**b**) Concentration–response profile for SRC(1–666) and wild-type Met(1–497) of *D. magna*. (**c**) Concentration–response profile for SRC and mutant Met. Vertical axis indicates fold activation, which is the luciferase activity normalized by solvent control (0.1% ethanol) of each experiment. Dotted lines indicate profiles of wild types. Each point represents the mean of triplicate determinations, and vertical bars represent the mean ± s.d.

interacting with Met–SRC complex, is expected to provide a more unified mechanistic understanding of the development and environmentally induced phenotypic plasticity across Arthropoda.

Methods

Animals. The *D. pulex* and *D. magna* clones used in the experiments were provided by the National Institute for Environmental Studies (NIES), Tsukuba, Japan. The clones were reared in the laboratory at 20 °C in aged tap water and fed unicellular green algae (Chlorella Industry Co. Ltd, Fukuoka, Japan) over generations in 1 l beakers in a temperature- and photocycle-controlled incubator (20 °C, 16-h light/8-h dark).

Cloning of Met and SRC. Amino-acid sequences of insect Met and SRC homologues were obtained using euGene's Arthropod genomes (http://insects.eugenes.org/arthropods/), and aligned by the ClustalX program[36] using default options. Based on the aligned sequences, conserved regions were identified and used to perform TBLASTN searches against the wFleaBase (http://wfleabase.org/) to identify *D. pulex* gene models. PCR primers for cloning were designed based on

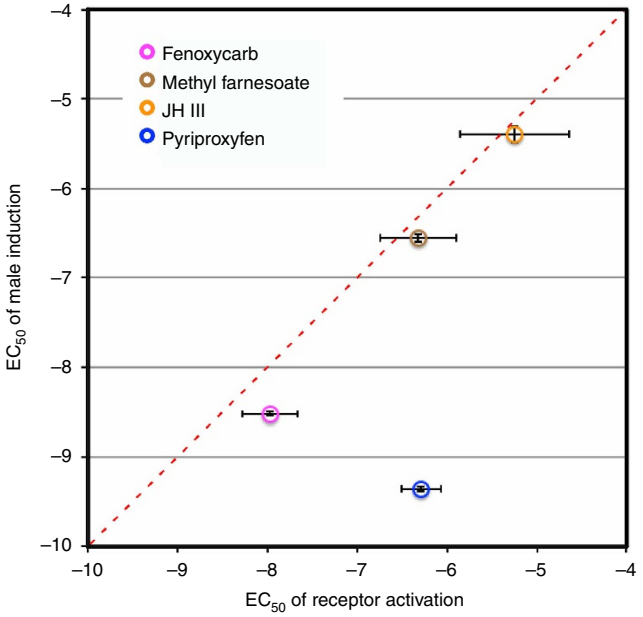

Figure 4 | Logarithmic relationships between EC_{50} of receptor activation and juvenoid induction of males in *D. magna*. Error bars indicate 95% confidence intervals. Dotted line indicates log ratio (1). EC_{50} values of male induction are based on the data of previous studies (Tatarazako *et al.*[10] and Oda *et al.*[22] (see Table 1)).

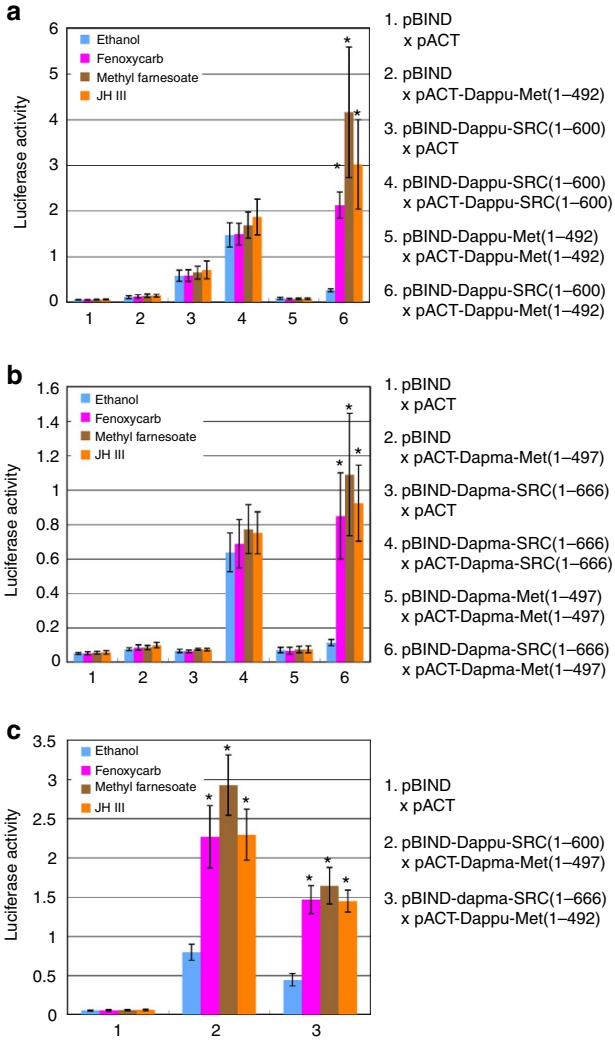

Figure 5 | Transcriptional activations with various vector combinations. (**a**) Response profile of *D. pulex*. (**b**) Response profile of *D. magna*. (**c**) Response profile for SRC and Met from different *Daphnia* species compared with each other. Vertical axis indicates luciferase activities. Each point represents the mean of triplicate determinations, and vertical bars represent the mean ± s.d. Asterisks indicate significant differences ($P<0.05$, *t*-test) from ethanol treatment of each vector combination. Concentrations of juvenoid (M) used in this experiment are as follows: fenoxycarb (3.32E − 08), methyl farnesoate (3.99E − 06) and JH III (3.75E − 05).

sequences of these gene models. Total RNA was extracted from ~20 adult *D. pulex* and *D. magna* using an SV Total RNA Isolation System (Promega, Madison, WI) and converted to complementary DNA using Superscript III and random primers (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Partial sequences of *Met* and *SRC* were obtained by PCR amplification, and full-length cDNAs were obtained by GeneRacer Kit (Life Technologies). Domain and motif searches were performed with obtained sequences using the European Bioinformatics Institute (EBI) InterPro database (http://www.ebi.ac.uk/Tools/InterProScan/).

Phylogenetic analysis. The amino-acid sequences were aligned by the MEGA5 software using default options[37]. The Maximum Likelihood tree was constructed from this alignment using a 1,000 replicate bootstrap analysis, along with complete deletion options.

RNA interference. Knockdown of *Dapma-Met* and *Dapma-SRC* was performed using RNA interference technique according to our previous study[38]. *Escherichia coli malE* gene was used as a negative control. Double-stranded RNA (dsRNA) was synthesized using the MEGAscript RNAi Kit (Life Technologies). Eggs were obtained from *D. magna* at 2 weeks of age just after ovulation and placed in ice-cold M4 medium containing 80 mM sucrose (M4-sucrose). The synthesized dsRNA (2 mg ml^{-1}) was mixed with 2 mM Chromeo 494 fluorescent dye (Active Motif Chromeon GmbH, Tegernheim, Germany), which was used as a visible marker for injection. Mixture containing 1 mg ml^{-1} dsRNA was injected to eggs under a microscope. Then, injected eggs were transferred into new Petri dishes and incubated at 20 °C.

Construction of the reporter system. We used a two-hybrid system to detect heterodimerization of Met and SRC. Chinese hamster ovary cells were transfected according to the instructions[32] with slight modifications. Dappu-Met(1–492) and Dapma-Met(1–497) were cloned into pBIND, and Dappu-SRC(1–600) and Dappu-SRC(1–666) (bHLH–PAS domain) were cloned into pACT (Promega). For transfections, Chinese hamster ovary cells were grown in 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 containing 10% fetal bovine serum, and incubated at 37 °C in a 5% CO_2 atmosphere. The day before transfection, cells were transferred to a 24-well plate. The transfection was performed using FuGENE HD (Promega), according to the manufacturer's protocols. Each well received 0.05 mg pBIND, 0.05 mg pACT and 0.2 mg pG5luc, which has luciferase gene under the control of GAL4-binding site. All juvenoids were dissolved in ethanol and added to the medium after 24 h, and cells were harvested after 48 h. Reporter activities were measured using the Dual-Luciferase Assay kit (Promega), according to the manufacturer's protocols. All transfections were performed at least three times, employing triplicate sample points in each experiment. The values shown are mean ± s.d. from three separate experiments, and dose–response data and EC_{50} were analysed using GraphPad Prism (Graph Pad Software, Inc., San Diego, CA).

References

1. Nijhout, H. F. & Wheeler, D. E. Juvenile hormone and the physiological basis of insect polymorphisms. *Q. Rev. Biol.* **57,** 109–133 (1982).
2. Nijhout, H. F. *Insect Hormones* (Princeton University Press, 1994).
3. Nijhout, H. F. Control mechanisms of polyphenic development in insects. *Bioscience* **49,** 181–192 (1999).
4. Hartfelder, K. & Emlen, D. J. *Comprehensive Molecular Insect Science* Vol. 3 (eds Gilbert, L. I., Iatrou, K. & Gill, S. S.) 651–703 (Elsevier, 2005).
5. Truman, J. W. *et al.* Juvenile hormone is required to couple imaginal disc formation with nutrition in insects. *Science* **312,** 1385–1388 (2006).
6. Cornette, R., Gotoh, H., Koshikawa, S. & Miura, T. Juvenile hormone titers and caste differentiation in the damp-wood termite *Hodotermopsis sjostedti* (Isoptera, Termopsidae). *J. Insect. Physiol.* **54,** 922–930 (2008).

7. Riddiford, L. M. Juvenile hormone action: a 2007 perspective. *J. Insect. Physiol.* **54,** 895–901 (2008).
8. Olmstead, A. W. & LeBlanc, G. A. Juvenoid hormone methyl farnesoate is a sex determinant in the crustacean *Daphnia magna*. *J. Exp. Zool.* **293,** 736–739 (2002).
9. Olmstead, A. W. & LeBlanc, G. A. Insecticidal juvenile hormone analogs stimulate the production of male offspring in the crustacean *Daphnia magna*. *Environ. Health Perspect.* **111,** 919–924 (2003).
10. Tatarazako, N., Oda, S., Watanabe, H., Morita, M. & Iguchi, T. Juvenile hormone agonists affect the occurrence of male *Daphnia*. *Chemosphere* **53,** 827–833 (2003).
11. Arias, J. R. & Mulla, M. S. Morphogenetic aberrations induced by a juvenile hormone analogue in the mosquito *Culex tarsalis* (Diptera: Culicidae). *J. Med. Entomol.* **12,** 309–316 (1975).
12. Ali, A., Xue, R. D. & Lobinske, R. Efficacy of two formulations of the insect growth regulator, pyriproxyfen (Nylar or Sumilarv), against nuisance Chironomidae (Diptera) in man-made ponds. *J. Am. Mosq. Control Assoc.* **9,** 302–307 (1993).
13. Laufer, H., Ahl, J. S. B. & Sagi, A. The role of juvenile hormones in crustacean reproduction. *Am. Zool.* **33,** 365–374 (1993).
14. Helvig, C., Koener, J. F., Unnithan, G. C. & Feyereisen, R. CYP15A1, the cytochrome P450 that catalyzes epoxidation of methyl farnesoate to juvenile hormone III in cockroach corpora allata. *Proc. Natl Acad. Sci. USA* **101,** 4024–4029 (2004).
15. Shinoda, T. & Itoyama, K. Juvenile hormone acid methyltransferase: a key regulatory enzyme for insect metamorphosis. *Proc. Natl Acad. Sci. USA* **100,** 11986–11991 (2003).
16. Minakuchi, C., Namiki, T., Yoshiyama, M. & Shinoda, T. RNAi-mediated knockdown of juvenile hormone acid O-methyltransferase gene causes precocious metamorphosis in the red flour beetle *Tribolium castaneum*. *FEBS J.* **275,** 2919–2931 (2008).
17. Niwa, R. *et al.* Juvenile hormone acid O-methyltransferase in *Drosophila melanogaster*. *Insect. Biochem. Mol. Biol.* **38,** 714–720 (2008).
18. Rider, C. V., Gorr, T. A., Olmstead, A. W., Wasilak, B. A. & LeBlanc, G. A. Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. *J. Exp. Biol.* **208,** 15–23 (2005).
19. Eads, B. D., Andrews, J. & Colbourne, J. K. Ecological genomics in *Daphnia*: stress responses and environmental sex determination. *Heredity* **100,** 184–190 (2008).
20. Miyakawa, H. *et al.* Gene up-regulation in response to predator kairomones in the water flea, *Daphnia pulex*. *BMC Dev. Biol.* **10,** 45 (2010).
21. Oda, S., Kato, Y., Watanabe, H., Tatarazako, N. & Iguchi, T. Morphological changes in *Daphnia galeata* induced by a crustacean terpenoid hormone and its analog. *Environ. Toxicol. Chem.* **30,** 232–238 (2011).
22. Oda, S., Tatarazako, N., Watanabe, H., Morita, M. & Iguchi, T. Production of male neonates in *Daphnia magna* (Cladocera, Crustacea) exposed to juvenile hormones and their analogs. *Chemosphere* **61,** 1168–1174 (2005).
23. Kleiven, O. T., Larsson, P. & Hobaek, A. Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65,** 197–206 (1992).
24. Charles, J. P. *et al.* Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proc. Natl Acad. Sci. USA* **108,** 21128–21133 (2011).
25. Li, M., Mead, E. A. & Zhu, J. S. Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. *Proc. Natl Acad. Sci. USA* **108,** 638–643 (2011).
26. Zhang, Z. L., Xu, J. J., Sheng, Z. T., Sui, Y. P. & Palli, S. R. Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. *J. Biol. Chem.* **286,** 8437–8447 (2011).
27. Miura, K., Oda, M., Makita, S. & Chinzei, Y. Characterization of the *Drosophila* Methoprene-tolerant gene product. Juvenile hormone binding and ligand-dependent gene regulation. *FEBS J.* **272,** 1169–1178 (2005).
28. Konopova, B. & Jindra, M. Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle *Tribolium castaneum*. *Proc. Natl Acad. Sci. USA* **104,** 10488–10493 (2007).
29. Abdou, M. A. *et al. Drosophila* Met and Gce are partially redundant in transducing juvenile hormone action. *Insect Biochem. Mol. Biol.* **41,** 938–945 (2011).
30. Godlewski, J., Wang, S. L. & Wilson, T. G. Interaction of bHLH-PAS proteins involved in juvenile hormone reception in *Drosophila*. *Biochem. Biophys. Res. Commun.* **342,** 1305–1311 (2006).
31. Regier, J. C. *et al.* Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. *Nature* **463,** 1079–1083 (2010).
32. Kato, Y. *et al.* Cloning and characterization of the ecdysone receptor and ultraspiracle protein from the water flea *Daphnia magna*. *J. Endocrinol.* **193,** 183–194 (2007).
33. Yin, C. M. *et al.* Identification of juvenile hormone III bisepoxide (JHB_3), juvenile hormone III and methyl farnesoate secreted by the corpus allatum of *Phormia regina* (Meigen), *in vitro* and function of JHB_3 either applied alone or as a part of a juvenoid blend. *J. Insect Physiol.* **41,** 473–479 (1995).
34. Darrouzet, E., Rossignol, F. & Couillaud, F. The release of isoprenoids by locust corpora allata *in vitro*. *J. Insect Physiol.* **44,** 103–111 (1998).
35. Betts, M. J. & Russell, R. B. *Bioinformatics for Geneticists* (eds Barnes, M. R. & Gray, I. C.) 289–316 (Wiley, 2003).
36. Thompson, J. D., Higgins, D. G. & Gibson, T. J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22,** 4673–4680 (1994).
37. Tamura, K. *et al.* MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28,** 2731–2739 (2011).
38. Kato, Y. *et al.* Development of an RNA interference method in the cladoceran crustacean *Daphnia magna*. *Dev. Genes Evol.* **220,** 337–345 (2011).
39. Leo, C. & Chen, J. D. The SRC family of nuclear receptor coactivators. *Gene* **245,** 1–11 (2000).

Acknowledgements

The *D. magna* and *D. pulex* sequence data were provided by The Center for Genomics and Bioinformatics at Indiana University, which is supported in part by the METACyt Initiative of Indiana University, funded in part through a major grant from the Lilly Endowment, Inc. and distributed via wFleaBase in collaboration with the *Daphnia* Genomics Consortium (http://daphnia.cgb.indiana.edu). This study was partly supported through a grant from the Ministry of Education, Culture, Sports, Science and Technology (T.I.), the Ministry of the Environment of Japan (T.I.), a grant of Long-Range Research Initiative by Japan Chemical Industry Association (T.I.) and a grant from National Institute for Basic Biology (T.I.). H.M. and K.T. were supported by the Japan Society for the Promotion of Science (JSPS) research fellowship for young scientists. This study benefits from and contributes to the *Daphnia* Genomics Consortium.

Author contributions

H.M., S.M., Y.O. and T.I. designed the experiments. H.M. and K.T. performed the experiments. H.M., K.T., I.H. and S.O. analysed the data. S.O., N.T., T.M. and J.K.C. discussed/commented on results and edited the manuscript. H.M., J.K.C. and T.I. wrote the paper. All authors have read and approved the final manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Miyakawa, H. *et al.* A mutation in the receptor Methoprene-tolerant alters juvenile hormone response in insects and crustaceans. *Nat. Commun.* 4:1856 doi: 10.1038/ncomms2868 (2013).