Electronic supplementary material

MATERIAL AND METHODS

Protocol for electroantennogram (EAG) recordings in *Coenobita clypeatus*

EAG recordings were performed by mounting an excised lateral flagellum of an antennule between a reference and a recording electrode. Electrical contact was improved by the use of electrically conductive gel (Blågel, Cefar, Lund, Sweden). The recording electrodes were connected to a pre-amplification EAG-probe (×10) (Syntech, Hilversum, the Netherlands), in turn connected to an Intelligent Data Acquisition Controller (IDAC-4; Syntech). The digitally converted EAG signal was monitored, stored and analyzed on a PC using software EAGPro version 4.3 (Syntech). The antennular preparation was placed into the widened end (12 mm inner Ø) of a silanized glass tube (7 mm inner Ø) and throughout an experiment, a continuous flow of charcoal-filtered, humidified air (1.5 l min^{-1}) passed over the excised antennule. Compounds screened for EAG-activity were used at 10^{-1} dilutions (solid compounds at 100 mg ml^{-1}). Pure solvent served as control. Test stimulus solution (1 µl) was applied onto a strip of filter paper (ca. 5×20 mm; Sartorius Stedim Biotech GmbH, Göttingen, Germany), which was inserted into a glass Pasteur pipette. Test stimuli were presented by inserting the tip of the Pasteur pipette into a small hole in the glass tube with the continuous air stream (ca. 10 cm from the antennular preparation) and subsequently puffing the air volume of the Pasteur pipette (containing the volatiles of the test stimuli) for 0.5 s at 1.0 l min^{-1}, into the continuous air stream, using a stimulus controller (CS-55, Syntech). A compensatory air stream alternated with the stimulus puff to guarantee that the stimulus did not change the total air flow over the antennule.

An experimental protocol started with a puff from a clean, empty Pasteur pipette to test for mechanical responses, followed by a control (solvent alone) and a standard stimulus (isovaleric acid) that had shown to elicit repeatable responses for a prolonged period of time. Only preparations that responded ≥ 200 µV to the standard stimulus were used for further testing. Test stimuli were thereafter presented in randomized order at ascending concentrations. An interstimulus interval of at least 30 s was used for all stimuli. The presentations of the control and the standard stimulus were applied regularly during the recording sessions to check the reliability of the preparation. Fresh stimuli were prepared for each recording session and the wide end of the Pasteur pipettes were capped with sealed 1 ml pipette tips until use to reduce evaporation of the test compounds. Carbon dioxide stimulation was performed by extracting headspace over dry ice with a syringe and injecting this headspace into the constant airstream. Here, air of the same volume served as control. EAG experiments were performed at room temperature from 20-25°C. For analysis, control values were subtracted from the test stimuli values. Only one antennule per crab was used and each stimulus/concentration was tested only once on each antennule.

EAG recordings at high and low humidity levels

Odorants were tested for EAG activity at high and low humidity levels in *C. clypeatus* by switching on and off the humidifying step of the constant air stream between recording sessions. By increasing the humidified, continuous air stream to 3.5 l min^{-1}, while decreasing the stimuli/compensatory air flow to 0.30-0.40 l min^{-1}, and at the same time heating the water within the gas-washing bottle to 30°C (range 29.4-30.5°C), whereafter the air stream was cooled down through the tubing before it reached the antennular preparation, it was possible to obtain 86-91% relative humidity (RH) without changing the temperature of the air stream. At the lower humidity level, the humidifying step was omitted, thus leaving the air stream at room RH levels (32-35% RH). Humidity and temperature of the continuous air stream were measured at the outlet of the air stream with a humidity/temperature data logger (EL-USB-2, Lascar).

Isovaleric acid, propionic acid, triethylamine and propylamine were tested at dilutions from 10^{-4} to 10^{-1} in a recording session at the higher humidity (initial high humidity recordings), whereafter the humidifying step was removed and the humidity level was allowed to stabilize at the lower level for ca. 20 min, and the recording session was repeated (low humidity recordings). Thereafter the continuous air stream was reconnected to the humidifying step and allowed to stabilize for another 20 min, after which a final recording session was performed (final high humidity recordings) to make sure the antennular preparation was still active. These humidity tests were run on five antennular preparations (n = 5).

EAG recordings in *Drosophila melanogaster*

A 4-6 days old, mated female fly, was immobilized in a pipette tip, with only the head partially protruding. A glass microelectrode filled with standard insect saline solution was placed on the third antennal segment of the fixed fly. The reference electrode filled with the same saline solution was inserted into the fly's eye. The fly was positioned 1 cm from the outlet of the glass tube with the continuous air stream. Propionic acid, triethylamine and isoamyl acetate were presented at 10^{-1} dilution in a recording session (as described above), recording from totally six flies (n = 6). The EAG signal (transferred via Ag-AgCl wires) was pre-amplified (×10) with a probe connected to a high-impedance DC-amplifier (EAG-probe, Syntech) and digitally converted, monitored and recorded as described for *C. clypeatus.*

Bioassay with *C. clypeatus*

Twenty plastic boxes (52×43×28 cm) were used as test arenas, each containing a centrally placed shelter (half a ceramic flowerpot, 6.5 or 8.5 cm inner Ø, depending on crab size) and one pit-fall on each short side of the arena. A pit-fall consisted of a plastic cup (12 cm height, 40 cl volume) placed underneath a 4.5 or 5.5 cm Ø hole, depending on crab size. A crab that had fallen into a pit-fall could not exit. To avoid positional effects, the position of the treatment and control pit-falls were alternated between the 20 arenas run at the same time. The bioassays were conducted under controlled temperature (24-26˚C day, 20-22˚C night temperature) and humidity

(60-70% RH). Crabs were tested only once for each test odorant and individual crabs were tested at the most once per week. Between experiments, crabs were kept in their rearing terraria.

Water and food odour sources tested in the bioassay: Water vapour from distilled water (10 ml), ASW (Instant Ocean, Aquarium Systems, Sarrebourg, France; 32‰; 10 ml), banana (ripe, but not overripe: 10g), overripe banana (10 g), biodynamically farmed salmon (raw filet with skin, 10g) and ground maize-peanut snacks “peanut snacks” (Clarkey’s; ground maize 60%, ground peanuts 32%, sunflower oil, sea salt 1.8%, natural pepper aroma; 2 g), ground peanut snacks (2 g) in combination with distilled water (10 ml) using distilled water (10 ml) as control. Both cups in a setup were always covered with perforated lids to avoid visual stimuli, while allowing odour stimuli.

Headspace banana volatile collection: Volatiles were collected from 100 g banana added to each of six 250 ml E-flasks. Air was sucked through the flasks at 300 ml min^{-1}, using Teflon tubing for connection. Incoming air was filtered with an activated charcoal filter and volatiles in the out flow were collected in glass columns with Super-Q filters (volatile collection trap*s,* 25 mg, Alltech, Deerfield, USA). Odour collection was made for 4 h before changing all filter columns and thereafter for another 4 h. Immediately after collection, the trapped volatiles in the filters were rinsed with dichloromethane (200 µl) into cone shaped 2.5 ml glass vials and concentrated to ca. 20-30 µl by gently flushing the samples with nitrogen. The banana extract from 12 filters were pooled into one batch (ca. 300 µl), which was stored in two glass micro inserts (250 µl) at -18˚C until use in the assay. Just before use in the bioassay, 55 µl banana extract was dissolved in 2100 µl mineral oil to a concentration analogous to ca. 10g banana per 100 µl mineral oil, constituting the banana extract test solution.

RESULTS

Physiologically inactive compounds

Compounds scored as inactive in the EAG screening, i.e. elicited activity in less than 2 out of 5 preparations, or did not elicit an average response > 30 µV or < -30 µV ($n \geq 5$). Compounds marked with (*) were tested also at the higher humidity level.

Acids: octanoic acid*, decanoic acid, DL-lactic acid, L(+)-lactic acid, benzoic acid*, 3-indoleacetic acid, 3-indolebutyric acid, hydrochloric acid*.

Aldehydes: hexanal*, cis-3-hexenal, nonanal.

Amines: indole*, skatole*.

Alcohols: 1-propanol*, 2-propanol, 1-butanol*, 1-pentanol, isoamyl alcohol, 1-hexanol*, cis-3-hexen-1-ol, trans-3-hexen-1-ol, octanol*, 1-octen-3-ol*.

Esters: methyl acetate, ethyl formate, ethyl acetate, methyl propionate, ethyl propionate, methyl butyrate, butyl acetate, isobutyl acetate, n-amyl acetate, isoamyl acetate*, cis-3-hexenyl acetate, butyl butyrate, ethyl hexanoate*.

Lactones: β-propiolactone, γ-butyrolactone, γ-hexalactone, γ-octalactone, γ-nonalactone*, δ-decalactone, γ-decalactone.

Ketones: acetone, 2-butanone*, 3-methyl-2-butanone, pentanone, acetyl acetone, hexanone*, 2-heptanone*, 3-heptanone, cyclohexanone.

Aromatics: benzene*, toluene*, m-xylene, phenol*, p-cresol, 3-ethyl phenol, phenylacetaldehyde*, benzyl alcohol*, 2-phenyl ethanol, benzyl acetate*, phenylethyl acetate, methyl salicylate, anisole*.

Cyclic terpenes: menthol, I-menthone, cis-jasmone*, methyl jasmonate, d-limonene*, γ-pinene*.

Ethers: diethylether , butyl methyl ether, 1,4-dioxane.

Furans: tetrahydrofuran, furfural*.

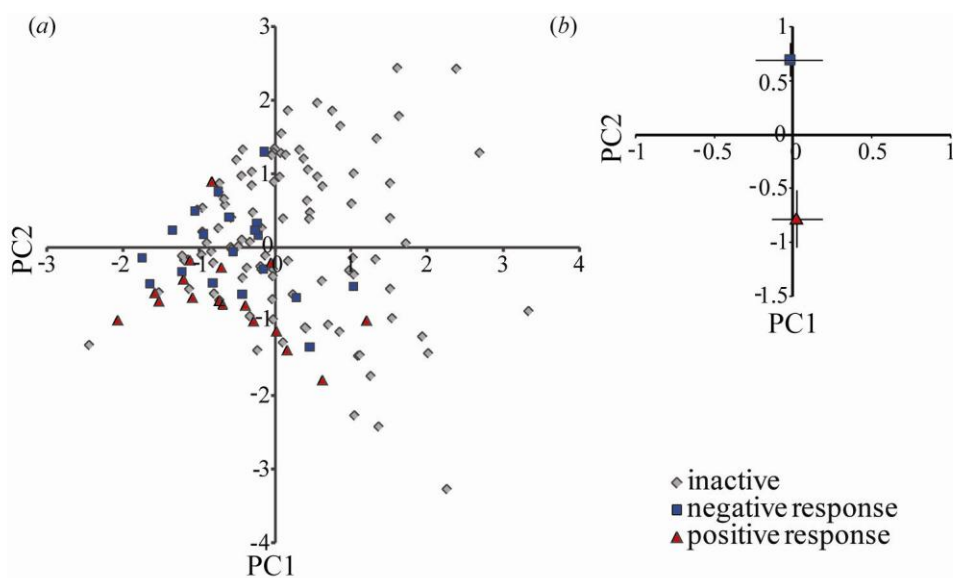
Sulphur compounds: dimethyl sulfide, dimethyl disulfide* , dimethyl trisulfide*.

Thiocyanates: mustard oil*.

Amino acids: glycine, alanine, 2-aminobutyric acid, serine, proline, taurine, leucine, isoleucine, L-asparagine, aspartic acid, glutamine, L-glutamic acid, methionine, histidine, cysteine, phenylalanine, arginine, tyrosine, tryptophan, L-lysine.

Influence of an odorant's physicochemical properties on the EAG responses provoked by this odorant

About half of the EAG-active odorants elicited positive EAG responses, while the rest elicited negative responses. When performing a principal component analysis (figure S1) we found that whether an odorant elicits a positive or negative response depends on its physicochemical properties.

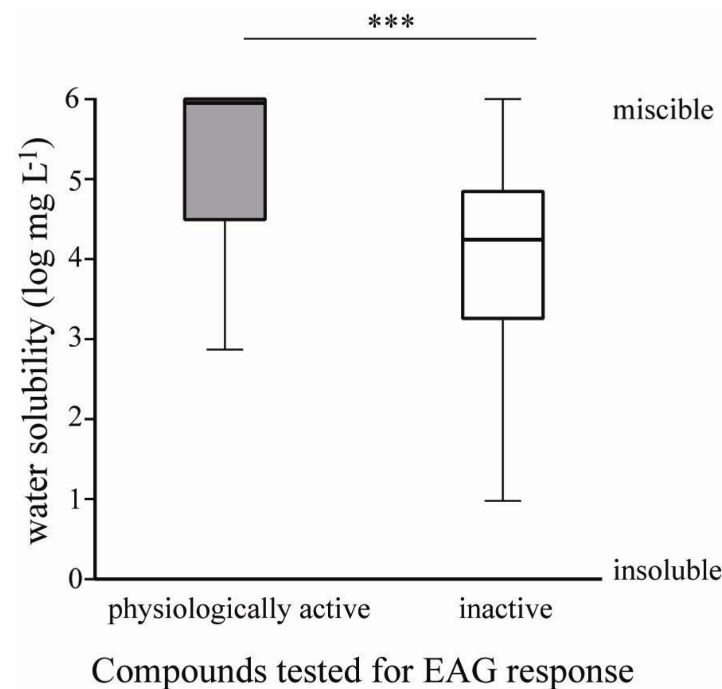


Supplementary Figure S1. Mapping of compounds screened for EAG activity in *Coenobita clypeatus*, into a physicochemical odour space based on Euclidean

distances of chemical descriptors obtained from DRAGON (descriptors normalized). (*a*) Principal component (PC) scatter plot with representation of all screened compounds divided into three classes, i.e. physiologically inactive compounds (grey diamond) and compounds generating negative (blue square) and positive (red triangle) EAG responses. Shown here are the first two principal components of the multi-dimensional physicochemical space (PC1 explains 35.4% and PC2 10.8% of the total variance). (*b*) Compounds eliciting negative and positive EAG responses are separated in their odour space representation (PC1 explains 41.4% and PC2 14.1% of the total variance; error bars represent SEM, n = 19 and 17 for compounds eliciting negative and positive EAG responses, respectively).

Influence of an odorant's water solubility on the EAG responses provoked by this odorant

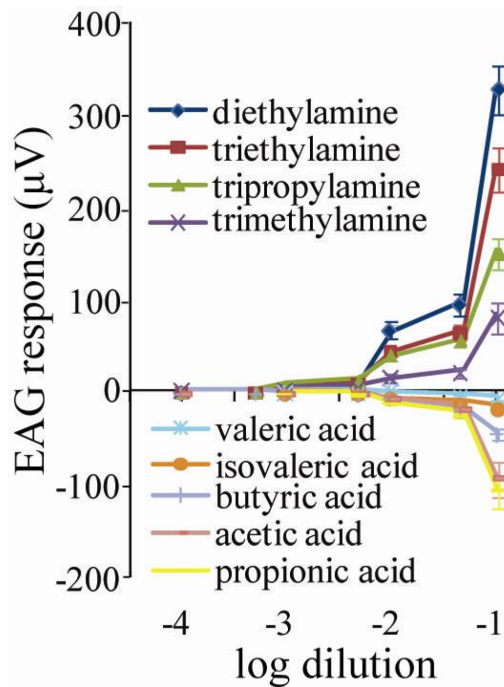
We found that in *Coenobita clypeatus* water solubility of an odorant was necessary but not sufficient to induce EAG activity (figure S2).



Supplementary Figure S2. Water solubility of compounds screened for EAG response in *Coenobita clypeatus*, illustrating that mainly water-soluble compounds are physiologically active, while inactive compounds can be of any solubility. Horizontal lines within boxes represent the median, boxes represent the lower and upper quartiles, and whiskers represent the minimum and maximum values (Mann-Whitney U-test, *** $p < 0.001$, n = 35 and 91 for the active and inactive compounds, respectively).

EAG responses of *Coenobita compressus* to amines and acids are dose-dependent

For EAG active component tested in *Coenobita compressus* we found strict relationship between stimulus intensity (i.e. odorant concentration) and strength of the EAG response (Figure S3).



Supplementary Figure S3. Dose-dependent EAG responses to selected odorants in the land hermit crab *Coenobita compressus*. Odorants (1 µl) were presented to the antennule in ascending steps (error bars represent SEM; n = 10-14).