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Supplementary Information for

The oxytocin/vasopressin-like peptide inotocin regulates cuticular hydrocarbon synthesis and water balancing in ants

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Supplementary Methods

Peptide extraction

Body parts were homogenized in 400μL of acidified MtOH (MtOH:water:formic acid=90: 9.9:0.1). After 30 min incubation on ice, samples were centrifuged at 15,000 rpm for 10 min at 4°C. After collecting the supernatant, the precipitate was extracted again under the same conditions, and the supernatant was combined with the first extract. The extracts were centrifuged with 400μl of hexane, and after removal of the organic upper layer, the aqueous layer was completely evaporated. The pellets were suspended in 150μl of acidified acetonitrile (Acetonitrile:water:formic acid=90:9.9:0.1). After incubation on ice for 30min and centrifuging, the supernatant was transferred and evaporated again. The pellets were re-suspended in 100μl of water containing 0.1% formic acid and loaded to the solid-phase extraction column (GL-tip SDB, GL science). After centrifuging, the tip was washed with 50μl of 0.1% formic acid, and eluted by 40μl of 20% acetonitrile in 0.1% formic acid, and used in the quantification with LC-MS/MS.

DNA constructs

Inotocin precursor sequence was infused into *pUAST-7Xmyc* vector (generously provided by T. Chihara) with In-Fusion HD Cloning Kit (Clontech). IntR and OXTR fragments were first amplified with PCR, then inserted into *pmCherry-N1* (Clontech) vector to produce *pmCherry-intR* and *pmCherry-OXTR* vector. Flag-tagged Gα15 fragment was inserted into *pIRESpuro3* (Clontech) with In-Fusion HD Cloning Kit. We next infused the piggy back fragment amplified from *pPB-CAG-Tet3G-IRESneopA* vector (generously provided by Y.Yamaguchi) into *pIRESpuro3-Gα15-FLAG* vector digested with *Mlu* I (Takara) and *Xho* I (Takara) to produce *pPB-Gα15-FLAG-IRES-puroR* vector. *intR-mcherry* or *OXTR-mcherry* fragment was infused into *pPB-Gα15-FLAG-IRES-puroR* vector which was digested with *Hpa* I (Takara) to produce *pPB-intR-mcherry* and *pPB-OXTR-mcherry*.

Transfection

S2 cells were grown at 26°C in Schneider's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells in six-well plates (1×10^6 cells per well) were transfected using the Effectene Transfection Reagent (Qiagen) according to the manufacturer's instructions. We used 100ng of *Actin-Gal4* as a driver and 300ng of *UAS-int-7Xmyc* and *UAS-GFP* per well.

Protein sample preparation

The tissues dissected in ant saline or cell lysate were lysed with SDS sample buffer (2% SDS (wt/vol), 80 mM Tris-HCl (pH 6.8), 15% glycerol (vol/vol), 0.0025% Brilliant blue FCF (wt/vol), 2% 2-mercaptoethanol (vol/vol)) incubated for 1hour at 37°C and used for the western blot analysis.

Immunohistochemistry

Brains were dissected and cleaned by removing tracheae and fat tissues in cooled ant saline (1). They were then fixed in ZnFA (18.4mM $ZnCl_2$, 109mM NaCl, 1.2% 8.3mM sucrose, and 1% formaldehyde) prepared with the same osmolarity (273.4 mOsm) as ant saline, and kept overnight at 4°C. After fixation, the preparations were rinsed three times in ant saline and incubated with 1mg/ml collagenase/dispase (Sigma-Aldrich) in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 15min at room temperature. After rinsing with the ant saline, brains were dehydrated with 80% methanol in DMSO for 2 hours and 100% methanol for 1 hour under agitation, and rehydrated with 90%, 70%, 50% and 30% methanol in 0.1M Tris buffer (pH 7.4) into Tris buffer (10 min at each concentration). The brains were pre-incubated with 5% normal donkey serum in PBSd (1%DMSO, 0.05% NaN_3 in 0.1M PBS) for 1 hour with agitation and then incubated with rabbit anti-int antibody (diluted 1:1000 in PBSd-NDS) and anti-synapsin (SYNORF1) antibody (DSHB, 3C11, diluted 1:50 in PBSd-NDS) at 4°C for a week. Following incubation in primary antibodies, brains were rinsed in PBSd and then incubated with Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch; diluted 1:200 in PBSd-NDS) and Alexa 488-conjugated secondary anti-mouse secondary antibody (Thermo Fisher Scientific; diluted 1:100 in PBSd-NDS) at 4°C for 2 or 3 days. Brains were then rinsed three times (2hours each) in PBSt (0.2% TritonX-100 in 0.1M PBS) and dehydrated in ascending grades of ethanol series (30%, 50% 70%, 90%, 95%, and 2 times at 100%) for 30 min for each concentration. After the removal of ethanol, samples were kept in focus clear (FC-101, CEDARLANE), then imaged with confocal microscopy.

The ventral nerve cords and fat body plus oenocyte were dissected from ants fixed on a dissecting table made of a mixture of paraffin and beeswax, then fixed overnight with 4% formaldehyde/PB without agitation. After washing with PBST and blocking with PBST-NDS, the tissues were incubated with a primary antibody overnight at 4°C and a secondary antibody for 2 hours at room temperature. For the immunostaining of the ventral nerve cord (Fig. 3G-3J), the same antibodies were used as described above for the brains. For the staining of fat body plus oenocyte (Fig. 4C), the following dyes and antibody were used; hoechst (1μM), BODIPY 493/503 (1μM, Thermo Fisher Scientific) as the probe for the lipid droplet and anti-CYP4G1 antibody (diluted 1:100).

In situ hybridization

Using RNAscope® Multiplex Fluorescent Reagent Kit 2.0 according to the manufacturer's instructions (Advanced Cell Diagnostics), brains were fixed as described above with ZnFA and dehydrated through graded ethanol solutions (25, 50, 75, and 100%) for 10 min each. Brains were subjected to reagent protease III, and then hybridized overnight with probes at 40°C. Brains were then sequentially treated with a series of probe signal amplification steps at 40°C for 30 min, rinsed with wash buffer 3 times for 15 min between each step and finally counterstained with DAPI and mounted with slowfade Gold (Thermo Fisher Scientific). In a combined experiment using fluorescence in situ hybridization and immunohistochemistry, whole brains were prepared with ZnFA fixation and collagenase treatment. After dehydration and RNAscope procedures, the immunohistochemistry was sequentially performed using the same methods as described above.

For the expression analysis of *intR* mRNA in the fat body plus oenocyte, digoxigenin (DIG)-labelled sense or anti-sense probes for the full length of *intR* (1128bp) were synthesized by in vitro transcription with a DIG RNA Labeling Mix (Roche). The fat body plus oenocyte was dissected in ant saline and fixed in 4% formaldehyde in PB for 20 min at room temperature with agitation. The tissues were kept overnight in methanol at -20°C. After rehydration with decreasing series of methanol concentrations (75%, 50%, and 25%) and PTw (0.1% Tween20 in 0.1M PBS) for 10 min each, the tissues were moved into PTw and then digested with 20μg/ml proteinase K for 3 min (the digestion was stopped by adding 2 mg/ml glycine in PTw twice for 15 min). After rinsing with PTw three times (5 min each), the tissues were post-fixed in PTw with 4% PFA for 20 min with agitation. The tissues were then transferred to 50% PTw in a hybridization buffer (50% formamide, 5XSSC, 0.1% Tween20, 1X Denhardt's solution, 1mg/ml tRNA, 50μg/ml heparin and 0.1mg/ml herring sperm DNA) for 10 min, and prehybridized with hybridization buffer for 2 hours at 60°C. The tissues were hybridized overnight with labelled anti-sense or sense RNA probes at 60°C. The tissues were then washed with decreasing concentrations of hybridization buffer (75%, 50%, 25%) in 2X SSC for 30 min each at 60°C, then incubated with 2x SSC and 0.2X SSC for 30 min each. The tissues were then treated with DIG buffer I (100 mM Tris-HCl pH 7.5, 150 mM NaCl) for 5 min, 1.5% blocking reagent (Roche) for 1 hour, and anti-DIG antibody conjugated with alkaline phosphatase (1:1000; Roche) in DIG buffer I for 1 hour. After washing in DIG buffer I twice for 15 min, the tissues were treated with DIG buffer III (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, and 0.005% Tween 20) for 3 min. NBT/BCIP stock solution (Roche) was applied for 1 hour and then changed to stop solution for 3 min. The samples were washed with DIG buffer III twice for 10 min and PTw for 5 min. All steps

were performed at room temperature. The tissues were mounted with 70% glycerol and images were acquired with Axiophot (Carl Zeiss).

Generation of stable cell line

CHO cells were cultured in DMEM (10% FBS and 1% penicillin/streptomycin) at 37°C, 5% CO_2. *pPB-int (OXT) R-mCherry*, *pPB-Gα15-FLAG-IRES-puroR*, and PiggyBac transposase vector (*pCAGGS-PBase*, generously provided by Y. Yamaguchi) were mixed in the following ratio (45: 45:10 or 15:75:10, total 4μg) and transfected to CHO cells with Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's instructions. Transfected cells were selected during four rounds of selection with 30μg/ml puromycin (Sigma-Aldrich) in the medium. Individual cell colonies were picked and separated with 0.05% Trypsin-EDTA（Thermo Fisher Scientific) then cultured with 100μl of DMEM. Stable cell lines were selected from the mcherry signal (excitation; 515-560 nm, emission; >590 nm) using the fluorescence microscopy (Leica DMI4000B) and the activity assay against 100nM of inotocin or oxytocin (Sigma-Aldrich) peptide with plate reader (PerkinElmer ARVO X5). The expression of Gα15 was confirmed with western blotting using anti-flag antibody (1:2000, Sigma-Aldrich).

Cell preparation for the calcium assay

Cultured cells were washed with PBS, trypsinized by 2.4ml 0.05 % Trypsin-EDTA, and resuspended in 2 ml of loading buffer with 1μM Fluo-4 AM (Dojindo), 0.04% Pluronic F-127 (Sigma-Aldrich), 2.5mM Probenecid (Thermo Fisher Scientific) and 20mM HEPES (Thermo Fisher Scientific) in HBSS (Thermo Fisher Scientific). To load the fluorescent probe into the cells, the cell suspension was incubated for 30 min at 37°C with light shielding. After centrifugation and removal of the supernatant, the precipitate was re-suspended in 8ml of recording buffer (2.5mM Probenecid and 20mM HEPES in HBSS). The cells were dispensed for 20μl/well using Multidrop Combi (Thermo Fisher Scientific) to the 384-well assay plate (Greiner), where 100 nL of 1mM compound solution had been dispensed in advance. After 1 hour of incubation at room temperature with light shielding, a calcium assay was performed.

Chemical screening

We performed the chemical screening with 222,400 compounds obtained from the Drug Discovery Initiative (DDI, The University of Tokyo). For 1st screening, we measured the fluorescent intensity before and after the 25nM inotocin injection (final concentration; 5nM). 2nd and 23rd rows were used as the positive control. 1st and 24th rows were used as the negative control in which only buffer (20mM HEPES in HBSS) was applied. The Inhibition Rate (IR) was given by

$$\mathrm{IR}(\%) = \left(1 - \left(\frac{\mathrm{R_{Sample}} - \mathrm{Average}(\mathrm{R_{negative\ controls}})}{\mathrm{Average}(\mathrm{R_{positive\ controls}}) - \mathrm{Average}(\mathrm{R_{negative\ controls}})}\right)\right) \times 100.$$

We used Z'-factor to assess the quality of the assay, and adopted the data from plates showing their Z' values greater than 0.5. Z' factor was given by

$$\mathrm{Z}' = 1 - \frac{3 \times \left(\mathrm{SD}\left(\mathrm{R_{positive\ controls}}\right) + \mathrm{SD}\left(\mathrm{R_{negative\ controls}}\right)\right)}{\mathrm{Average}\left(\mathrm{R_{positive\ controls}}\right) - \mathrm{Average}\left(\mathrm{R_{negative\ controls}}\right)},$$

where SD is the standard deviation.

We then picked up the 902 compounds whose inhibition rate against inotocin signaling was over 50% for the 2nd screening. In the 2nd screening, 200nl of compound solution （2mM, 1mM, 500μM, 250μM and 125μM） was dispensed into the 384 well plate. Final concentrations were 20μM, 10μM, 5μM, 2.5μM and 1.25μM, respectively. Using the same method as for the 1st screening, we measured the response of CHO/intR to 25nM inotocin, CHO/OXTR to 750nM oxytocin (final concentrations were 5nM and 250nM respectively). Inotocin was dissolved in the buffer (20mM HEPES in HBSS) and oxytocin in the buffer containing 0.01% BSA (Sigma-Aldrich) to avoid the absorption of oxytocin to the tip.

Binding assay with TR-FRET

Using the Tag-lite® assay (cisbio), the fluorescent ligand was prepared with dilution in Red ligand assay buffer in 40nM concentration, and transient Tag-lite receptor-expressing cells were diluted five times in Tag-lite buffer. 10μl of labelled cells, 5μl of atosiban (1.95μM-2mM) and 5μl of fluorescent ligands were loaded and kept for 24 hours in the plate with light shielding at room temperature. Fluorescent levels were measured with the wavelength bands centered at 337nm (excitation), 665 nm and 620nm (emission) with PHERAstar FS (BMG LABOTECH). To exclude non-specific signals, fluorescence was counted from 60μs to 460μs after laser emission, then the sum value of photon counting during the 400μs was used as Intensity in the following formula:

$$\mathrm{Ratio} = \frac{\mathrm{Intensity}\ \ (665\mathrm{nm})}{\mathrm{Intensity}\ \ (620\mathrm{nm})} \times 10^4$$

and the FRET inhibition rate was given by

$$\mathrm{Inhibition\ rate}\ (\%) = \left(1 - \frac{\mathrm{Ratio_{Sample}} - \mathrm{Ratio_{min}}}{\mathrm{Ratio_{max}} - \mathrm{Ratio_{min}}}\right) \times 100.$$

Cuticular hydrocarbon analysis

The extraction and quantification of CHCs was performed as described previously (2) with some modifications. To extract the CHCs, ants were immersed in approximately 500μl of *n*-hexane for 3 min. 500ng of Tetracosane (Wako) was added as the internal standard. For hydrocarbon fractions, the crude extract was chromatographed on 0.5 g of silica gel (0.040–0.063 mm, Merck

Millipore). Hydrocarbons were eluted with 2 ml of *n*-hexane at 48 hours after the injection of each compound. The extracts and fractions were concentrated and re-dissolved in 50μl of *n*-hexane. GC analyses were carried out using a GC-17A interfaced to a QP-5000 (Shimadzu, JAPAN). The GC was fitted with a DB-5 column (30 m×0.25 mm×0.25 μm; J &W Scientific, USA), programmed from 80°C for 1 min, 30°C/min to 170°C, 5°C/min to 300°C and held for 5 min. Helium was used as a carrier gas and the column head pressure was 123.8 kPa. Injection and detector were at 300°C. Data were collected and calculated using CLASS-5000 software (Shimadzu, JAPAN). The MS interface temperature was 250°C. We used 3 major peaks of normal alkane and 7 major peaks of branched alkane for statistical analysis.

Survival analysis with antagonist feeding

Seven >5-mo-old workers were placed in an insect breeding dish (Bio Medical Science) containing a light-shielded nest box (3) and a feeding tray made with aluminum foil. To maintain a steady supply of comp. B or control DMSO, we laced a small amount (20 μl) of sugar water with either 1μM comp. B or 1% DMSO. This small amount of solution was provided once daily in the 'feeding tray' to provide sufficient doses of treatment and control, while minimizing the opportunity for the ants to obtain water. In all cases the solution was consumed by the ants. For the expression analysis for *CYP4G1*, after 10 days of antagonist feeding, RNA was extracted from the abdomen of each worker and used for qRT-PCR. During the survival tests, there was no supply of extra water or food. Rearing boxes were kept at 55%RH in the incubator or at 85%RH in a tupperware container with the humidity being controlled with a water chamber. All boxes were kept at 30°C under 12h light-12 h dark cycles. We analyzed the survival of ants with a cox mixed-effects model fit by maximum likelihood in R (R 3.4.4.) with two fixed factors (humidity and treatment) and two random factors (colony of origin and box id).

Statistical analysis

The expression levels of each gene were compared with two-way ANOVA using Prism 7 software (GraphPad, San Diego, CA, USA) in Fig. 1, S1, and Table S1. To analyze the expression of each gene, we fitted a GLMM using R (R 3.4.4), with two fixed factors (age and task allocation in Fig. 2C, 2D, age and tissue in Fig. 3L and 4A) or one fixed factor (treatment in Fig. 6A, 6E, and S7A) and one random factor (colony of origin) to account for variation between colonies. We first tested whether the data were normally-distributed with Shapiro tests and diagnostic qqPlots. When the residuals were not normally-distributed, we applied the log transformation, which normalized the residuals. The interaction between the age and foraging propensity is tested with pairwise Fisher test in Fig. 2B. To evaluate the effect of atosiban, comp. A, B, and C, non-linear regression analyses were performed to generate dose-response curves and calculate EC50 or IC50 values

with Prism 7 software in Fig. 5A-5C and 5H.

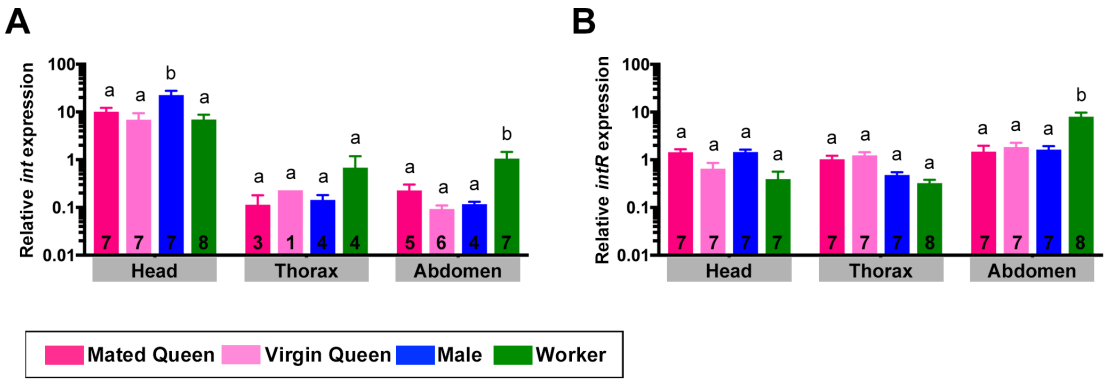

Fig. S1 Relative expression of *inotocin* and *inotocin receptor* in different castes and tissues of *C. fellah*

Relative expression (mean ± SEM) of *inotocin* (*int* in A) and *inotocin receptor* (*intR* in B) in the head, thorax and abdomen of mated queens (red), virgin queens (pink), males (blue) and workers (green). The expression of each gene was scaled by the average value in each graph and is therefore not comparable between the graphs. Number of individuals is shown in each box. The relative expression levels of each gene were tested with two-way ANOVA. Groups differing significantly (p<0.05) are marked with different letters.

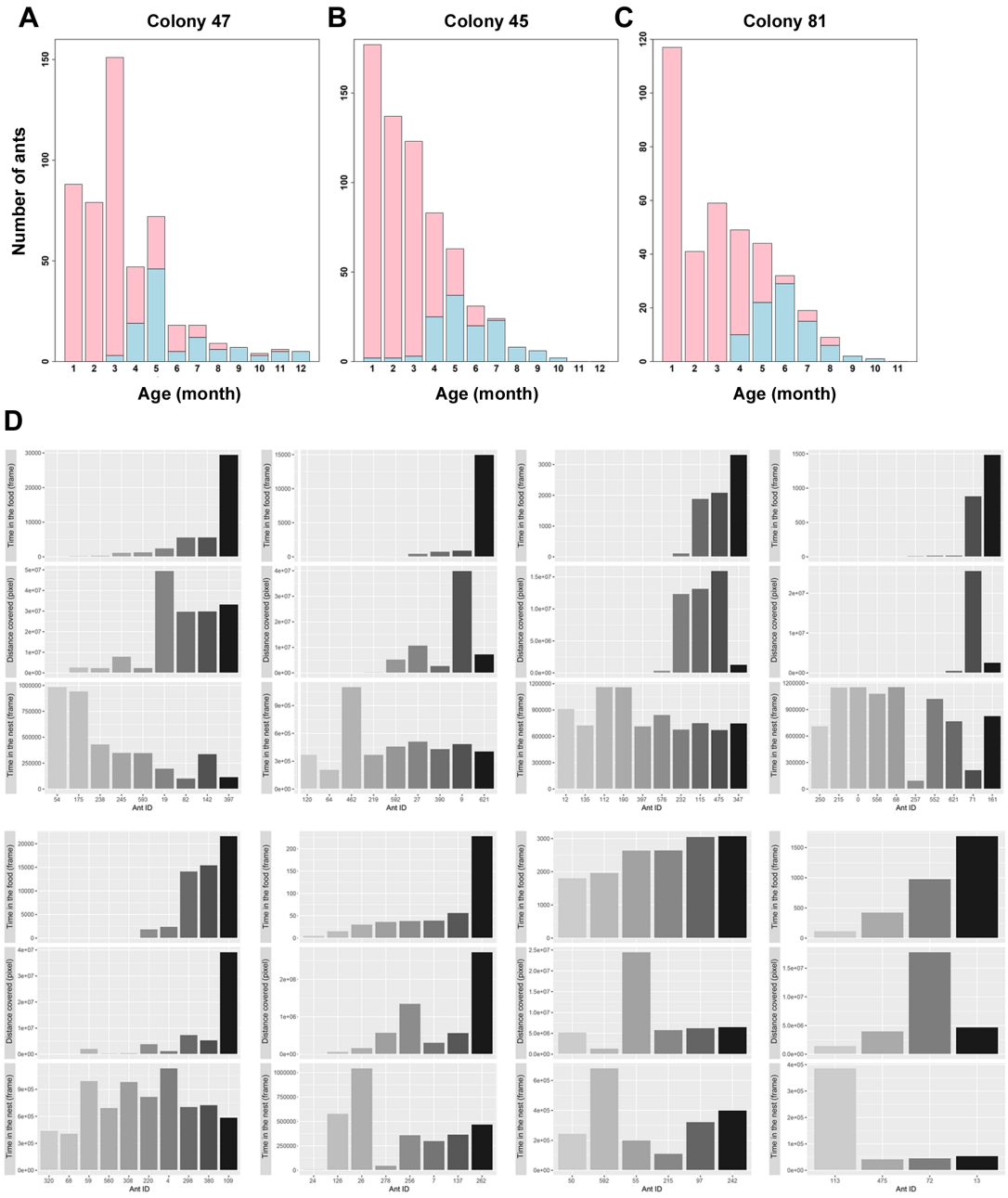

Fig. S2 Task allocation in the laboratory-reared colony and in the single-cohort treatment (A-C) Age-dependent division of labor in 3 independent colonies. Nurses are shown in pink and foragers in light blue. (D) Behavioral parameter for 10 workers in the 6-days of behavioral tracking in 8 independent boxes. Time spent in the food region, distance covered, and time spent in the nest are presented in each graph. Workers that died during the 6 days of behavioral tracking were not included.

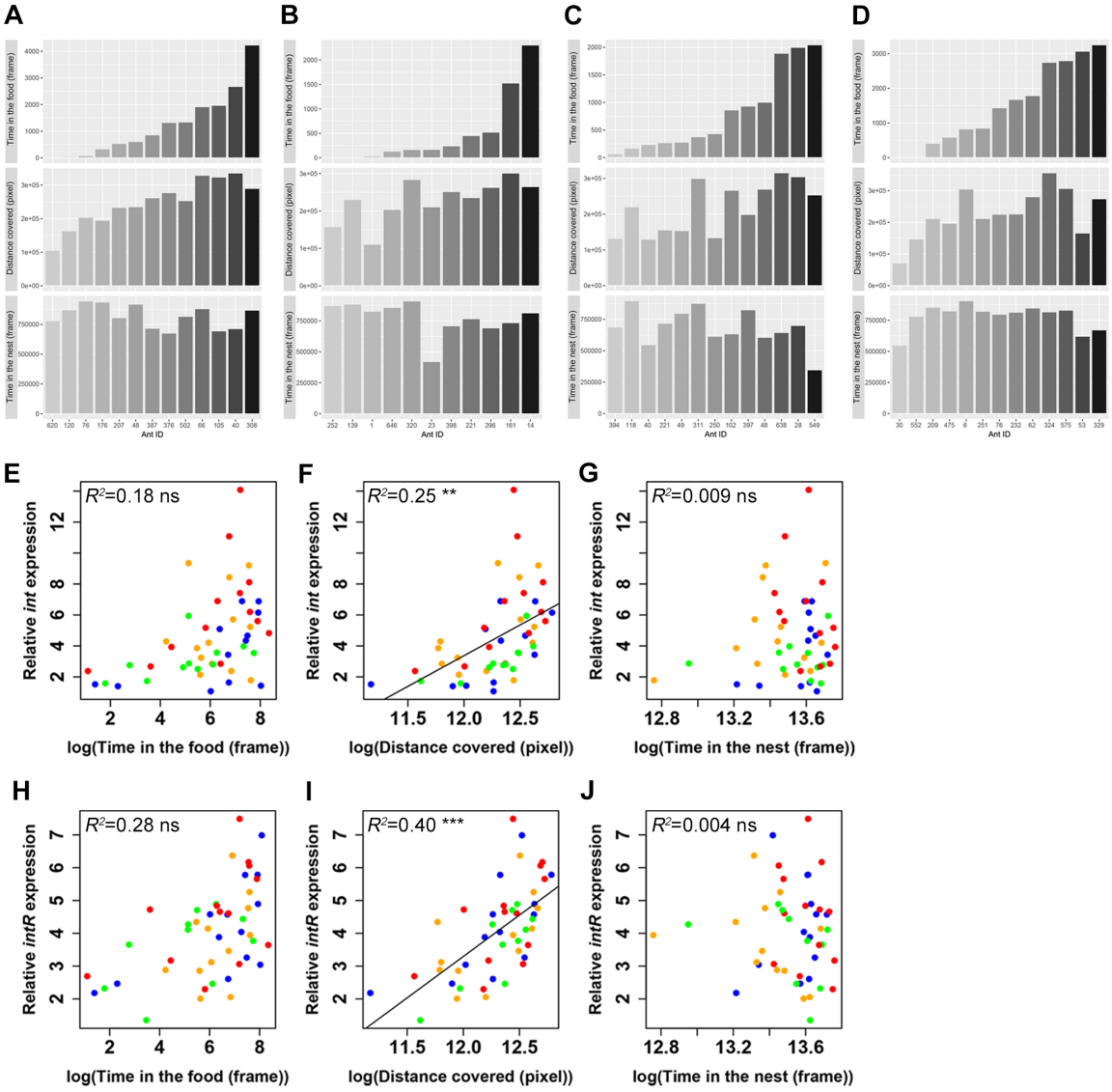

Fig. S3 The expressions of *inotocin* and *inotocin receptor* correlate with foraging behavioral parameters in 1- to 2-mo-old workers

(A-D) Time spent in the food region, distance ant covered, and time spent in the nest for 1- to 2-mo-old workers in the 6-days of behavioral tracking in 4 independent boxes. Workers that died during the 6 days of behavioral tracking were not included. (E-J) Relationship between the behavioral parameters and *int* (E-G) and *intR* expression (H-J). Different colors indicate the colony of origin of workers. R^2 and *p*-values are shown on the top left of graphs. The correlation between the expression of *int* or *intR* and behavioral parameters were tested with GLMMs. ns, $p>0.05$; **$p<0.01$; ***$p<0.001$.

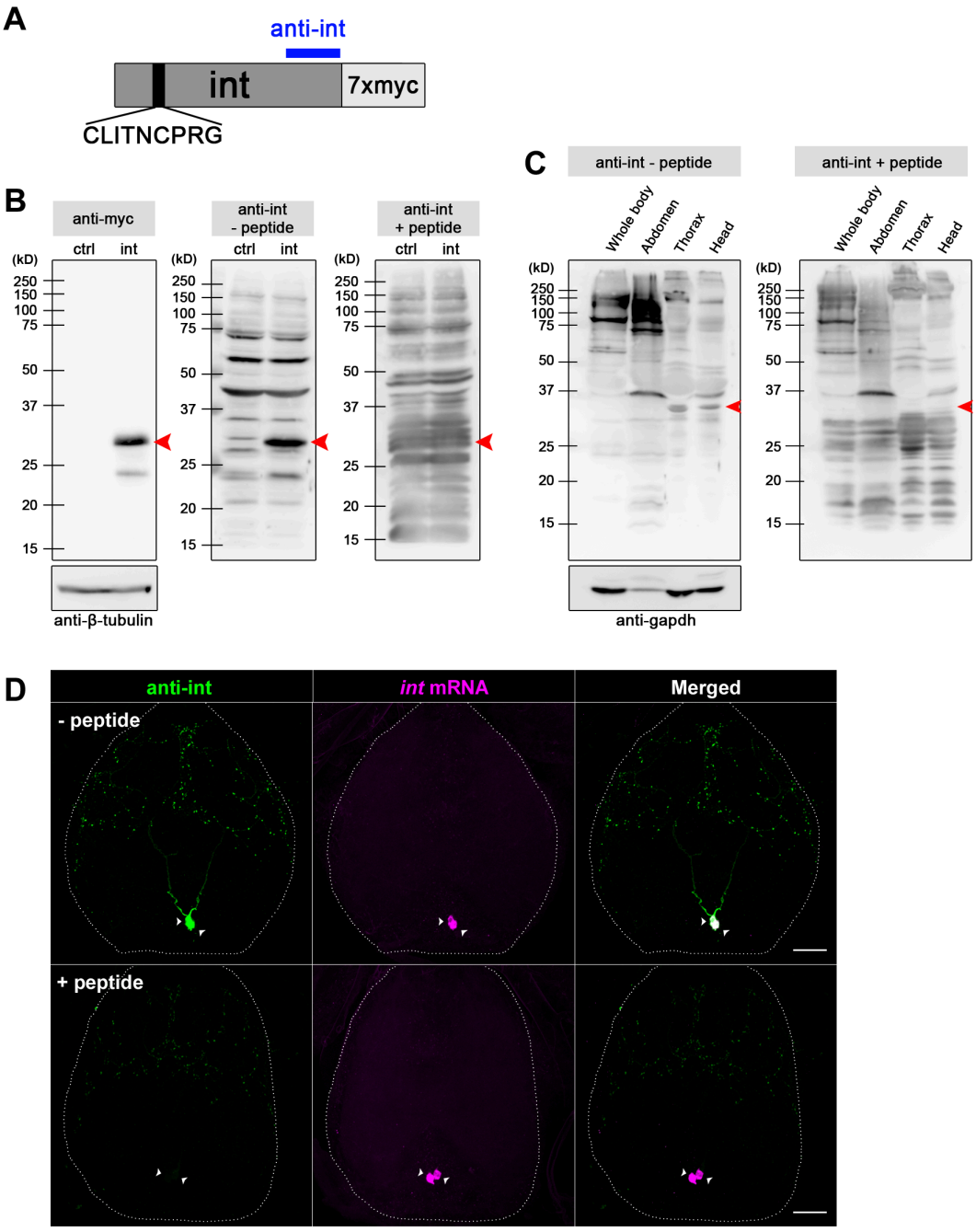

Fig. S4 Characterization of anti-inotocin antibody

(A) Structure of inotocin proprotein tagged with 7x myc in C-terminus. Anti-inotocin (anti-int) antibody was targeted to the C-teminus of inotocin proprotein. (B) Inotocin proprotein (arrowhead) with myc tag overexpressed in S2 cell was detected by anti-myc antibody (red arrowhead in left panel), anti-int antibody without peptide absorption (middle panel), but not with

anti-int antibody with peptide absorption (right panel). (C) In the lysates from head or thorax of workers, inotocin proprotein (indicated with red arrowhead) was detected with anti-int antibody (left panel), but not with anti-int antibody after the peptide absorption (right panel). (D) Sequential staining with fluorescence in situ hybridization using specific *int* antisense probe (magenta) and immunohistochemistry with anti-int antibody (green). One pair of neurons (indicated with arrowheads) in the subesophageal zone (outlined with white dotted line) was positive both with inotocin proprotein and *int* mRNA. Merged image is shown on the right panel. No signal was observed when using an anti-int antibody which is pre-absorbed with the antigen peptide as the negative control (bottom left panel). Scale bar, 50 μm.

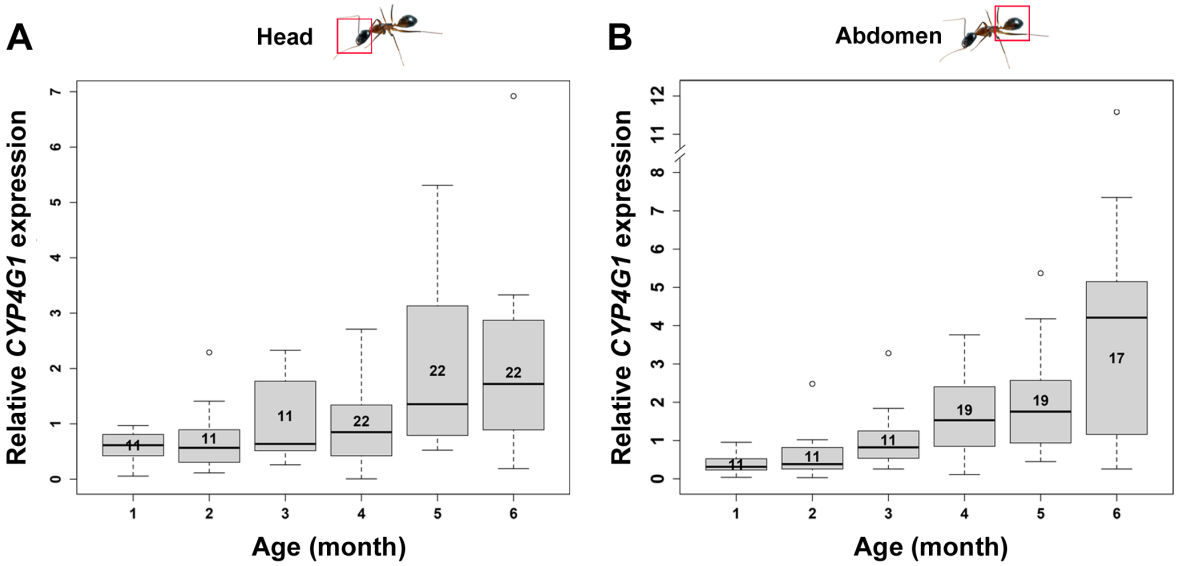

Fig. S5 Age-dependent up-regulation of *CYP4G1* in workers

Box-plots of the relative expression levels of *CYP4G1* in the head (A) and abdomen (B) for each age class. Number of individuals is shown in each box.

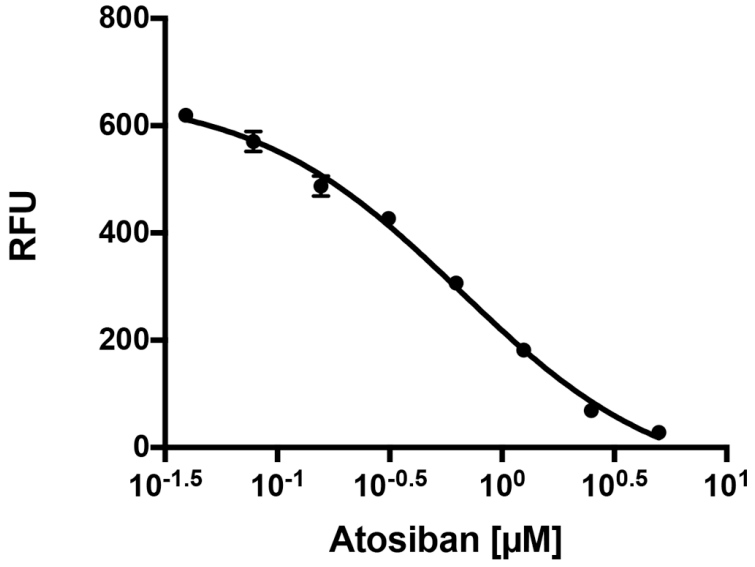

Fig. S6 Inhibitory effect of atosiban against oxytocin signaling

Relative fluorescence units (RFU) of Fluo-4 probe (mean ± SEM, n=3) in response to oxytocin in CHO cells expressing oxytocin receptor treated with different dose of atosiban (IC_{50}=0.65μM).

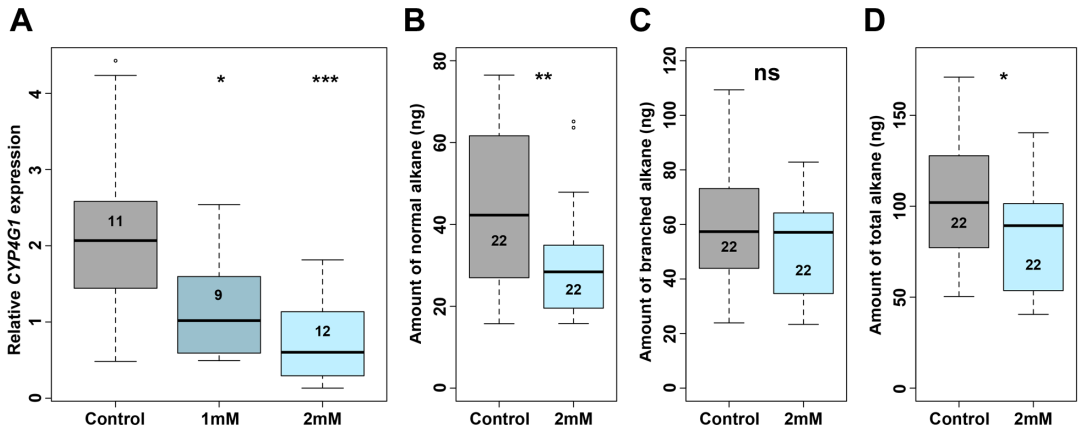

Fig. S7 Effects of Atosiban on *CYP4G1* expression and cuticular hydrocarbon production

(A) *CYP4G1* expression (qRT-PCR) for control (grey), 1mM (dark blue), and 2mM (light blue) atosiban-injected 4-mo-old workers. The data were tested with GLMM with Dunnet post hoc tests. (B-D) The quantities of normal (B), branched (C) and total alkanes (D) in control (grey) and 2mM atosiban (light blue) >5-mo-old injected workers. Number of individuals is shown in each box. The data were tested with GLMM. ns, $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$.

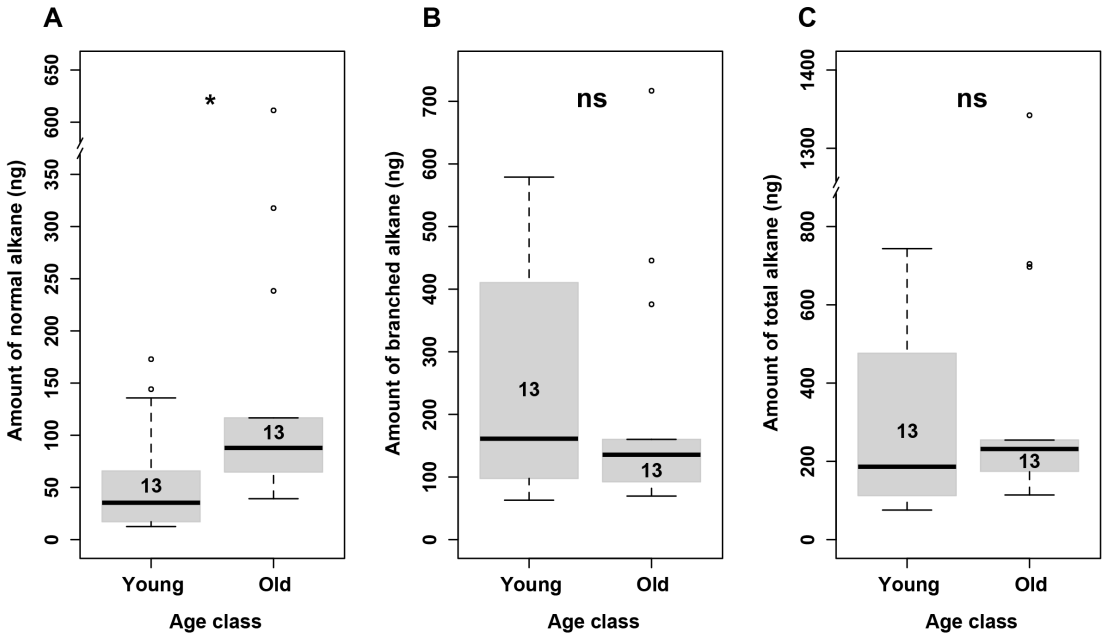

Fig. S8 Cuticular hydrocarbon profiles in workers

The quantities of normal (A), branched (B) or total alkanes (C) in 1- to 2-mo-old and 6-mo-old workers. The data were analyzed with GLMM. ns, $p>0.05$; *$p<0.05$.

Table S1. Summary statistics for the expression of *int* and *intR* in body parts (head, thorax and abdomen) in the different castes (mated queens, virgin queens, males and workers) of the *Camponotus* species studied

Species	year	gene	Source of variation	*DFn, DFd*	*F*	*P*	Figures
Camponotus japonicus	2016	*int*	body parts	2,49	47.3	<0.0001	1A
			castes	3,49	30.2	<0.0001	
			interaction	6,49	31.4	<0.0001	
		intR	body parts	2,53	10.96	0.0001	1B
			castes	3,53	18.3	<0.0001	
			interaction	6,53	12.24	<0.0001	
Camponotus japonicus	2015	*int*	body parts	2,18	84.21	<0.0001	1C
			castes	2,18	15.75	0.0001	
			interaction	4,18	6.114	0.0027	
		intR	body parts	2,18	8.359	0.0027	1D
			castes	2,18	11.54	0.0006	
			interaction	4,18	8.804	0.0004	
Camponotus fellah	2018	*int*	body parts	2,51	25.91	<0.0001	S1A
			castes	3,51	2.036	0.12	
			interaction	6,51	3.023	0.013	
		intR	body parts	2,74	19.41	<0.0001	S1B
			castes	3,74	5.543	0.0017	
			interaction	6,74	11.58	<0.0001	

Table S2. Summary statistics for the expression of *int* in the head and of *intR* in the abdomen in nurses and foragers in the same age class

age class	tissue	gene	Source of variation	*DFn, DFd*	*F*	*P*	Figure
4-month	head	*int*	task	1, 35.4	2.95	0.095	2C
5-month	head	*int*	task	1, 30.4	4.77	0.037	2C
6-month	head	*int*	task	1, 38.3	1.31	0.26	2C
4-month	abdomen	*intR*	task	1, 35.1	0.02	0.89	2D
5-month	abdomen	*intR*	task	1, 33.6	10.8	0.0024	2D
6-month	abdomen	*intR*	task	1, 30.6	5.3	0.028	2D

Table S3 Sequence of primers used in this study

qRTPCR

Gene name	Primer Type	Sequence (5' to 3')
ef1a	Forward	CCACCAGGCCGACTGATAAG
	Reverse	AGGTACTGTTCCAATACCACCAATC
rp2	Forward	TGGGTCATCGCGTCAAAGT
	Reverse	GCGATGTGCAGCTCAGGTT
gapdh	Forward	TCATGACGACTGTACATGCGATT
	Reverse	GCGCCATAACTTGCCAGAA
int	Forward	TTCATAGGAACACCTGAAACATACC
	Reverse	ATATCCAGCGATACAAGGCTTAATG
intR	Forward	GGATGTGAACTCTGGGCAAC
	Reverse	AAAGCTGTCAACGCTGTACG
CYP4G1	Forward	GCTGTAGCTCTTGAATATCACAAA
	Reverse	GGGCGCTATTGCCTTCCTAT

dsRNA synthesis

Gene name	Primer Type	Sequence (5' to 3')
intR	Forward	TAATACGACTCACTATAGGGATGTTTTACGACTCG
	Reverse	ATTATGCTGAGTGATATCCCCTCGTATGAGAATAT
EGFP	Forward	TAATACGACTCACTATAGGGGTGATGCAACATACG
	Reverse	ATTATGCTGAGTGATATCCCAAAGGGCAGATTGTG

References

1. Nishikawa M, Watanabe H, & Yokohari F (2012) Higher brain centers for social tasks in worker ants, *Camponotus japonicus*. *J Comp Neurol* 520(7):1584-1598.
2. Hojo MK, *et al*. (2009) Chemical disguise as particular caste of host ants in the ant inquiline parasite *Niphanda fusca* (Lepidoptera: Lycaenidae). *Proc Biol Sci* 276:551-558.
3. Koto A, Mersch D, Hollis B, & Keller L (2015) Social isolation causes mortality by disrupting energy homeostasis in ants. *Behav Ecol Sociobiol* 69(4):583-591.