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Temporal characteristics of electrophysiological activity of olfactory neurons in sensilla on the maxillary palp of the malaria mosquito, *Anopheles gambiae*

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1. Contents

2. Summary

Malaria mosquitoes in search of a blood meal locate their hosts primarily by olfactory cues. Close to a human host, an odour plume is homogeneous in concentration. Downwind, turbulence in the atmosphere will have caused the odour plume to fluctuate in concentration, resulting in an intermittent signal.

In this study we investigated the effect of a repetitive stimulus on two neurons of the capitate peg sensillum on the maxillary palps of the malaria mosquito, *Anopheles gambiae*. To that end we repeatedly stimulated the capitate peg sensillum at 1-5 Hz with carbon dioxide or 1-octen-3-ol.

We show that both stimulated neurons could distinguish between subsequent stimuli of 0.2 s for frequencies of 1-3 Hz. Stimulated at 4 Hz with a stimulus duration of 0.2 s, not all neurons were able to distinguish between the first 3 stimuli. After some adaptation, those neurons could follow the stimuli applied after the first three. When stimulated at 5 Hz with a stimulus duration of 0.1 s, some neurons sensitive to carbon dioxide were unable to distinguish between the first two stimuli. After adapting, distinguishing between subsequent stimuli was possible. Neurons sensitive to 1-octen-3-ol showed, when stimulated at 5 Hz with a stimulus duration of 0.1 s, similar distinguishing capabilities as when stimulated at 4 Hz with 0.2 s. However, the number of neurons that were unable to distinguish between subsequent stimuli had decreased relative to stimulation at 4 Hz. We conclude that in the capitate peg sensillum the neuron stimulated by carbon dioxide and the neuron stimulated by 1-octen-3-ol were able to distinguish between subsequent stimuli up to 3 Hz. Distinguishing between subsequent stimuli at frequencies of 4 Hz and 5 Hz could, after adaptation in the first three stimuli, also be distinguished.

3. Introduction

Female malaria mosquitoes, *Anopheles gambiae* Giles *s.s.*, in search of a blood meal, locate their host by physical and chemical cues from their environment. Physical cues, such as moisture and temperature, and chemical cues are used for short distance host localisation (De Jong & Knols 1995). Host localisation over long distances is primarily mediated by olfactory input (Takken 1991). Olfactory inputs are carbon dioxide (CO_2) and organic compounds originating from human breath, skin, and urine (Takken 1991).

Olfaction in *An. gambiae* takes place in the antennae, maxillary palps and proboscis by a variety of sensilla. The antennae have six different types of sensilla, of which two are mechanosensors, two are thermosensors and two are olfactory sensilla. The maxillary palps have three different types of sensilla. Of these three palpal sensilla, two are mechanosensors and one is an olfactory sensillum: the capitate peg sensillum (sensillum basiconicum) (Qiu 2005). Capitate peg sensilla have three olfactory receptor neurons (ORNs) and each tunes to different odorants. All three ORNs respond to organic compounds, but one is also a CO_2 receptor (Lu *et al.* 2007, Syed & Leal 2007, Grant *et al.* 1995). The sensitivity to CO_2 is the highest at concentrations that will be encountered when nearby a host (Lu *et al.* 2007, Grant *et al.* 1995). A small rise in CO_2 concentration results in a response which is hardly influenced by background CO_2 levels. When an increase in CO_2 is sensed, behavioural sensitivity to organic compounds increases (Dekker *et al.* 2005). It is suggested that an increase in CO_2 starts the active search for a host and is then assisted by other available odorants (Grant *et al.* 1995, Takken 1991).

For a number of moth species, it was shown that fluctuations in concentration of pheromone blends was necessary to sustain upwind flight (Vickers 2006, Barrozo & Kaissling 2002, Marion-Poll & Tobin 1992, Kaissling 1986). For *An. gambiae*, Dekker *et al.* (2001) showed that fluctuations in CO_2 concentration increased upwind flight relative to a homogeneous increased CO_2 level. The same holds for other mosquito species (Geier *et al.* 1999, Omer 1979). Skin odour however, increased upwind flight when in a homogeneous odour plume. An explanation is found in the odour plume originating from a large object such as a human. Then, an odour plume will have little fluctuations in concentration of odorants close to the body; so olfactory cues close to the body are not an intermittent signal. Further away however, turbulence will have caused concentrations to fluctuate. The CO_2 concentration, which is determined predominately by exhaled air, will show the fluctuations needed for sustained upwind flight sooner then skin emanations, due to inhalation and exhalation (Cardé 1996, Takken 1991).

Animals orientating to odour plumes normally encounter pulses of odours of a few milliseconds up to a second and reach frequencies of 2 Hz or more (Vickers 2006, Murlis 1986). This is further influenced by the flight track and speed of the mosquito. ORNs of the antennae of several species can distinguish between repeated stimuli at least at 3-8 Hz (*Antheraea polyphemus*: 20 ms stimuli, 5 Hz; *Manduca sexta*: 20 ms stimuli, 3 Hz; *Bombyx mori*: 50 ms stimuli, 8 Hz) (Vickers 2006, Barrozo & Kaissling 2002, Marion-Poll & Tobin 1992, Kaissling 1986). Electroantennograms of three different moth species (*Cadra cautella, Pectinophora gossypiella, Spodoptera exigua*) suggested ORNs to be able to follow frequencies even up to 33 Hz at stimulus durations of 20 ms (Bau *et al.* 2002). Single projection neurons of *M. sexta* and *Heliothis virescens* could follow stimuli of single compounds up to stimulus delivery rates of 3 Hz. When a pheromone blend was used, stimulus delivery rates up to 10 Hz could be resolved (Vickers 2006). Whether the central nervous system can follow frequencies of 33 Hz is not known. Characteristics of other neurons and transfer characteristics between neurons then become more important.

Through this research we aim to describe the temporal characteristics of the capitate peg sensillum of *An. gambiae*. We will evaluate this by applying 10 repeated stimuli of CO_2 and 1-octen-3-ol at 1-5 Hz, combined with single sensillum electrophysiological recordings.

4. Materials and Methods

4.1 Experimental animals

Malaria mosquitoes (*A. gambiae s.s.*; colony established in laboratory in 1987; originating from Suakoko, Liberia (courtesy of Professor M. Coluzzi)) were reared at 27 °C with a relative air humidity of 70% and a 12h:12h light:dark photoperiod, shifted to have dawn at 12:30 during daytime. Larvae were fed Tetra MikroMin (Tetra Werke, Ulrich Baensch Gmbh, Melle, Germany). Pupae eclosed in polypropylene/polyethylene/nylon insect rearing cages (30x30x30 cm; BugDorm-1; Megaview Science Education Services CO. Ltd., Taichung, Taiwan). Male and female adult mosquitoes were kept together in the cages they eclosed in. They were fed with a 6 % glucose solution. Female mosquitoes of 6-9 day old were used for our experiments. All experiments are carried out between the last 4 hours of the dark period and the first 4 hours of the light period of the mosquitoes' photoperiod.

4.2 Experimental procedure

Prior to the experiments, 5-10 female mosquitoes were selected by being attracted to a human hand and transferred to a Perspex cylinder (10 cm, Ø 6 cm). A single mosquito with its legs removed was then mounted on the bottom of a Perspex cube (1.1x1.2x1.5 cm) by carefully sticking it to transparent double-sided tape. The palps were spread, pointing away from the proboscis at an angle of 45°. Scales on the palps were removed by gently stroking them with transparent double-sided tape. The animals left for minimally 10 minutes before the first recording was made.

The preparation was mounted on an inverted microscope (Olympus IX51S8F-3; Olympus Nederland B.V., Zoeterwoude, Netherlands). An indifferent electrode was inserted into the eye of the mosquito. A recording electrode was inserted at the base of a single capitate peg sensillum on one of the palps. Electrodes were made from tungsten wire (H. Drijfhout & Zoon's Edelmetaalbedrijven B.V., Amsterdam, Netherlands; Ø 0.25 mm) electrolytically sharpened by repeated dipping in a saturated KNO_2 solution in H_2O at 4 V. The recording electrode was connected to a data acquisition controller (IDAC-4-USB; Syntech, Kirchzarten, Germany). The signal was then transmitted to an Intel Pentium 4 computer (1.5 GHz, 512 Mb RAM) and recorded by the program AutoSpike 32 (version 2.2, Syntech, Kirchzarten, Germany).

At a distance of 0.4-0.7 cm of the sensillum to be recorded, a continuous flow (18-20 °C, 700 ml min^{-1}) of synthetic air (80% N_2, 20% O_2; Hoekloos, Schiedam, The Netherlands) was applied through a Teflon coated tube (18 cm long, ø 5 mm). An additional flow containing an odorant could be added four cm before the ending of the tube. The synthetic air was directed through water to be moisturized. An air pump (Ametek MG-4; Ametek Lloyd Instruments, Fareham, Hants, UK) operating at 300 ml min^{-1} generated an airflow from an airbag (24.1x25.4 cm; Tedlar Sample Bag, 231-series; SKC Ltd., Blandford Forum, Dorset, UK). This airbag contains an odorant or a control. The flow was directed through Teflon tubing from the airbag through the air pump towards a stimulus controller containing a valve (STC-1/S; Syntech, Kirchzarten, Germany). This valve was controlled by a pulse generator (Hewlett-Packard 3311A; Hewlett-Packard Nederland B.V., Amstelveen, Netherlands). Normally, the valve directed the flow away, to aspiration. When the stimulus controller received a pulse however, the valve directed the flow through Teflon tubing (test, air with odorant) or silicon tubing (control, air without odorant) towards the main stream of the synthetic air. The two streams added up and a stimulus was applied at 1000 ml min^{-1}, 300 ml min^{-1} above the continuous airflow.

We tested CO_2 (Hoekloos, Schiedam, The Netherlands) as stimulus to neuron A and 1-octen-3-ol (OCT) (Fluka, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) as stimulus to neuron B, as well as their controls. Carbon dioxide was directly mixed with synthetic air to generate a concentration of 1250 ppm in an airbag. The control of CO2 was synthetic air without any additions. One-octen-3-ol was first dissolved in paraffin oil to create a concentration of 0.001% (percentage of mass). Then, 6250 µl of the dilution was injected into an airbag that was then filled with 2.5 l synthetic air. As control for OCT we injected 6250 µl paraffin oil into an airbag. During the course of our experiments, the OCT airbag and paraffin oil airbag were refilled three times with 2.5 l synthetic air. After (re)filling the OCT airbag and paraffin oil airbag, the paraffin oil - odorant mixture or paraffin oil could evaporate into the airbag for at least one day, while the airbag was inside the test room. The concentrations of CO_2 and solved OCT were taken at the half maximal effective concentration (EC_{50}) of the capitate peg sensillum (Lu et *al*. 2007). The amount of odorant solution injected into the airbag was scaled linearly from Lu et *al*. (2007) as well (10 µl in 4 ml scaled to 6250 µl in 2500 ml).

We tested sensilla with stimuli repeated at frequencies of 1 Hz, 2 Hz, 3 Hz, 4 Hz and 5 Hz. At 1-4 Hz we used a stimulus duration of 0.2 s. At 5 Hz we used a stimulus duration of 0.1 s. At each frequency, 10 successive stimuli were applied. The response was recorded for 20 seconds and the first stimulus was applied at the start of the third second in this 20 second recording. Between every group of 10 stimuli, a one-minute break was presented. A recording and its control were only used if both signals were not burst-like (a silent period followed by a sudden short period with a high spike rate) and the spontaneous activity did not change much within one recording. This resulted in 7-13 relevant recordings per frequency for CO_2 and 4-6 relevant recordings per frequency for OCT.

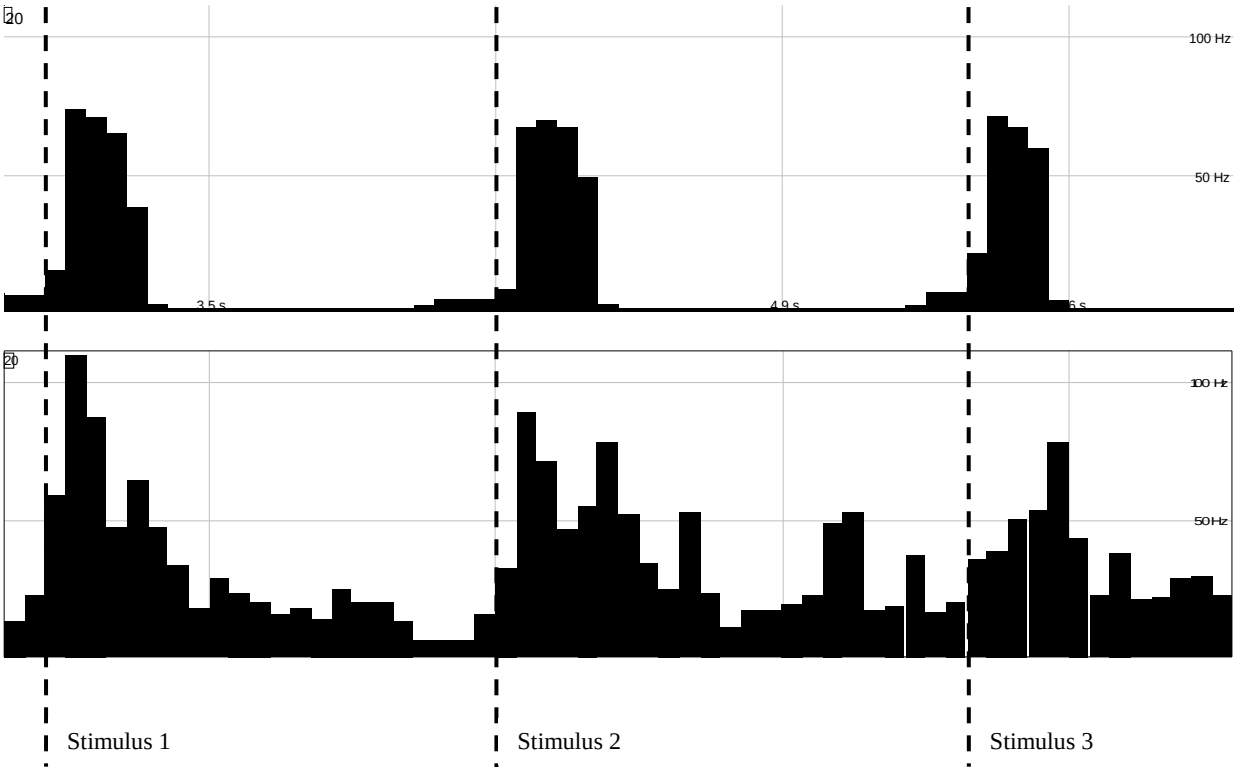


Figure 1: Spike frequency of neuron A (upper graph) and neuron B (lower graph). Stimulation was with OCT at 1 Hz and bin width was 50 ms. The beginning of the graph is at 3 seconds into the recording, the end is at 6 seconds into the recording. The dashed lines indicate the onset of the response to the first, second and third stimulus as indicated. Neuron A showed a response due to mechanical stimulation (see 'Discussion' section for further explanation) and neuron B responds to stimulation by OCT.

The AutoSpike program was used to auto-detect spikes in the recorded signals. The timing and amplitude of the spikes and the digital signal were exported to the program MatLab (version 7.4; The Mathworks, Gouda, The Netherlands), which was used for further analysis. Statistical analysis was done using GenStat (edition 10.2; VSN International Ltd., Hemel Hampstead, UK). Based on their amplitude, we were able to discriminate spikes of neuron A (amplitude relative to maximum amplitude in signal: 0.8-1) and spikes of the combined response of neurons B and C (amplitude relative to maximum amplitude in signal: < 0.6).

4.3 Data analysis

From the suitable data, for every stimulus the latency was calculated as the difference in stimulus onset and first spike as a reaction to the stimulus. The first spike was visually determined in the original signal, as the spike where spike rate of neuron A increased substantially (figure 1). Neuron B showed a sustained response to OCT, so the latency of neuron B cannot be estimated. We therefore assumed the latency of neuron B to be identical to neuron A, as neuron B had its increase in firing rate at the same moment as neuron A (figure 1). Neuron A responded to every stimulus, since we did not compensate for the change in airflow. This will be discussed in the 'Discussion'-section.

After the first spike, the spike pattern was split in 100 ms bins, until the onset of the next stimulus. For 3-5 Hz, the last bin in which the response to the next stimulus started had the bin width adapted so that the end of the bin was the start of the next stimulus (figure 2): for 1 Hz, we had 10 bins of 100 ms; for 2 Hz, we had 5 bins of 100 ms; for 3 Hz, we had 3 bins of 100 ms and the final bin was 35 ms on average; for 4 Hz, we had 2 bins of 100 ms and the final bin was 39 ms on average; for 5 Hz, we had 1 bin of 100 ms and the final bin was 93 ms on average. The final bin of stimulus 10, which had no subsequent stimulus, was 20 ms for 3-4 Hz and 100 ms for 5 Hz. For every signal, the spontaneous activity averaged over the first 3 seconds of the recording was subtracted. Then, for every bin the increase in spike rate, relative to the control, was calculated

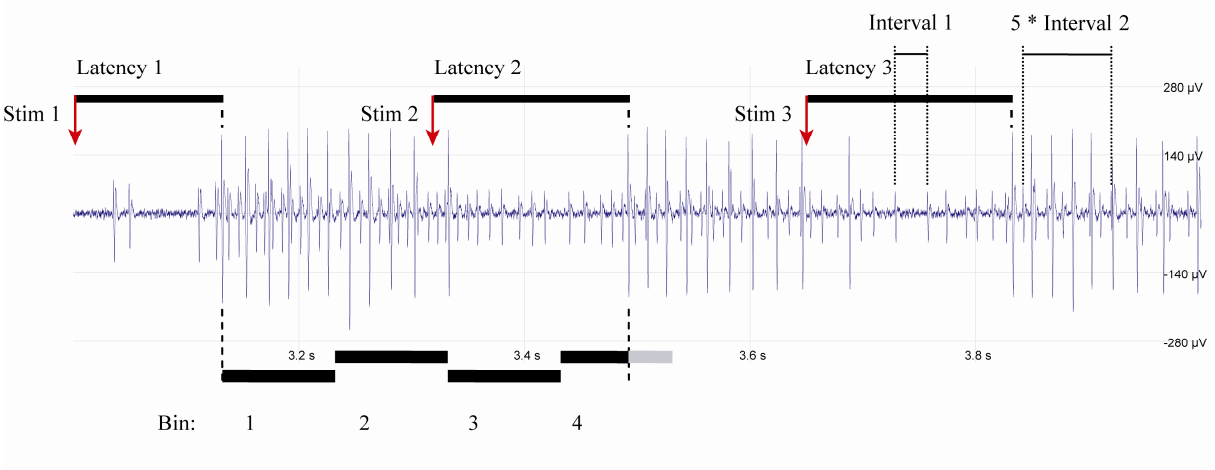


Figure 2: Electrophysiological recording of a capitate peg sensillum. Stimulation was done with OCT at 3 Hz. The beginning of the graph is at 3 seconds into the recording, the end is at 4 seconds into the recording. Red arrows indicate stimulus onset of stimuli 1-3. The latency is indicated by the black bar between the red arrow and the first spike of neuron A as a response to the stimulus. Below the graph the distribution of bins is displayed for stimulus 1. We can see three complete 100 ms bins as well as a fourth bin that could not complete the 100 ms. Instead, it is adapted so the onset of the response to the next stimulus marks the end of the fourth bin. Above the graph, 'Interval 1' indicates the largest inter-spike interval between two subsequent stimuli. 'Interval 2' indicates the average of the first five inter-spike intervals of the stimulus subsequent to interval 1.

For a single recording, the resolution of frequency calculated per bin was too low to accurately compare the last bin of a stimulus with the first bin of a subsequent stimulus. With an increased bin width this resolution would increase, but the effect of a change in spike rate would disappear. We therefore measured the largest inter-spike interval between two subsequent stimuli (henceforth interval 1), as well as the mean of the first three (CO_2) or five (OCT) inter-spike intervals during the latter of the two stimuli (henceforth interval 2). If there were less than three (CO_2) or five (OCT) inter-spike intervals available, the mean inter-spike interval of the maximum amount of available inter-spike intervals was taken (figure 2). The ratio between interval 2 divided by interval 1 gave an indication whether it is probable if a neuron could distinguish between two subsequent stimuli. If the ratio of interval 2 / interval 1 is close to or above 1, it is probable that the spike-rate does not change much and the neuron has difficulty distinguishing between subsequent stimuli. If the ratio of interval 2 / interval 1 is close to 0, it is probable that there is a large difference in spike-rate and the neuron can distinguish between subsequent stimuli.

For statistical analysis we used a generalized linear model (GLM) with Poisson distribution and a logarithmic link function. This GLM was applied to data on the latency, the increase in spike-rate per bin per stimulus per frequency and the ratio of interval 2 / interval 1.

5. Results

A typical electrophysiological recording is shown in figure 1. The spontaneous activity of the neurons can be seen in the period before the response to stimulus 1. Due to a main airstream without any CO_2, some sensilla show no spontaneous activity in neuron A, where others do show spontaneous activity. We did not further investigate this difference in spontaneous activity.

Shortly after the onset of every stimulus, densely packed spikes of neuron A were visible (figure 3A, B). In between the stimuli as well as the two seconds after stimulus 10, the spontaneous activity of neuron A was inhibited. Approximately 2 seconds after stimulus 10, the spontaneous activity reappeared and returned to the spike rate before stimulation. The spontaneous activity of neuron B and C was unaltered when stimulated with CO_2 (figure 3A). When stimulated with OCT however, neuron B and C showed a high spike rate relative to the spontaneous activity (figure 3B). The time it took for the combined spike rate of neuron B and C to return to its original spontaneous activity after stimulation with OCT varied from 2 to >10 seconds.

In figure 4 the mean latency of neuron A stimulated with CO_2 is shown. As we can see, the mean latency started for every stimulus at approximately the same level (128-134 ms). After 10 stimuli, stimulation at 1 Hz had resulted in an increase in latency of 15%, stimulation at 2 Hz had resulted in an increase of 44% and stimulation at 3 Hz had resulted in an increase of 61%. When stimulated above 3 Hz, the increase in latency decreased. Stimulation at 4 Hz resulted after 10 stimuli in an increase of 52% and stimulation at 5 Hz resulted after ten stimuli in an increase of 46%. It should be noted that the stimulation at 5 Hz had a stimulus duration of 0.1 s and all other frequencies of stimulation had a stimulus duration of 0.2 s. The interval between stimuli at 5 Hz roughly doubled relative to 4 Hz (± 0.05 s at 4 Hz; ± 0.1 s at 5 Hz). For the control, i.e. neuron A responding to a change in flow, a similar relation existed with higher starting and ending values. This difference may originate from a different length of silicon tubing and can, in this study, not be ascribed to the influence of CO_2.

Statistical analysis showed all frequencies of stimulation to differ significantly from each other ($p < 0.05$), except for frequencies of 2 and 5 Hz with $p = 0.16$. Stimulation at 3 Hz showed latencies above latencies associated with stimulation at 2 and 4 Hz, so a peak latency had been passed around 3 Hz. Within one frequency of stimulation, the latency of neuron A varied significantly to a number of stimuli limited to the beginning of the ten repeated stimuli: at 1, 2, 3, 4 and 5 Hz, there was no longer a significant difference in latency after stimulus 3, 4, 4, 4 and 5 respectively ($p > 0.05$). The latency thus increased to a maximum.

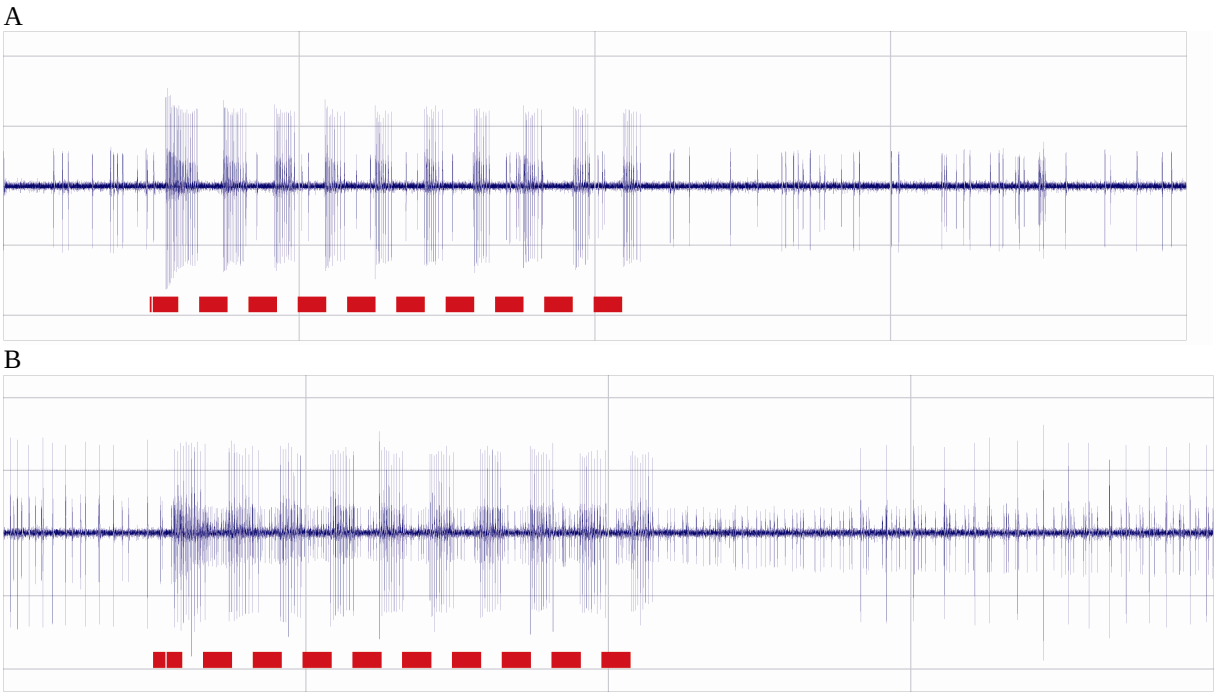


Figure 3: Two recordings of two different capitate peg sensilla stimulated 10 times at 3 Hz, with CO2 (A) and OCT (B). The x-axes indicate time in the recording, starting at 2 seconds into the recording and ending at 8 seconds into the recording. The y-axes indicate the response of the sensilla and vary from -260 to +260 µV (A) and -300 to +300 µV (B). The spikes with amplitude >140 µV originate from neuron A. Spikes with amplitude <140 µV originate from neuron B and C. The red bars show when the stimuli were applied.

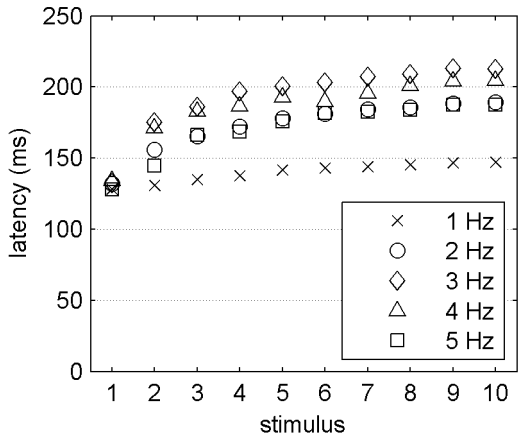


Figure 4: Mean latency of neuron A for different frequencies of stimulation. Stimulation was with 1250 ppm CO_2. The different symbols belong to different frequencies of stimulation, as indicated by the legend. This latency comprises the time before the stimulus actually reaches the mosquito and the time it takes for the neuron itself to respond.

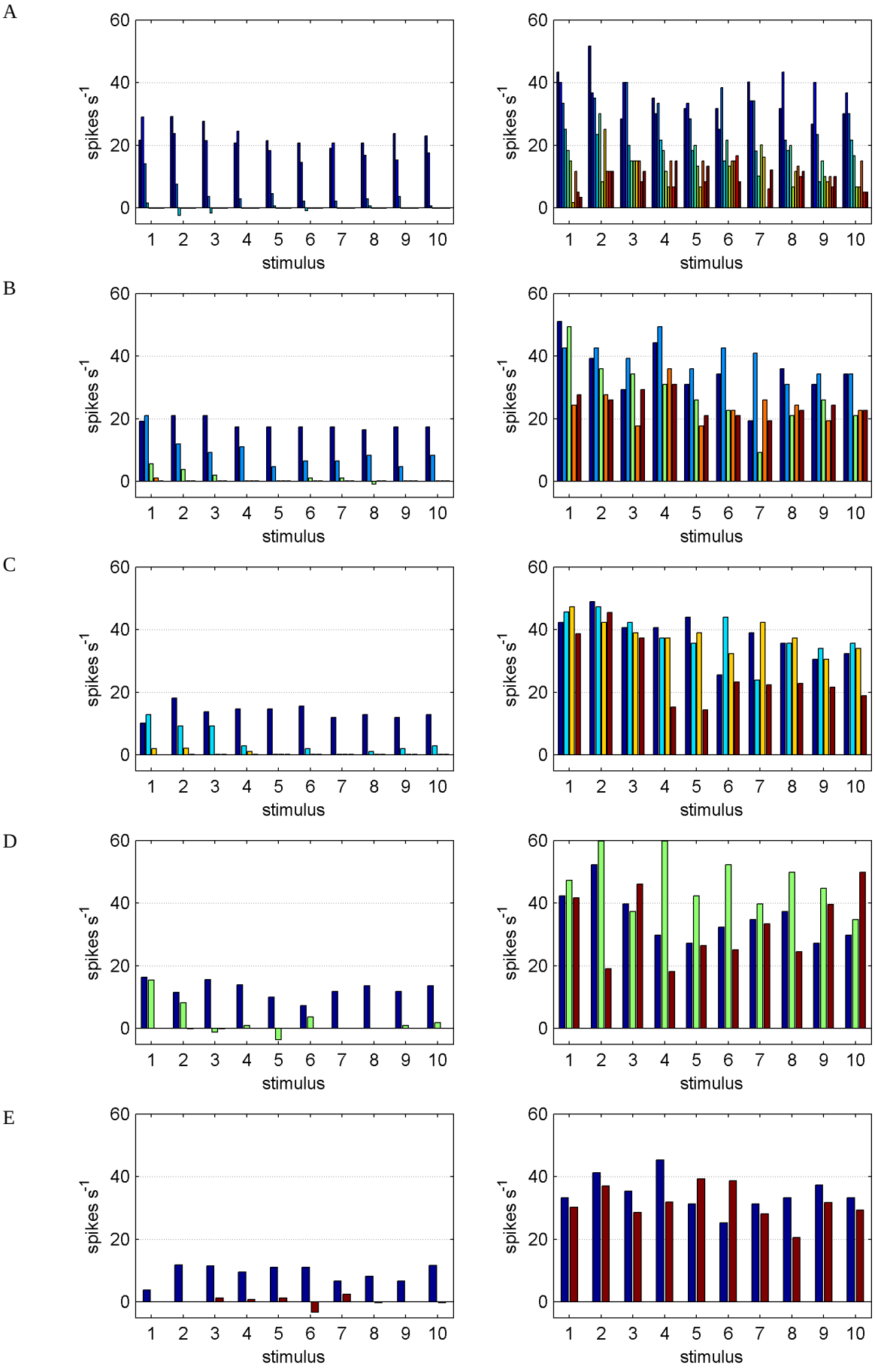
When neuron A was stimulated with CO_2 at a frequency of 1, 2 or 5 Hz, an increase in spike rate was observed for all stimuli for the duration of the stimulus (figure 5A, B, E). A stimulus with CO_2 at a frequency of 3 Hz and 4 Hz, resulted in an increase in spike rate for the duration of the stimulus for stimuli 1-4 and 1-2 respectively. Afterwards, the neuron responded to stimulation with an increase in spike rate roughly the first 100 ms of the stimulus (figure 5C, D). Only neurons stimulated at 1 Hz showed an increase in spike rate for all stimuli in the third bin and all other bins showed an increase in spike rate that is near zero or zero. When stimulated at 2 or 3 Hz, there was some increase in spike rate in the third bin. This ended at stimulus 4 for 2 Hz and at stimulus 3 for 3 Hz, when this increased spike rate was near zero, as were all other bins. When neuron A was stimulated at 4 or 5 Hz, there was no increase in spike rate after the stimulus had ended.

By a statistical analysis, every frequency was revealed to differ significantly from all other frequencies ($p < 0.05$). When stimulated at 1 Hz, stimulus 3 and the subsequent stimuli did not show significant differences in increase in spike rate from each other ($p > 0.10$), except for stimulus 6 that differed significantly from stimulus 3 ($p < 0.05$). When stimulated at 2 Hz, stimulus 3 and above did not differ significantly from each other in increase in spike rate ($p > 0.11$). Stimulus 2 already showed some significant similarity, but some significant differences as well. Stimulated at 3 Hz, we found no significant increase in spike rate between subsequent stimuli ($p > 0.07$) and the same holds for stimulation at 5 Hz ($p > 0.39$). Stimulation at 4 Hz showed no significant differences in increase in spike rate after stimulus 2 ($p > 0.16$)

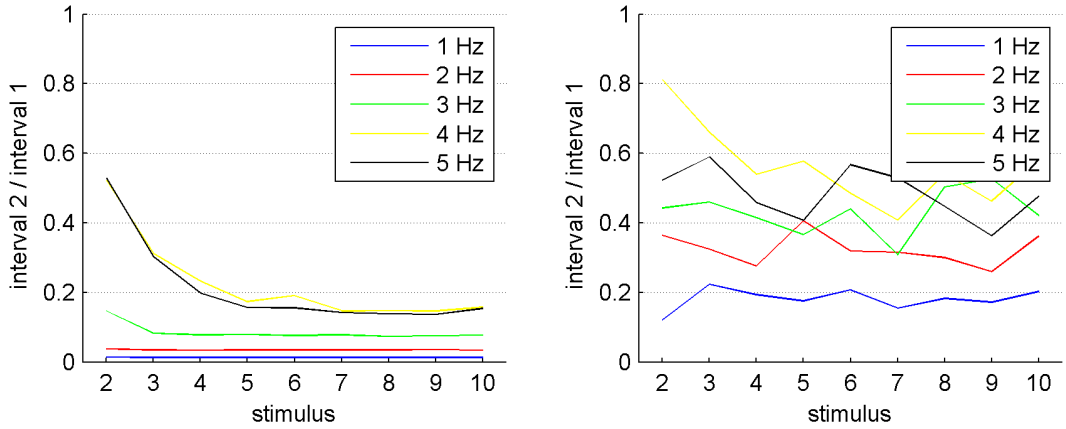
In contrast to neuron A stimulated with CO_2, neuron B stimulated with OCT showed an increase in spike rate for all frequencies for all bins (figure 5). Where a stimulation at 1 Hz showed a significant difference in increase in spike rate between the first and last bin of one stimulus ($p < 0.05$), stimulation at 5 Hz did not ($p = 0.49$). After stimulation at 4 Hz, only the middle bin differed significantly in increase in spike rate ($p < 0.05$), probably due to the low number of replicates. Especially with stimulation at 5 Hz, where spike rate did not differ significantly from bin to bin and stimuli did not significantly differ from each other, a constant firing rate might have been reached. This way a sensillum would be unable to distinguish between subsequent stimuli.

Figure 5 (next page): Mean increase in spike rate per bin per stimulus. The left plots correspond to activity of neuron A stimulated with CO_2, the right plots correspond to activity of neuron B stimulated with OCT. (A) stimulus delivery at 1 Hz, (B) stimulus delivery at 2 Hz, (C) stimulus delivery at 3 Hz, (D) stimulus delivery at 4 Hz, (E) stimulus delivery at 5 Hz. The height of the bars shows the increase in spike rate relative to the control. Each stimulus has separate bars for the number of bins (1 Hz - 10 bins; 2 Hz - 5 bins; 3 Hz - 4 bins; 4 Hz - 3 bins; 5 Hz - 2 bins). A negative value can occur due to subtraction of spontaneous activity in a bin with no activity, as well as by a higher activity in the control than in the test.

Figure 5



A



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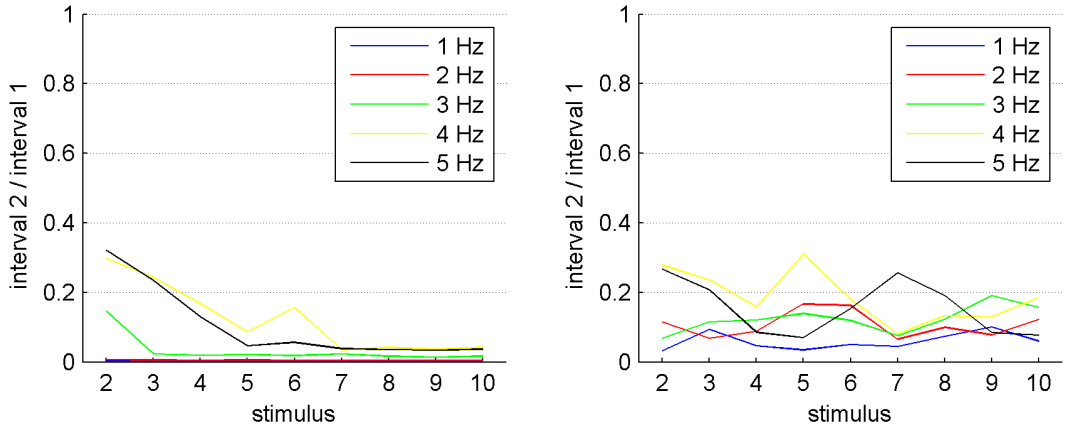

Figure 6: Ratio of interval 2 / interval 1. The left graphs correspond to the response of neuron A after stimulation with CO_2, the right graphs correspond to neuron B after stimulation with OCT. The different colours of the traces refer to different frequencies of stimulation as shown in the legend. The values on the x-axes indicate the stimulus during which interval 2 was measured. (A) The mean ratio of interval 2 / interval 1. A ratio of 1 means that the intervals are equal in duration. A ratio close to 0 means that interval 1 is much larger than interval 2. (B) Standard deviation of the mean ratio of interval 2 / interval 1.

The mean of the ratio interval 2 / interval 1 is displayed for both neuron A, stimulated by CO_2, and neuron B, stimulated by OCT, in figure 6A. Neuron A had a low mean ratio for all stimuli at frequencies 1-2 Hz. By a statistical analysis the ratio was shown not to vary significantly ($p > 0.12$). At 3, 4 and 5 Hz however, significantly higher ratios were found for stimulus 2 with stimuli at 3 Hz and stimuli 2-3 with stimuli at 4 and 5 Hz ($p < 0.05$). Neuron B had relatively low values for stimulation at 1 and 2 Hz and ratios above 0.4 for higher frequencies. With a higher mean ratio, a higher standard deviation existed (figure 6B). So spreading increased when the mean ratio increased. This resulted in a decrease in significance. None of the frequencies had a structural significant difference between its stimuli, except for stimulus 1 of stimulation at 1 Hz ($p < 0.05$ with 5 out of 8 stimuli).

Since the values shown in figure 6 do not give the actual maxima, interval 2 is plotted against interval 1 for stimulation at 3, 4 and 5 Hz in figure 7A, B, C respectively. The graph is limited to data below 100 ms, since ratios of 1 occurred in that limited region. In table 1 the spreading of interval 1, interval 2 and the ratio between them is given. As we can see, for frequencies of stimulation of 1 and 2 Hz the ratio of interval 2 / interval 1 did not approach 1, which is associated with a large difference in spike rate between two stimuli and spike rate at the start of the last of these two stimuli.

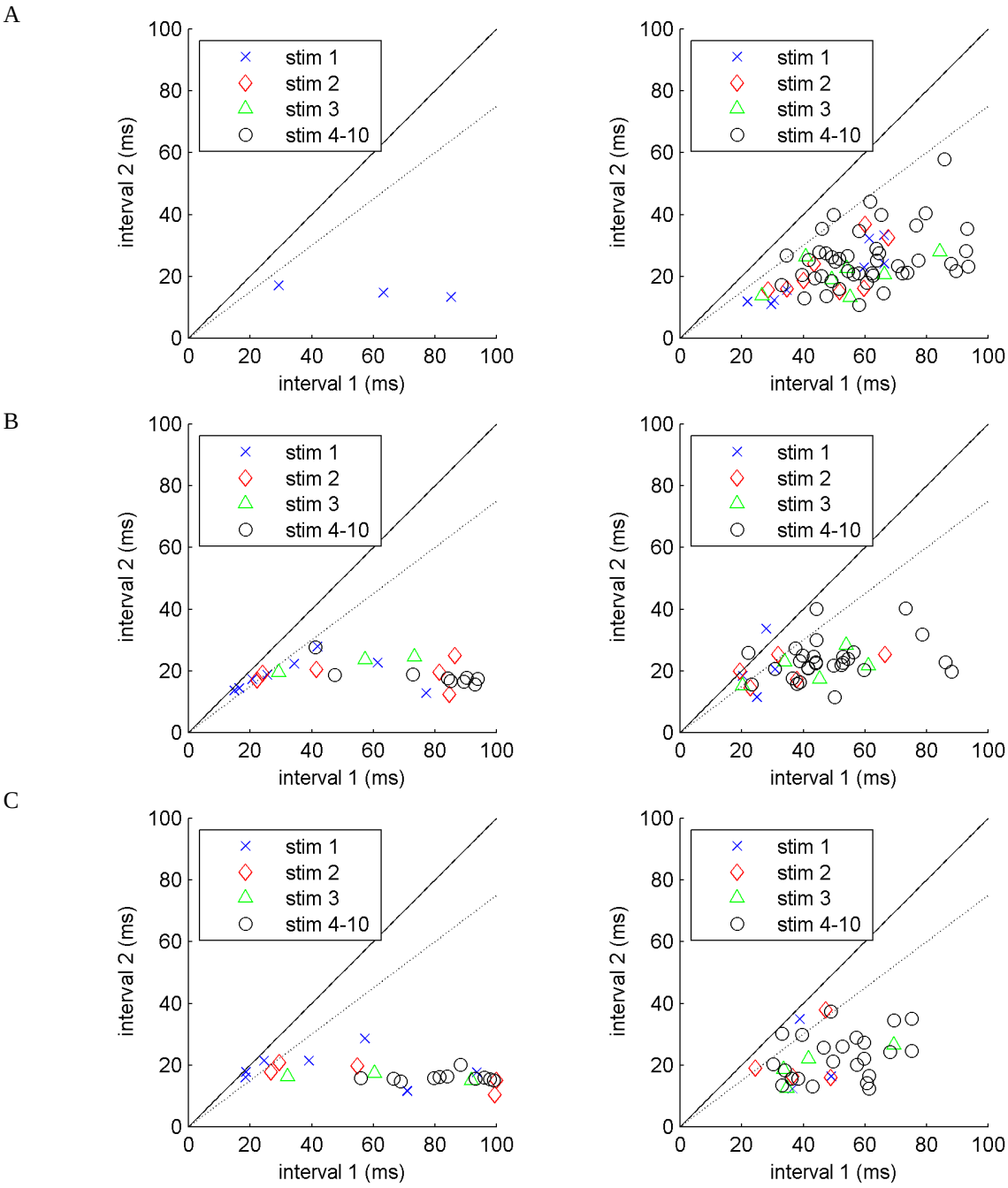

Figure 7: Interval 2 plotted against interval 1 for all replicates of the tests, with the limitation interval 1 ≤ 0.1 s. The left graphs correspond to the response of neuron A stimulated with CO2, the right graphs correspond to the response of neuron B stimulated with OCT. We distinguish between stimulus 1, stimulus 2, stimulus 3 and stimulus 4-10, with interval 1 measure after the stimulus. Thus 'stimulus 1' in the legend has interval 1 after stimulus 1 and interval 2 during stimulus 2. The legend shows the symbols associated with the different stimuli. The solid line is the line interval 2 = interval 1. This corresponds to a value of 1 for the ratio of interval 2 divided by interval 1. The dashed line is the line interval 2 = 0.75*interval 1. This corresponds to a value of 0.75 for the ratio of interval 2 divided by interval 1. (A) Stimulation frequency of 3 Hz. (B) Stimulation frequency of 4 Hz. (C) Stimulation frequency of 5 Hz

Where we see a ratio that did not reach the value 1 in figure 6, in figure 7 we see that this did happen with some but not all neurons (both neuron A and B at stimulus frequency of 4 and 5 Hz). At a stimulus frequency of 3 Hz, neuron B passed the ratio of 0.75, giving the indication that some neurons B might have had difficulty in distinguishing between subsequent stimuli (figure 7A). Neuron A did not yet show this. At a frequency of stimulation of 4 Hz however, some neurons A and some neurons B might not have been able to distinguish between subsequent stimuli as the ratios got close to or passed 1. For neuron A stimulated at 4 Hz, especially distinguishing between stimuli 1 to 3 will have been be difficult, as interval 1 and interval 2 were then nearly

identical. For neuron B stimulated at 4 Hz, also other stimuli have a ratio close to 1 and neuron B thus might have had difficulty distinguishing between subsequent stimuli later on during the repeated stimulus. When the frequency of stimulation is 5 Hz, both neurons seemed to have less difficulty in distinguishing between the subsequent stimuli than when stimulated at 4 Hz: the markers are positioned further away from a ratio >1, though still above a ratio of 0.75.

The major difference between neuron A, stimulated with CO2, and neuron B, stimulated with OCT, was interval 1 (table 1). The lowest values of interval 1 of neuron A occur at 4 and 5 Hz at the transition of stimulus 1 to stimulus 2. Then, the ratio of interval 2 / interval 1 was largest as well (figure 7). With neuron B, small intervals 1 were found throughout the recording, as firing continues at a high rate between two subsequent stimuli (figure 5).

The neurons with high ratios of interval 2 / interval 1 are not linked to a certain segment of the palps or certain mosquito. It seems that on a single mosquito, neurons with different responses are present.

Frequency of stimulation		1 Hz	2 Hz	3 Hz	4 Hz	5 Hz
CO2	Interval 1 (ms)	763-1004	235-448	29-289	15-195	18-176
	Interval 2 (ms)	8-16	9-17	9-30	12-38	10-29
	Ratio	0.009-0.019	0.023-0.048	0.043-0.582	0.080-0.921	0.081-0.958
OCT	Interval 1 (ms)	74-374	44-155	22-138	19-105	18-85
	Interval 2 (ms)	11-56	11-57	11-58	11-40	12-38
	Ratio	0.063-0.436	0.146-0.757	0.183-0.800	0.225-1.209	0.201-0.916

Table 1: Spreading of interval 1, interval 2 and ratio of interval 2 / interval 1 for neuron A and neuron B.

6. Discussion

In our stimulus generation system, two errors arose. Since our pulse generator did not have discrete steps for frequencies of pulse generation, there was some deviance from the supposed frequencies of stimulation. The actual frequencies of stimulation were for 1, 2, 3, 4 and 5 Hz, 0.92 ± 0.02, 1.91 ± 0.02, 3.07 ± 0.05, 4.32 ± 0.04 and 5.33 ± 0.08 Hz (mean ± sd) respectively.

Stimulus delivery to the mosquito was not step-like. During the change of direction of the stimulus controller valve, airflow with odorant was split in two and was partly aspired and partly directed to the mosquito. This resulted in a gradual stimulus onset and offset. This gradual stimulus delivery was enhanced by the compressibility of air. When the valve switched, pressure had to build up inside the stimulus delivery tubing before the stimulus was actually delivered.

This gradual stimulus delivery could at high frequencies result in a more or less continuous stimulus, which never completely shut down, but fluctuated. This is probably not the explanation of the lower latencies found with stimulations at 4 Hz. Though the even lower latency for stimulation at 5 Hz might have some relation to the shorter stimuli, the time in between two subsequent stimuli is likely to be of great influence as well. This time between stimuli is less for 5 Hz than for 3 Hz. So the explanation must be sought elsewhere. The only explanation remaining is adaptation of the neuron to the repeated stimuli. In order to follow stimuli at higher frequencies with greater accuracy, the latency is lowered.

Though we did not discriminate between spikes of neurons B and C, interference of neuron C on the OCT test was low, since spontaneous activity of neuron C is low (0.8 ± 0.8 spikes s^{-1}). When the increase in spike rate was above 10 spikes s^{-1}, as is the case for all bins of frequencies 2-4 Hz and for most bins at 1 Hz, the influence was < 10%. (Lu *et al.* 2007).

While some neurons A in a continuous synthetic airflow did not show any spontaneous activity, other neurons A did. Also Lu *et al.* (2007) showed some activity of neuron A when a main stream of synthetic air was used. For female *Aedes aegypti* of 4-7 days old, Grant & O'Connell (2007) found no spontaneous activity, when a main stream of synthetic air was used. Females aged 0-2 days did show some spontaneous activity in the same research. A similar relation might exist with our research on *An. gambiae*, but the females used for our experiments were all 6-9 days old. So, the strict distinction made in *Ae. aegypti* will not hold for *An. gambiae*.

Another striking characteristic of neuron A is its response to a change in flow. The control of CO_2, i.e. adding 300 ml min^{-1} of synthetic air to a main stream of 700 ml min^{-1} of synthetic air, stimulated neuron A. When this increase in flow was maintained, the activity of neuron A slowly (>10 s) returned to the spontaneous activity before the change in flow. We ruled out contamination of the air and change in air humidity as possible causes of stimulation. Also, CO_2 is unlikely to be the stimulus, as synthetic air was the only air passing the mosquito.

The origin of this response to a mechanical stimulus is not known. A possible explanation is that other sensilla on the palps, mechanosensors, registered this mechanical input. As a response, the mosquito might have moved the palp in a for us invisible way, resulting in stimulation of neuron A. Another explanation includes movement of the capitate peg sensillum by the mechanical stimulus, which somehow stimulated neuron A. In that case, the capitate peg sensillum would also have a mechanosensory function. A final explanation might be found in CO_2. Though synthetic air passed the sensillum, turbulence might have interacted with the surrounding air and have taken some CO_2 from the surrounding air to the sensillum. Further investigation will have to reveal the exact origin of the response of neuron A to change in airflow.

The air pump, used to pump air from the airbag to the valve, sucked in a small amount of air from its surroundings. This resulted in an unknown decrease in concentration of odorant. Comparing our data to the data of Lu *et al.* (2007) for OCT, we can compare stimulus 1 of frequencies 1-4 Hz with 0.001% OCT in their study. We found roughly an increase in spike rate of 40-50 spikes s^{-1}. Lu *et al.* (2007) found an increase in spike rate of 80-90 spikes s^{-1}. This difference is not solely due to the pump, since the presence of CO_2 increases the sensitivity to organic compounds (Dekker *et al.* 2005). When neuron A was stimulated with CO_2, we found an increase in spike rate of 10-30 spikes s^{-1}. Again Lu *et al.* (2007) found a larger increase in spike rate: 40-45 spikes s^{-1}. Now we do not have the problem of sensitization, but in our study the response of neuron A to mechanical stimulation appeared. Neuron A was already loaded with a response to this mechanical stimulation, resulting in a relatively lower response in our study due to CO_2 stimulation, compared with Lu *et al.* (2007).

Another source of error to the increase in spike rate was due to the AutoSpike program. AutoSpike did not always auto-detect every spike (e.g. when spike shape was distorted by two simultaneous spikes). In our study, which had recordings of spikes of large amplitude and small amplitude (<75% of the large amplitude), this resulted in correct detection of the spikes of large amplitude only. The data on neuron A stimulated with CO_2 were therefore not subject to this error, but the data on neuron B stimulated with OCT had some missing spikes resulting in an underestimation. Since we used bins of 100 ms, for a single recording a decrease in spike rate of 10 spike s^{-1} per missing spike existed. If spikes were missing, it is most common to miss 1 spike, but sometimes up to 3 spikes were missing. However, the effect of missing spikes was largely counteracted by averaging over multiple recordings.

The missing spikes will also have been of influence to data on interval 1 and 2. If a missing spike was of influence to interval 1, the ratio between interval 1 and interval 2 would be closer to zero and vice versa. The first case will lead to the conclusion that the neurons can more easily distinguish between subsequent stimuli. As we have seen, this is not the case for all stimuli. If missing spikes influenced interval 2, then the averaging over 5 inter-spike intervals largely corrected for this error.

The ratio of interval 2 / interval 1 close to 1, with stimuli belonging to interval 2, was found in stimuli 2-3 for neuron A stimulated with CO_2 at 4 and 5 Hz. For neuron B stimulated with OCT, we saw a similar pattern plus some additional stimuli with a ratio of interval 2 / interval 1 close to 1. It is therefore probable that when stimulated at 4 Hz with 0.2 s stimuli, not all neurons A and B were always able to distinguish between the first three subsequent stimuli. The same holds for neuron B stimulated with OCT when stimulated at 5 Hz with 0.1 s stimuli. However, some neurons A stimulated at 5 Hz with 0.1 s stimuli with CO_2, were not able to distinguish between the first two stimuli only.

For neuron B this conclusion is supported by our finding of an increase in spike rate per bin per stimulus. The first and last bin, when stimulated at 4 Hz, and all bins when stimulated at 5 Hz, had no significant difference in increase in spike rate. So it is plausible that some neurons continued their activity at the same spike rate during the interval between two subsequent stimuli. For neuron A we did not find this relation. The influence of the response of neuron A to the mechanical stimulus was too large for any additional spike to be found relative to the control.

Next to the changing ratio of interval 2 / interval 1 that decreased roughly during the first 3 stimuli, there was also the change in latency, which changes roughly until the fourth stimulus. These are both indications of adaptation in the first 3 stimuli.

As stated before, pulsed CO_2 stimuli as well as continuous organic compound stimuli increase upwind flight in *An. gambiae* (Dekker *et al.* 2001). The present study shows neuron A to better distinguish between subsequent CO_2 stimuli than neuron B distinguishes between subsequent OCT stimuli. As a consequence, it is tempting to say that a causal relation exists between these properties. For this to hold, we would have to know the response and adaptation of all ORNs of all olfactory sensilla to other organic compounds as well. A strong ability to distinguish between subsequent stimuli for CO_2 as well as less adaptation to stimuli of organic compounds was also suggested by Geier *et al.* (1999) as a possible characteristic of ORNs

Whether the adaptation found in this study is an active process, i.e. actively desensitizing ORNs, or a passive consequence of physiological characteristics, such as depletion of odour binding proteins in the ORNs, is not known. It is clear that after some adaptation, neurons A and B of the capitate peg sensillum can distinguish between 0.2 s stimuli up to at least 4 Hz and 0.1 s stimuli up to at least 5 Hz of CO_2 and OCT respectively. Whether these frequencies can be reached at a higher level in the central nervous system is not known and the characteristics of other neurons come into play (Grant *et al.* 1995). It is proposed though, that different neurons working together can increase temporal resolution (Vickers 2006).

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