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Influence of blood meal on the responsiveness of olfactory receptor neurons in antennal sensilla trichodea of the yellow fever mosquito, *Aedes aegypti*

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Abstract

In female *Aedes aegypti* L. mosquitoes, a blood meal induces physiological and behavioral changes. Previous studies have shown that olfactory receptor neurons (ORNs) housed in grooved peg sensilla on the antennae of *Ae. aegypti* down-regulate their sensitivity to lactic acid, a key component driving host-seeking behavior, which correlates with observed changes in the host-seeking behavior of this species. In the present study, we performed electrophysiological recordings from the most abundant antennal sensillum type, *sensilla trichodea.* Our results indicate that the response spectra of ORNs contained within most trichoid sensilla do not change in response to blood feeding. However, we observe an increase in sensitivity to primarily indole and phenolic compounds in neurons housed within four of the five functional types of short blunt tipped II trichoid sensilla, both at 24 h and 72 h post blood feeding, which was more pronounced at 24 h than 72 h. Furthermore, sensitivity to undecanone, acetic acid and propionic acid was observed to increase 72 h post blood meal. Considering the timing of these changes, we believe that these neurons may be involved in driving the orientation behavior of female mosquitoes to oviposition sites, which are known to release these compounds.

Keywords

Olfaction, Oviposition, Gonotrophic cycle, Disease vector, Modulation

1. Introduction

For female mosquitoes, olfaction is the primary sensory modality used during the identification of energy-rich resources, like nectar and blood, as well as appropriate oviposition sites (Takken, 1991; Zwiebel and Takken, 2004). The behavioral response of female mosquitoes to host and oviposition cues generally changes sequentially in accordance with their physiological condition and behavioral status (Klowden, 1994). Young female mosquitoes have a preference for sugar-rich resources, and only develop competence to take blood meals 24-to-72 h after adult emergence (Klowden, 1990). Females that have taken a full blood meal, and thus commenced vitellogenesis, are generally inactive throughout the period of ovarian development; flight activity is reduced (Jones, 1981) and they do not respond behaviorally to host odors (Klowden and Lea, 1978, 1979a, 1979b; Takken, et al., 2001). In the yellow fever mosquito, *Aedes aegypti*, and the African malaria mosquito, *Anopheles gambiae,* the behavioral unresponsiveness is correlated with a change in the physiological response of olfactory receptor neurons (ORNs) tuned to host volatiles, such as geranylacetone and short chain carboxylic acids (Davis, 1984; Qiu et al., 2006). Furthermore, in *An. gambiae* a change in odorant receptor gene expression is observed 12 h post blood feeding, which correlates with the observed changes in behavior and physiology (Fox et al., 2001). Female mosquitoes thereafter display a pre-oviposition behavior, usually 48-to-72 h post blood meal, in which they are behaviorally attracted to potential oviposition sites and the associated olfactory cues (Klowden and Blackmer, 1987). The development of this behavioral competence also seems to be controlled through changes in sensitivity of ORNs tuned to these cues. In *An. gambiae,* ORN sensitivity to putative oviposition site cues, including indoles, phenols, ketones and carboxylic

acids, is increased 12 h post blood meal (Qiu et al., 2006). After oviposition, female mosquitoes gradually recover their behavioral and physiological response to host cues and are no longer behaviorally attracted to oviposition cues (Klowden, 1989).

The mosquito *Ae. aegypti* is the primary vector of dengue, yellow fever and other arboviruses in the world (Gubler, 2004; Gubler, 1988; Monath, 1988). Previous physiological analysis of the grooved peg sensilla on the antenna of this species has revealed that ORNs housed in these sensilla down-regulate their sensitivity to lactic acid, a key host attractant, post blood meal (Davis, 1984). Although it has been proposed that there is a direct causal relationship between this down-regulation of sensitivity and the observed behavioral change, i.e. down-regulation of the host-seeking behavior and up-regulation of the oviposition orientation behavior (Bowen, 1990; Bowen et al., 1988; Davis, 1984), it has been deemed untenable by other researchers (Bowen et al., 1994a, b). It is thus plausible that the physiological condition of *Ae. aegypti,* induced by blood feeding, triggers additional changes, such as further modulation of the sensory systems, that affect its behavior. In the present study, we investigate the response characteristics of *sensilla trichodea,* the most abundant antennal olfactory sensillum type of this species. Physiological recordings, 24 h and 72 h post blood meal, indicate significant changes in the response spectra and sensitivity of ORNs housed in four functional classes of *sensilla trichodea*, compared to non-blood fed female mosquitoes of the same age. Considering the timing of these changes we speculate that these neurons are involved in driving the orientation behavior of female mosquitoes to oviposition sites, which are known to release indoles, ketones and phenolic compounds.

2. Materials and Methods

2.1. Mosquitoes

A colony of *Aedes aegypti* (Rockefeller strain) was reared from larvae to adults at 27°C, 70-80% relative humidity and at a 12 h:12 h light:dark photoperiod. Mixed populations of male and female mosquitoes were maintained as previously described (Siju et al., 2008). Four-to-five day old female mosquitoes were offered a human blood meal, and allowed to feed until fully gorged; mosquitoes that had not taken a full blood meal were removed from the cage and not included in further analyses. The blood fed mosquitoes were used for physiological analyses 24 h and 72 h post blood meal. For controls, we used non-blood fed mosquitoes of the same age. All mosquitoes had *ad libitum* access to 10% sucrose throughout the experiments.

2.2. Preparation and single sensillum recordings

Preparation of animals and single sensillum recordings (SSR) were performed according to Ghaninia et al. (2007). Analysis of responses to stimulation were made manually using Autospike™ (Syntech, Germany). Spikes generated by the ORNs, innervating single sensilla, were differentiated by shape and amplitude, as either A or B, where A designates the ORN with higher spike amplitude and B the ORN with the smaller spike amplitude; for further details see Ghaninia et al. (2007). Response to stimulation was calculated by counting the number of A and B spikes 0.5 s post-stimulus and subtracting the number of these spikes during the 0.5 s pre-stimulus period; the frequency of spikes was then converted to spike/s.

2.3. Odor stimuli and delivery

Fourteen odorant compounds were used in the present study. The odorants used were selected because they have previously been described as host-volatile cues or associated with oviposition sites of different mosquito species, including carboxylic acids, ketones, phenolics, heterocyclics, bicyclic monoterpenes and alcohols (Bentley and Day, 1989; Cork, 1996; Miller et al., 1992; Qiu et al., 2006). All compounds were prepared in paraffin oil at the following dilutions: acetic acid (1:10 volume by volume), propionic acid (1:10), hexanoic acid (1:10), octanoic acid (1:10), thujone (1:10), phenol (1:100), 3-methylphenol (1:10), 4-methylphenol (1:100), 4-ethylphenol (1:100), indole (1:100), 3-methylindole (1:10), 2-undecanone (1:10), 2-butoxyethanol (1:10) and 4-methylcyclohexanol (1:10). In order to assess sensitivity changes in physiologically identified ORNs, we made serial dilutions of indole and phenol in paraffin (1:10 to 1:100000). All chemicals with the highest purity grade available were purchased from either Sigma-Aldrich or Fluka (Sweden). Aliquots of 10 µl of all compounds were transferred onto a filter paper (5 x 20 mm) and placed inside a Pasteur pipette; for control, 10 µl of paraffin was used. For odor stimulation, the tip of the Pasteur pipette was inserted into a charcoal-filtered and humidified air stream (1.5 l/min), which passed through a glass tube that terminated 10 mm from the antenna. By using a stimulus controller (Syntech, Germany), a 0.5 s (0.5 l/min) air-pulse was passed through the Pasteur pipette into the air stream. The impact of the stimulus pulse was reduced by a compensatory airflow.

2.4. Statistical analysis

To determine and verify the functional types of trichoid sensilla, previously identified by

Ghaninia et al. (2007), we performed a complete linkage cluster analysis, with squared Euclidean distances, to generate a dendrogram (Minitab Release 14.12. 0, Minitab Inc., State College, PA), which is representative of the relationships between each ORN response profile to all, or a subset, of the stimuli in multidimensional space. ORNs that cluster together are considered to be a functional class.

Analysis of variation (ANOVA) for repeated measures was used to test for differences in ORN responses to odors between the two feeding states as well as for the dose response. *Post hoc* comparisons were made with Bonferroni's method using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

The *sensilla trichodea* of *Ae. aegypti* are divided into four subtypes based on their external morphology: long sharp-tipped (lst), short sharp-tipped (sst), short blunt-tipped type I (sbtI), and short blunt-tipped type II (sbtII) (McIver, 1978, 1982; Ghaninia et al., 2007). Based on their response to a diagnostic set of odorants, these sensilla may be further subdivided into 11 distinct functional sensillum classes, each of which house two ORN types (Ghaninia et al., 2007, 2008).

A total of 239 successful recordings were made from trichoid sensilla in both non-blood fed and blood fed mosquitoes. Cluster analysis (data not shown) of ORN responses, to the panel of 14 odorants tested in the present study, in non-blood fed mosquitoes allowed us to identify and match all but one, sst2, of the functional sensillum types described by Ghaninia et al. (2007). Moreover, we were able to identify two novel func-

tional types of sensilla, sbtI3 and sbtII5. Cluster analysis of ORN responses in blood fed mosquitoes identified the same number of functional sensillum types.

3.1. Long and short sharp-tipped sensilla trichodea

None of the tested compounds were able to elicit any response in ORNs of the lst sensilla of neither non-blood fed nor blood fed mosquitoes (Fig. 1: lst1). Comparisons among the ORNs housed in sst trichoid sensilla at 24 h and 72 h post-blood meal also did not reveal any changes in sensitivity or specificity compared to non-blood fed mosquitoes of the same age (Fig. 1: sst1, sst3, sst4).

3.2. Short blunt-tipped type I sensilla trichodea

Single sensillum recordings from sbtI sensilla revealed three functional types, each with a unique response spectrum to the tested odorant panel. Two of these were matched to the sbtI1 and sbtI2 (Fig. 1: sbtI2) functional types described by Ghaninia et al. (2007); however, we only obtained two recordings from sbtI1 sensilla, one from non-blood fed mosquitoes at 24 h and one from blood fed mosquitoes 72 h post blood meal, and omit these from further analysis. The third, newly described, functional type, sbtI3 responded to stimulation with 2-butoxyethanol and 4-methylcyclohexanol; the B cell displayed a similar response spectrum but was less excited by these compounds (Fig. 1: sbtI3). There were no significant differences in response spectra or sensitivity of ORNs housed in the sbtI2 and sbtI3 sensilla at 24 h and 72 h post blood meal compared to non-blood fed mosquitoes (Fig. 1: sbtI2, sbtI3).

3.3. Short blunt-tipped type II sensilla trichodea

Olfactory receptor neurons housed in sbtII sensilla of blood fed mosquitoes generally exhibited an increased sensitivity to the tested odorants compared to ORNs housed in sbtII sensilla of non-blood fed mosquitoes (Figs. 2, 3). These differences were generally more accentuated in female mosquitoes 24 h post-blood meal compared to the same cohort 72 h post-blood meal (e.g. Figs. 2, 3: sbtII5A). Due to the inherent difficulties in matching the sbtII functional type sensilla of non-blood fed and blood fed mosquitoes we did not rely on cluster analysis to functionally group these sensilla in the present study but classified the types based on the response spectra of individual ORNs, which displayed little or no change in response to blood feeding. i.e. sbtII1A, sbtII2AB, sbtII4B and sbtII5B (ANOVA, $P>0.05$).

The A neuron of the sbtII1 sensilla displayed no significant changes in response spectra or sensitivity to any of the tested odorants in response to blood feeding (Fig. 2: sbtII1). In non-blood fed mosquitoes, the sbtII1B neuron showed a low response to a few of the tested odorants. However, at 24 h post blood meal, the sensitivity of the sbtII1B neuron to indole was significantly (ANOVA, $P<0.001$) higher compared to the non-blood fed cohort, a response that was maintained 72 h after blood feeding (ANOVA, $P<0.001$) (Fig. 3: sbtII1). Dose response relationships showed a significant increase in response to indole, but not to phenol, 24 h post blood meal (Fig. 4). In the second functional type, sbtII2, we did not observe any significant changes in response characteristics at 24 h and 72 h post-blood meal (Figs. 2, 3: sbtII2).

The ORNs of the third functional type, sbtII3, displayed a significantly increased sensitivity to some of the odorants tested. This change was most obvious for the sbtII3A

neuron, in which we observed an increased sensitivity 24 h post blood meal to especially indole (ANOVA, $P<0.001$), but also to phenol (ANOVA, $P<0.01$), 3-methylphenol (ANOVA, $P<0.05$), 4-ethylphenol (ANOVA, $P<0.05$) and 2-butoxyethanol (ANOVA, $P<0.05$) (Fig. 2: sbtII3). This increased sensitivity to indole and two of the phenols was maintained 72 h post blood meal (Fig. 3: sbtII3). In addition, an increased and decreased sensitivity to 2-undecanone was observed in sbtII3A (ANOVA, $P<0.001$) and sbtII3B (ANOVA, $P<0.05$), respectively, 72 h post blood meal (Fig. 3: sbtII3). We also observed an increased sensitivity in the sbtII3B neuron to indole (ANOVA, $P<0.05$) 24 h post blood meal but not after 72 h (Figs. 2, 3: sbtII3).

In the sbtII4 sensilla, the A neuron significantly increased its sensitivity to 4-ethylphenol (ANOVA, $P<0.05$) 24 h post blood meal, while at 72 h post blood meal the sensitivity was not significantly different from that observed in non-blood fed mosquitoes of the same age. However, we observed an increased sensitivity to acetic acid (ANOVA, $P<0.001$) and 3-methylphenol (ANOVA, $P<0.05$) in the sbtII4A neuron 72 h post blood meal. There were no significant changes in sensitivity in the sbtII4B to any compounds tested 24 h or 72 h after blood meal.

The A neuron of the novel functional type, sbtII5, displayed a significantly increased sensitivity to indole (ANOVA, $P<0.001$), 4-methylphenol (ANOVA, $P<0.01$) and phenol (ANOVA, $P<0.01$) 24 h post blood meal (Fig. 2). At 72 h post blood meal, the high sensitivity to indole (ANOVA, $P<0.001$) was maintained but the response to the phenols had returned to the level observed in non-blood fed mosquitoes. In addition, at 72 h post blood meal, we observed a significant increase in sensitivity of the sbtII5A neuron to acetic acid and propionic acid (ANOVA, $P<0.05$) (Fig. 3).

4. Discussion

In the present study we describe the effect of blood feeding on the sensitivity of ORNs contained within *sensilla trichodea* on the antenna of female *Ae. aegypti*. We assessed the sensitivity of the ORNs during two physiological states of female mosquitoes, i.e. non-blood fed and blood fed, 24 h and 72 h post blood meal. Previous experiments have shown that the host-seeking behavior of female *Ae. aegypti* mosquitoes is inhibited by a single blood meal, caused initially by distension of the abdomen following blood ingestion (Klowden and Lea, 1978; Klowden and Lea, 1979a) and later by factors released by the developing ovaries (Klowden and Lea, 1979b; Davis, 1984). The behavioral inhibition due to oocyte development is initiated 24-to-30 h post blood meal and reaches a maximum 36-to-72 h post blood meal (Klowden and Lea, 1979b). The female mosquitoes then display a pre-oviposition behavior, usually 48-to-72 h post blood meal, in which they are behaviorally attracted to potential oviposition sites and the associated olfactory cues (Klowden and Blackmer, 1987). Our results clearly show that the sensitivity of distinct functional classes of ORNs in sbtII trichoid sensilla, to cues released by potential oviposition sites, change in response to blood feeding more or less coinciding with the observed onset of the behavioral preference for oviposition sites. The response of ORNs of other sensillum types seems, however, to be static in their response.

Olfactory receptor neurons housed in lst, sst and sbtI sensilla did not change their sensitivity or specificity following a blood meal at either time period (24 h and 72 h) compared to non-blood fed mosquitoes. These results indicate that these sensillum types do not play a role in the olfactory-related physiological changes occurring in the mosquito after blood feeding. Indeed, it is perhaps not surprising to find ORN types in which

volatile sensitivity and selectivity do not change in response to a blood meal. These blood meal insensitive sensory neurons provide a consistent baseline between two physiological states that will allow the primary olfactory centre, the antennal lobe and in particular the projection neurons, to highlight those neurons which have changed their sensitivity with the altered physiological state (Kazama and Wilson, 2008; Olsen and Wilson, 2008). Therefore, the conservation of ORN function in many sensillum types would in fact be predicted.

Comparing the response spectra of all functional classes of sbtII sensilla at 24 h and 72 h post blood meal with those of non-blood fed mosquitoes of the same age, we found differences in ORN sensitivity in four of the five functional classes (sbtII1, sbtII3, sbtII4 and sbtII5). Olfactory receptor neurons of three of the four functional classes increased their response to indole 24 h post blood meal, an effect that was maintained also 72 h post blood meal. An increased response was also observed to phenolic compounds in response to blood feeding 24 h post blood meal, however, this increase was not always maintained 72 h post blood meal. Similarly, we found an increased response to 2-butoxyethanol 24 h post blood meal, but not at 72 h post blood meal. In contrast, we observed a significant increased sensitivity to 2-undecanone and the acids, acetic acid and propionic acid, 72 h post blood meal suggesting that the onset of ORN activation is state-, and age-dependent.

Indole and the phenolic compounds in our odorant panel are emitted from a wide variety of sources: plants emit indole as a secondary defense compound following herbivory (Frey et al., 2000); indole is the most abundant volatile, followed by many of the phenolic compounds, emitted by bacteria (Isenberg and Sundheim, 1958; Lindh et al.,

2008); and mammals, including humans, excrete feces in which the top malodorous compounds include indole and phenolic esters (Cork, 1996). These compounds are, therefore, encountered by mosquitoes regularly in their environment and their ratios contain valuable information for the mosquito concerning available hosts. In addition *Ae. aegypti*, as well as many other mosquito species, prefer oviposition sites near to human habitation that are commonly found to emit large quantities of indolic and phenolic compounds along with 2-undecanone (Bentley and Day, 1989; Cork, 1996; Miller et al., 1992). Other compounds, including acetic and propionic acid tested in the present study have been shown to be effective repellents to ovipositing mosquitoes (Bentley and Day, 1989). Considering the correlation between the change in ORN sensitivity to these compounds and the change in physiological state triggered by a blood meal, we believe that these neurons are involved in driving the orientation behavior of female mosquitoes to these oviposition sites. The odor blends associated with oviposition sites contain different ratios of these indolic and phenolic compounds, than that found in vertebrate host odors. It has previously been shown that during a gonotrophic cycle after a blood meal, the host-seeking behavior of female mosquitoes is inhibited while at the same time their ability to find oviposition sites is increased (Klowden, 1989; Klowden and Lea, 1978, 1979a, 1979b). This switch from host seeking to oviposition site-seeking behavior is supported by our study and by a previous electrophysiological study on *An. gambiae*. Qiu et al. (2006) demonstrated that, following a blood meal, female *An. gambiae* responsiveness to indole increased significantly while at the same time they showed a decrease in the responsiveness to cues associated with host-seeking in this species, such as ammonia. In the present study we find significant changes in the sensitivity to those compounds involved in both

host seeking and oviposition site-seeking behaviors (indole and phenols). Our panel of odorants was chosen to highlight those volatiles involved in both host-seeking and oviposition site-seeking behavior, so while we did not observe any significant decrease to exclusively host-associated chemical cues, we suggest that such down-regulation may occur and a different panel of odorants should be tested to address this question. In fact, Davis (1984) showed that there is a sensitivity difference in antennal ORNs to a host-related cue comparing blood fed and non-blood fed female mosquitoes. In his study, Davis (1984) investigated the variation in sensitivity of ORNs housed in grooved peg sensilla, and found that following a blood meal the sensitivity of ORNs responding to the host-volatile L-lactic acid is significantly decreased compared to ORNs of non-blood fed mosquitoes. Davis (1984) proposed that by altering the sensitivity of ORNs the mosquito could screen for behaviorally relevant compounds at the peripheral level, i. e. the first relay center for chemical information, without involving the central nervous system. Since then, models have been proposed to accomplish this peripheral modulation: one strongly favored mechanism of altering the peripheral sensitivity is by the up- or down-regulation of olfactory receptor gene expression in ORNs. Support for this model in mosquitoes arises from a previous study in *An. gambia*e, which demonstrated that 12 h after blood feeding the expression of the OR gene, AgOr1, was down-regulated (Fox et al., 2001). Heterologous expression of AgOr1 in the fly empty neuron system suggests that the ligands of this receptor are 3-methylphenol, 4-methylphenol, 4-ethylphenol and 4-methylcyclohexanol (Hallem et al. 2004). The pairing of more general cues, the phenol esters, with host-specific cues, one aldehyde and one alcohol, suggest that these Ors and their associated ORNs are host-specific; therefore, down-regulation of these receptors

post blood meal correlates well with the known behavioral results. In light of these findings it may be reasonable to suggest a similar mechanism whereby an up-regulation in the expression of olfactory receptors that are more tuned to oviposition attractant compounds in the antenna of *Ae. aegypti* may occur after a blood meal.

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Figure captions

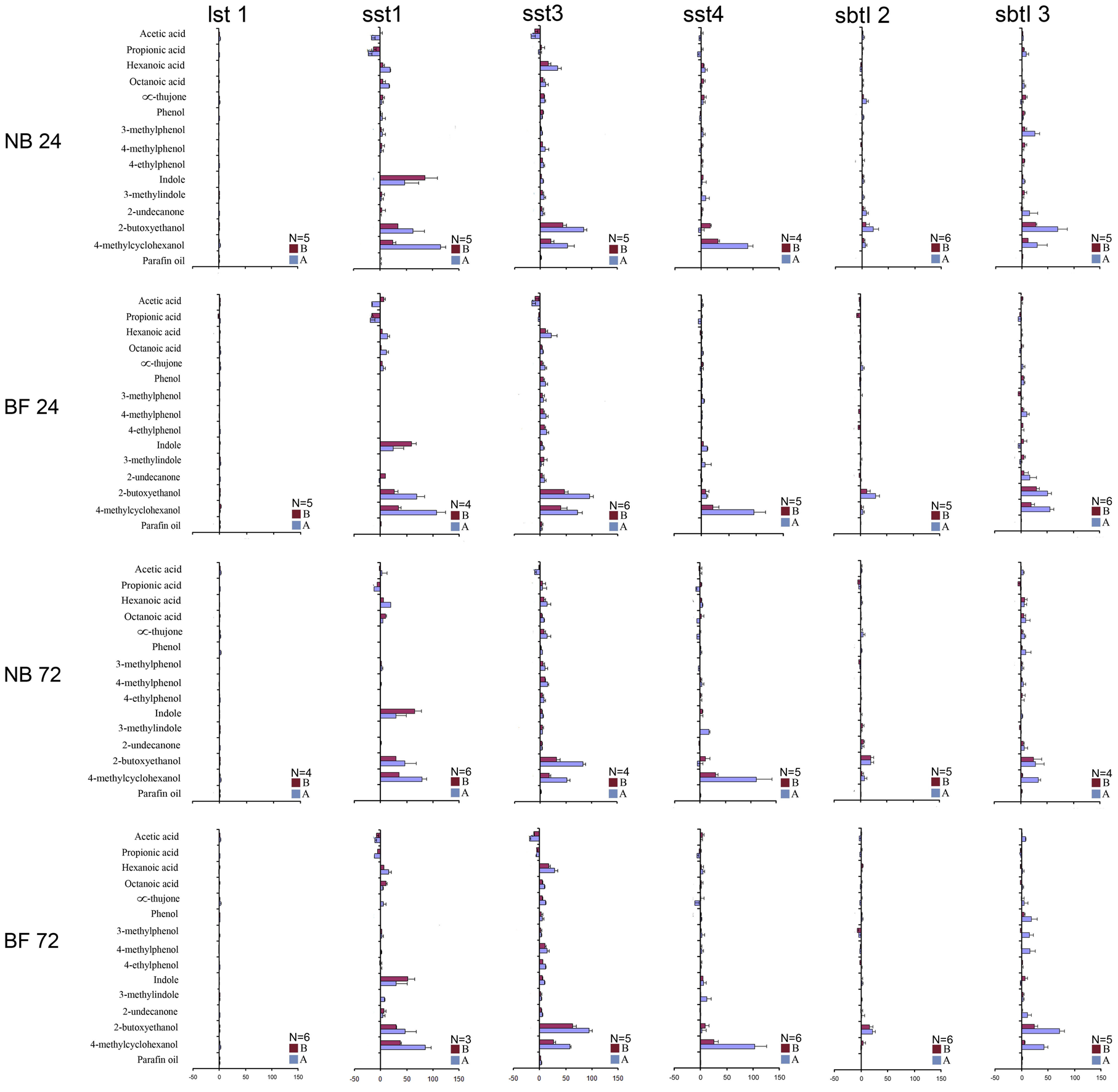
Fig. 1 Response spectra of the olfactory receptor neurons housed in three morphological types of *sensilla trichodea* of non-blood fed (NB) and blood fed (BF) female *Aedes aegypti* at 24 h and 72 h post blood meal. No differences were observed between NB and BF in any of the functional classes belonging to: lst, long sharp-tipped; sst, short sharp-tipped; sbtI, short blunt-tipped *sensilla trichodea*. The neuronal responses of the two neurons, i.e. A and B, housed in these sensillum classes are shown as an average over (N) replicates. Error bars indicate standard error of mean.

Fig. 2 Response spectra of the olfactory receptor neurons housed in the short blunt-tipped type II (sbtII) *sensilla trichodea* of non-blood fed (NB) and blood fed (BF) female *Aedes aegypti* at the 24 h time point. The neuronal responses of the two neurons, i.e. A and B, housed in the five functional sbtII sensillum classes are shown as an average over (N) replicates. Error bars indicate standard error of mean. The bottom panel depicts the difference in response spectra of the olfactory receptor neuron types, between BF 24 h and NB 24 h. Sensitivity differences were observed in the sbtII1, sbtII3 and sbtII5 functional

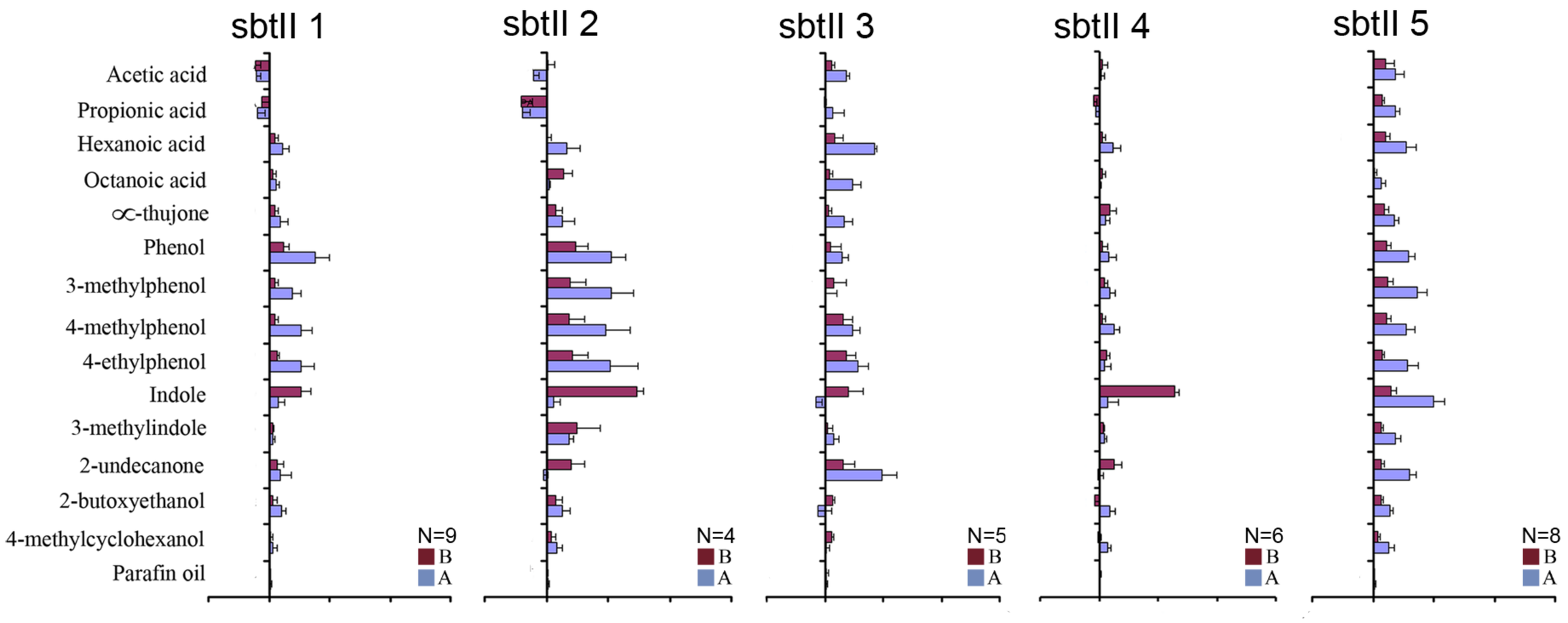
classes. Significant differences between BF and NB are indicated: *P<0.01; **P<0.001; ***P<0.0001 (ANOVA).

Fig. 3 Response spectra of the olfactory receptor neurons housed in the short blunt-tipped type II (sbtII) *sensilla trichodea* of non-blood fed (NB) and blood fed (BF) female *Aedes aegypti* at the 72 h time point. The neuronal responses of the two neurons, i.e. A and B, housed in the five functional sbtII sensillum classes are shown as an average over (N) replicates. Error bars indicate standard error of mean. The bottom panel depicts the difference in response spectra of the olfactory receptor neuron types, between BF 72 h and NB 72 h. Sensitivity differences were observed in the sbtII1, sbtII3 and sbtII5 functional classes. Significant differences between BF and NB are indicated: *P<0.01; **P<0.001; ***P<0.0001 (ANOVA).

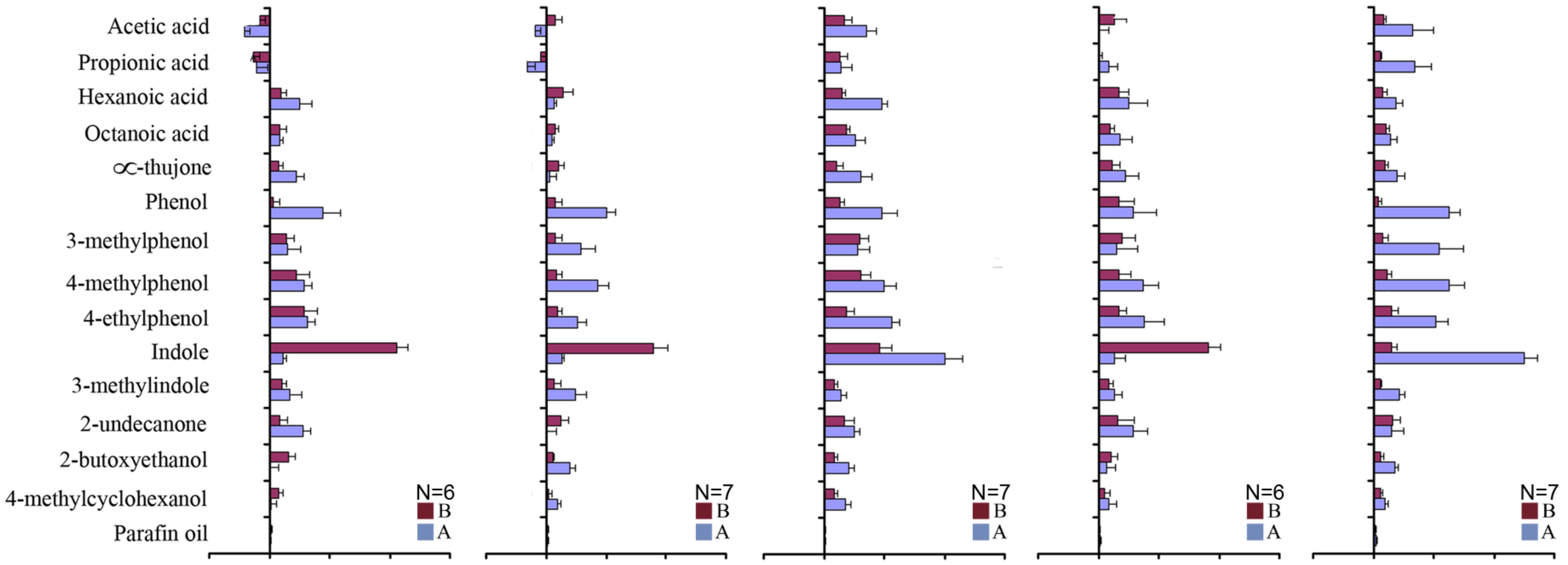
Fig. 4 Dose-response relationships for the short blunt-tipped II1B neuron responding to indole and phenol in blood fed (BF) and non-blood fed (NB) female mosquito at the 24 h time point. Error bars indicate standard error of mean. Significant differences in the response between BF and NB are indicated: *p<0.01; **p<0.001 (ANOVA). Sample size is 5.


lst 1
sst1
sst3
sst4
sbtl 2
sbtl 3
NB 24
BF 24
NB 72
BF 72
Acetic acid
Propionic acid
Hexanoic acid
Octanoic acid
∝-thujone
Phenol
3-methylphenol
4-methylphenol
4-ethylphenol
Indole
3-methylindole
2-undecanone
2-butoxyethanol
4-methylcyclohexanol
Parafin oil
N=5
N=4
N=6
N=3
B
A
-50
0
50
100
150
Response (spikes/s)

NB 24



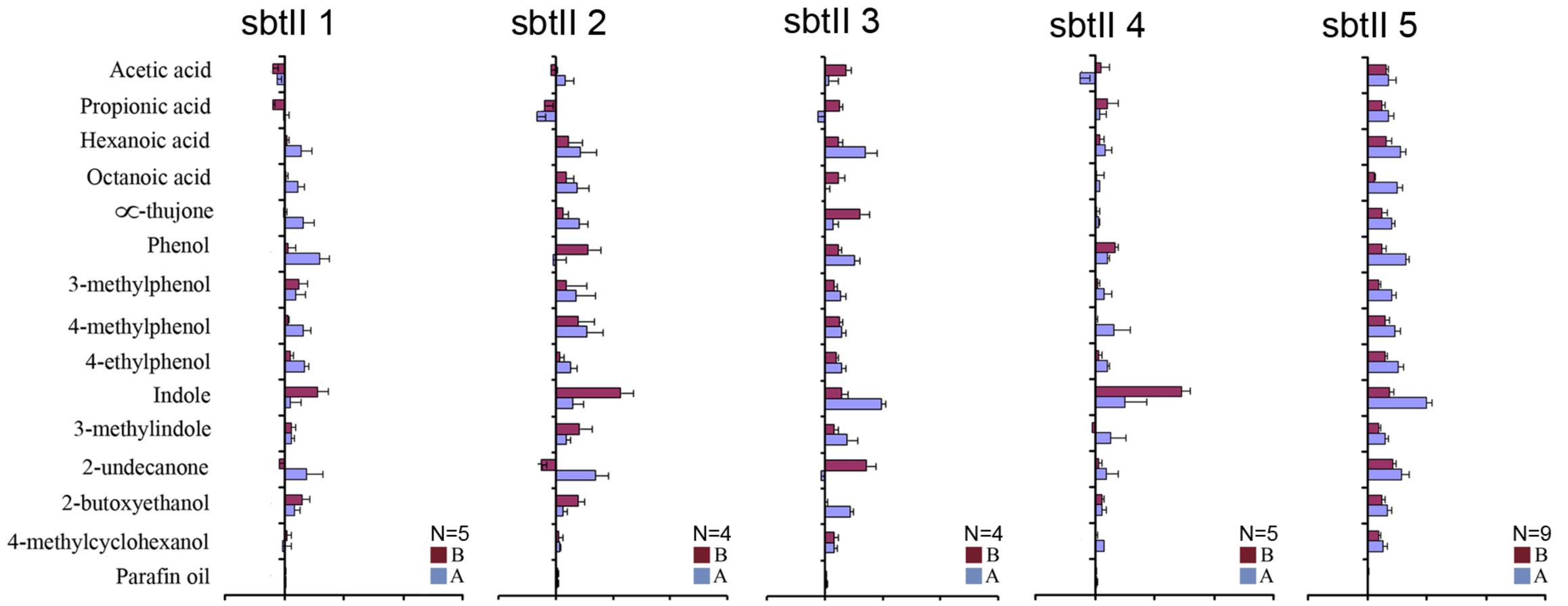
BF 24



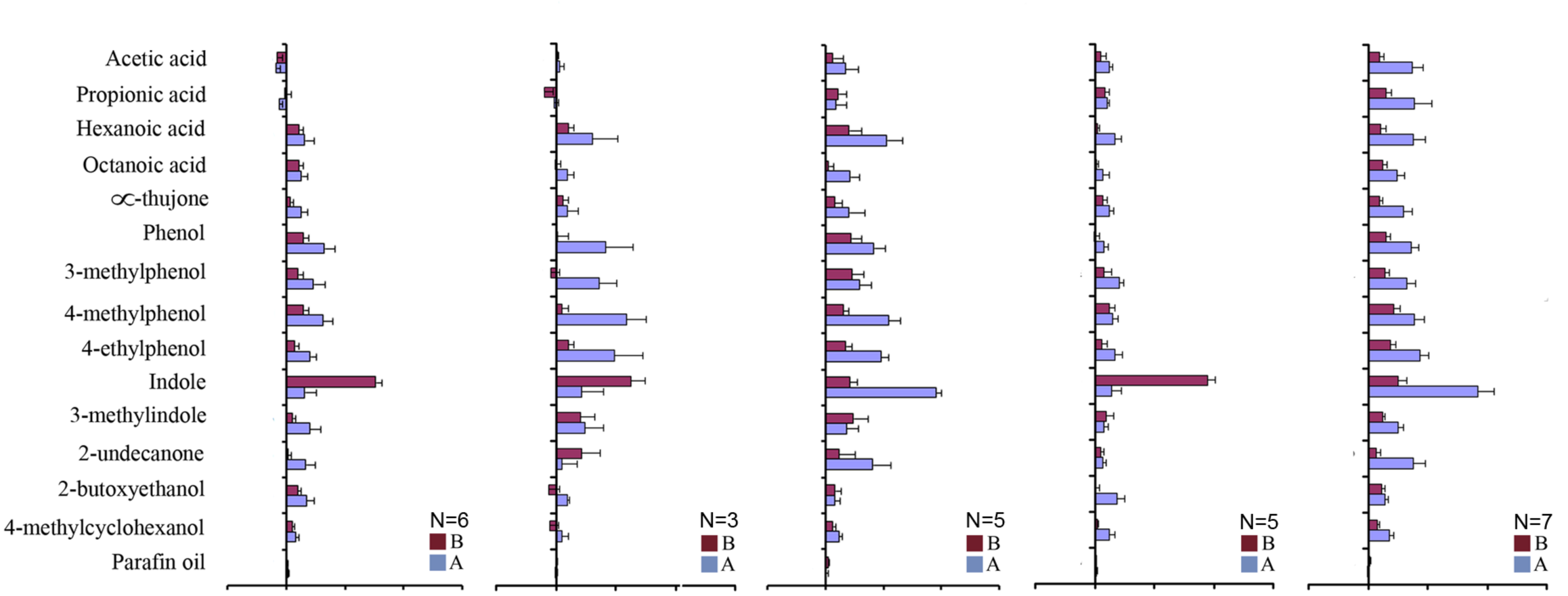
Δ BF-NB 24

Response (spikes/s)

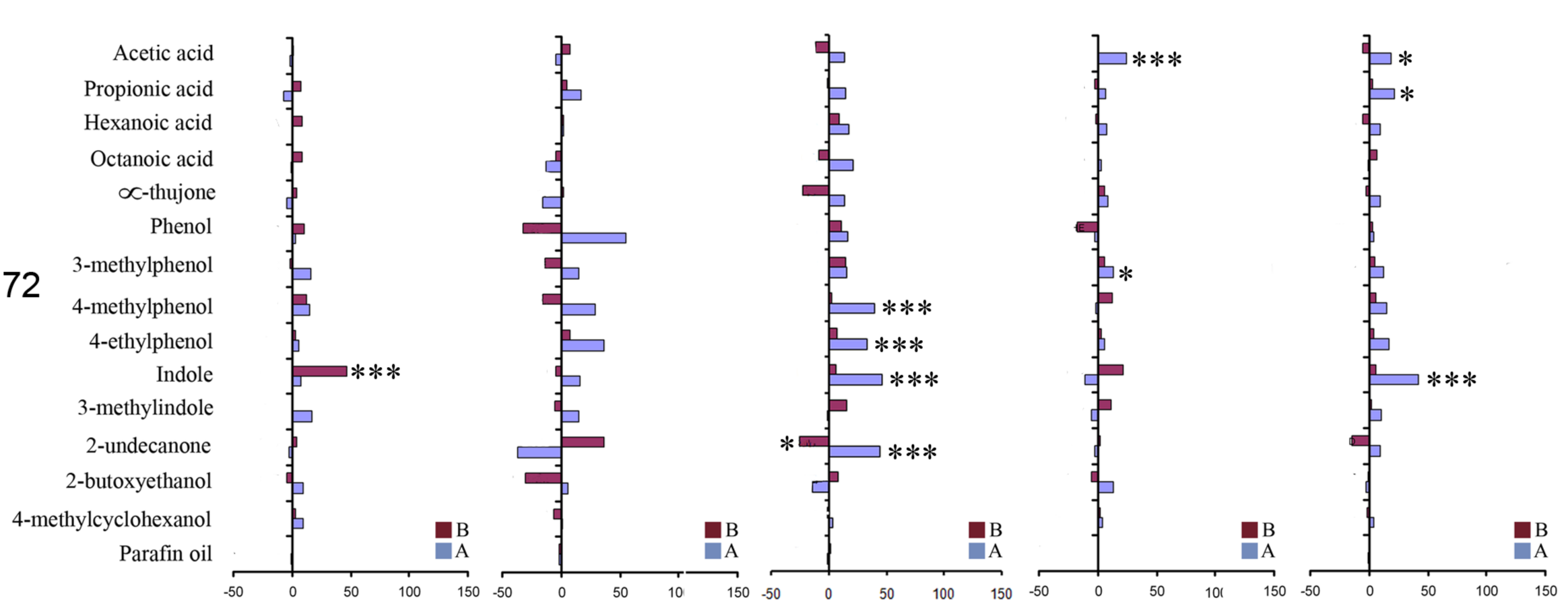
NB 72



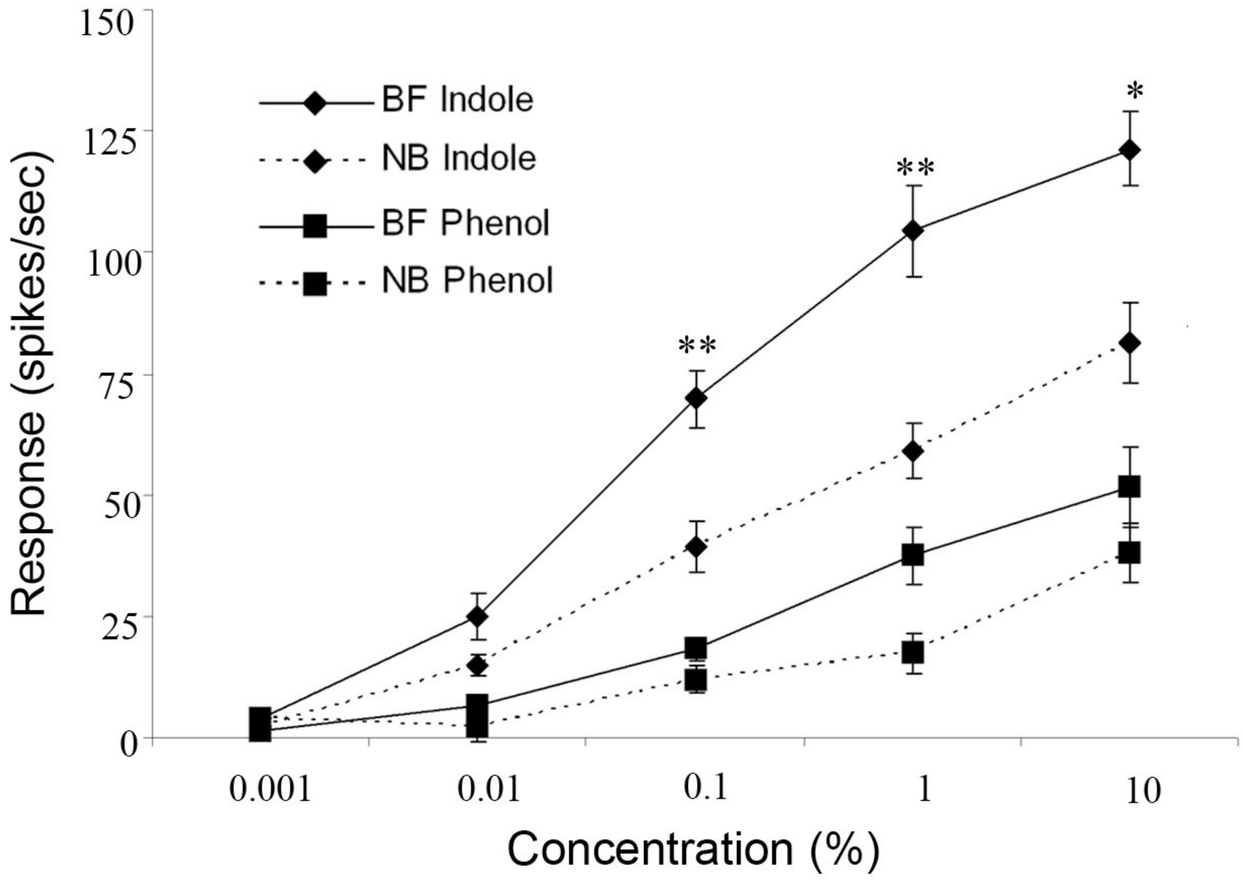
BF 72



ΔBF-NB 72



Response (spikes/s)


150
125
100
75
50
25
0
Response (spikes/sec)
BF Indole
NB Indole
BF Phenol
NB Phenol
**
**
*
0.001
0.01
0.1
1
10
Concentration (%)