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SPECTRAL CHARACTERIZATION OF CILIARY BEATING

TEMPERATURE DEPENDENCE ON SPECTRAL PARAMETERS

Dan ESHEL and Zvi PRIEL

Department of Chemistry, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

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Ciliary beating was optically examined in tissue cultures from frog palate epithelium. Consecutive segments of the analog signal were Fast-Fourier transformed. The three main parameters which define the spectrum, position of the peak maxium ($\bar{f}$), width of the spectral line ($\overline{S.D.}$), and area ($\bar{A}$) under the spectrum, were all measured as a function of temperature. These measurements were also examined as a function of the number of cilia by varying the examined area from 1.2 to 122 μm^2. It was found that: (a) all the parameters were exponentially temperature dependent; and (b) the average frequency was independent of the number of cilia examined, while $\overline{S.D.}$ was dependent on it. On a physiological level, we demonstrated that the ciliary fluctuation in frequency is temperature dependent, increasing with increase in temperature. Moreover, it was shown that where a relatively small number of cilia were measured ($d = 1.24$ μm), the area A under the observed spectrum was directly proportional to the amplitude of ciliary beating. Increasing the temperature decreases the amplitude and vice versa. According to our suggested model the dependence of $\bar{A}$ on $\bar{f}$ was predicted and verified experimentally. A mathematical model which simulates the $\overline{S.D.}$ as a function of examined area and temperature is suggested. The calculated results from the model are in a good agreement with our experimental findings.

1. Introduction

Temperature affects almost all biological processes and systems including ciliary activity. Most of the experimental work on ciliary activity and temperature has been performed on trachea of rabbit [1–7], rat [8,9] and guinea pig [10], and the human respiratory tract [11,12]. Among the methods employed were stroboscopic measurements [3,14], cinematography [1,6,8,12] and photoelectric techniques [2–5,7].

The results vary widely both with species and method. Even measurements by different groups on the same species, using essentially the same technique, have not given consistent results [3,5,7]. Therefore, it is not surprising that all conclusions about the effect of temperature on ciliary beat frequency are still unclear. Mercke et al. [5] claimed that frequency is linearly dependent on temperature and that increased temperature brought about a more uniform rhythm. Gabridge [13] and Kennedy and Duckett [4] claimed that the frequency is a sigmoidal function of temperature and that lower temperature brought about a more uniform rhythm [4,15].

Recently fast Fourier transform (FFT) has been adopted as a routine method of analyzing the frequency spectrum of ciliary beating [4,14]. This has resulted in more accurately measured and better defined frequencies. Furthermore, FFT analysis can yield additional information. The spectrum has three first-order parameters: the position of the spectral peak, the width of the peak and the area under the spectrum. Most ef-

forts until now have concentrated on the position of the spectral peak, which is the frequency of ciliary beating. We have shown [15] that the width of the spectral peak indicates the level of synchronization of cilia in the measured area. To our knowledge, no one has either discussed or used the third parameter, the area under the spectrum.

The aim of this work is to examine the temperature dependence of the three above-mentioned parameters, with the intention of determining their biological meaning.

2. Materials and methods

2.1. Preparation

Experiments were performed on locally supplied frogs (*Rana ridibunda*). The animals were decapitated and the palate was excised. The handling of preparation was described in detail previously [14]. Before the measurement the medium over the tissue culture was changed several times, until a total depletion of mucus was achieved. Therefore during the measurements there was no mucus over the cilia, so that the signals observed were derived directly from them.

2.2. Optical measurements of ciliary beating and data analysis

The preparation was viewed by an inverted microscope (Olympus, IMT), with a 50 μm diameter optic fiber placed in the focal plane of the ocular (Gama Scientific, 700-10-36A) in the manner described previously [14].

The following objectives employed were: $\times 4$, $\times 10$, $\times 20$ and $\times 40$, which correspond to field diameters of 12.5, 5, 2.5 and 1.25 μm, respectively. Assuming that on a single ciliary cell ($\sim 75\ \mu m^2$) there are about 50 cilia [16], and that the cilia density is uniform, the above-mentioned field diameters reflect 80, 15, 3 and 1 cilia, respectively, whose bases lie within the field. Collection and analysis of data and calibration of the system were the same as described previously [14,15].

2.3. Temperature control system

A hollow ring of glass was embedded in the petri dish. The preparation was positioned in the middle of the ring so the light could pass freely. Temperature-controlled water (cooled by a Haake KT33 cooling bath or heated by a Haake F4391 heating bath) was pumped through the ring. The temperature, measured by a termistor (GB35P8, Fenwal Electronics) positioned in the dish, was in the range 12.5–27.5°C.

3. Results and discussion

The three main parameters which define the spectrum ($\bar{f}$, $\overline{\text{S.D.}}$ and $\bar{A}$) were measured as a function of temperature which varied from 12.5 to 27.5°C. Within this range the effects were found to be reversible. Over this range of temperatures the viscosity of the medium changes by 30%, which definitely influences the ciliary beating. However, the effects which are presented and discussed below are about one order of magnitude larger. Therefore, the changes brought about through the direct influence of viscosity variation may be neglected to a first approximation.

3.1. Frequency

The frequency of ciliary beating is defined as the position of the spectral peak maximum. As seen in fig. 1, the frequency is independent of the dimensions of the examined area at all measured temperatures. This means that, at a given temperature, the cilia viewed were beating around the same average frequency value. However, the average frequency is exponentially temperature dependent, at least within the range of temperature scanned. As temperature increases, the spread around the average frequency increases. The average frequency as a function of temperature can be represented by the following equation:

$$\bar{f} = \hat{f} e^{-x/T} \tag{1}$$

where T is the absolute temperature, x defined by eq. 2, $\hat{f}$ a hypothetical frequency of ciliary beating

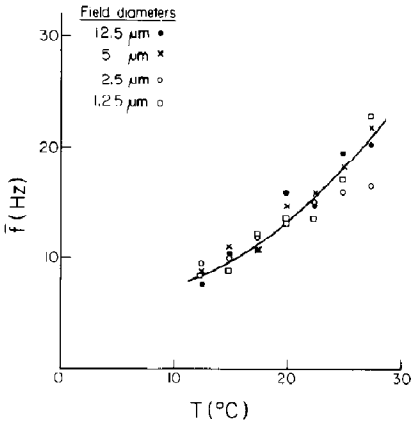

Fig. 1. Average frequency vs. temperature for various field diameters. Each point represents an average of values from 200 10-s spectra.

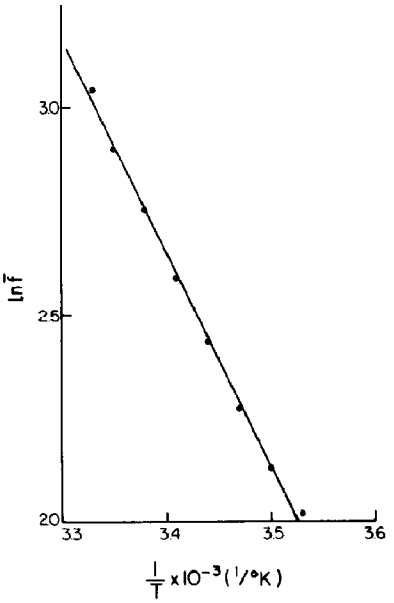

Fig. 2. ln $\bar{f}$ vs. $1/T$ (K). The $\bar{f}$ values were taken according to the average curve in fig. 1.

at temperature $T \to \infty$, and $\bar{f}$ the measured average frequency.

According to eq. 1, ln $\bar{f}$ has to be a linear function of $1/T$. Fig. 2 shows that it is. Moreover, eq. 1 is of the general form of the Arrhenius equation for a chemical reaction, where

$$x = \frac{\Delta E}{k} \tag{2}$$

in which ΔE is the activation energy and k Boltzmann's constant.

Calculating the slope (x) of the straight line in fig. 2 and using eq. 2 we otain an activation energy (ΔE) of 10 kcal/mol, a typical value for a chemical reaction. Changing the temperature by 10°C changed the ciliary beating frequency by a factor of two, e.g. (fig. 1), at 15°C the frequency was 9.8 Hz, while at 25°C it was 18 Hz. This all seems to indicate that a change in temperature influences the rate of some chemical reaction involved in the mechanism of ciliary beating which then changes the frequency.

3.2. *Width of the peak (S.D.)*

The definition of S.D. is:

$$\text{S.D} = \sqrt{\frac{\sum s_i (f_i - \bar{f})^2}{\sum s_i}} \tag{3}$$

where f_i is the i-th frequency and s_i its power. We have previously shown that $\overline{\text{S.D.}}$ represents fluctuations in frequency and/or phase distribution of the metachronal wave in the examined area [15]. Moreover, it was shown [15] that when a relatively small area is examined (1.2 μm^2) the $\overline{\text{S.D.}}$ represents mainly fluctuations in frequency. As can be seen (fig. 3) the fluctuations in frequency are temperature dependent, decreasing with decreasing temperature. Over the range of measured temperatures the magnitude of fluctuations decreased by almost a factor of two. Additional support for this can be found on examining the behaviour of the average frequency vs. temperature plot (fig. 1). At higher temperature the spread of experimental results around its average is considerably higher than at the low temperature. Moreover, the decrease in the spreading of frequencies is a monotonic function of temperature. Furthermore, when an area of 2.5 μm field diameter is examined it is still small enough to neglect reasonably the phase distribution. Therefore, for any practical purpose these results (fig. 4) also represent ciliary frequency fluctuations as a func-

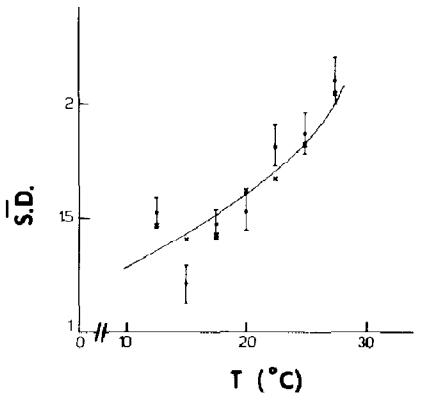

Fig. 3. The average linewidth of the spectral peak ($\overline{\text{S.D.}}$) vs. temperature at a constant field diameter ($d = 1.25$ μm). (●) Average values based on 200 experimental spectra for each temperature. (×) Calculated values according to the mathematical model, with parameter values given in table 1.

tion of temperature. But, according to the ergodic hypothesis [17], at every temperature the $\overline{\text{S.D.}}$ has to become smaller due to increasing numbers in the ensemble. As was indeed found, at any given temperature the $\overline{\text{S.D.}}$ measured at 2.5 μm field diameter (fig. 4) is lower than that at 1.25 μm field diameter (fig. 3).

To conclude, the frequency fluctuations of a cilium are an intrinsic property strongly dependent on temperature and increasing with increase in temperature. When the examined area is further increased to 5 μm field diameter (fig. 5) we can no longer neglect the phase distribution due to the existence of the metachronal pattern as was previously discussed [15].

At all measured field diameters, the $\overline{\text{S.D.}}$ is an exponential function of temperature. Therefore, a plot of ln $\overline{\text{S.D.}}$ vs. $1/T$ gives a straight line with an activation energy around 8 kcal/mol, a typical graph being reproduced in fig. 6. The activation energy obtained is very similar to that derived from the slope of ln f vs $1/T$ in fig. 2. The $\overline{\text{S.D.}}$ also appears to depend on the dimension of the examined area (fig. 7). We previously showed [15] at 23°C that such a curve represents two simultaneously measured effects: (a) fluctuations in frequency with time; and (b) phase distribution among the cilia comprising the metachronal wave. In our present study we examined these effects at temperatures in the range 12.5–25.5°C. Three representative curves are reproduced in fig. 7. The resultant curves at all temperatures were almost identical in shape, though translated vertically on

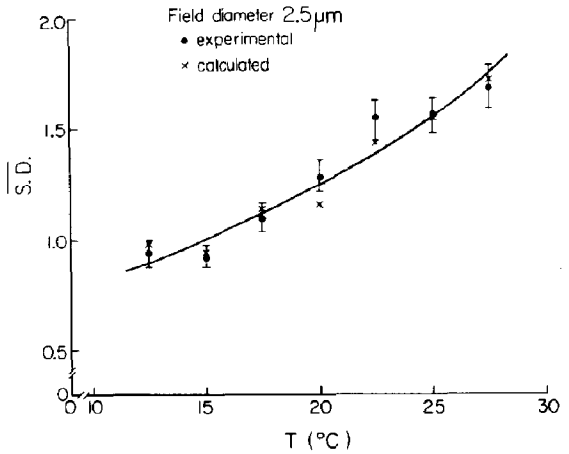

Fig. 4. The average linewidth of the spectral peak ($\overline{\text{S.D.}}$) vs. temperature at a constant field diameter ($d = 2.5$ μm). (●) Average values based on 200 experimental 10-s spectra for each temperature, (×) calculated values according to the mathematical model, with parameter values as appearing in table 1.

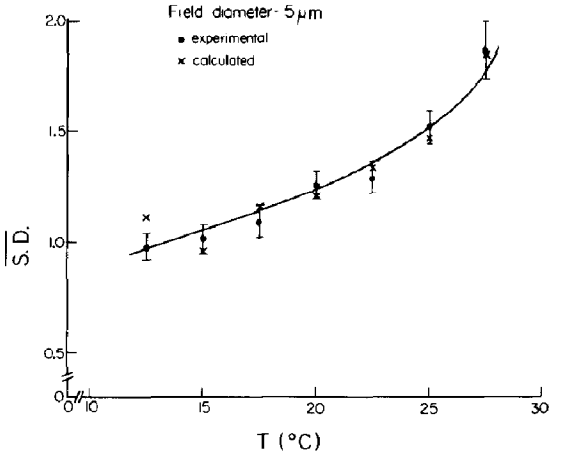

Fig. 5. The average linewidth of the spectra peak ($\overline{\text{S.D.}}$) vs. temperature at a constant field diameter ($d = 5$ μm). (●) Average values based on 200 experimental 10-s spectra for each temperature. (×) Calculated values according to the mathematical model, with parameters values as appearing in table 1.

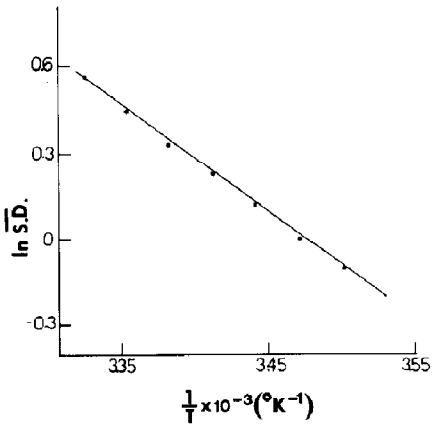

Fig. 6. ln S.D. vs. $1/T$. The S.D. values were taken according to the average curve in fig. 3.

the graph with increasing temperature. At lower temperatures the frequency fluctuations are significantly smaller.

Therefore the S.D. values should, accordingly, also be smaller at all field diameters. It is interesting to note that although the average frequency is independent of the size of the measured area the width of the peak is strongly dependent on it.

In order to explain the S.D. dependence on

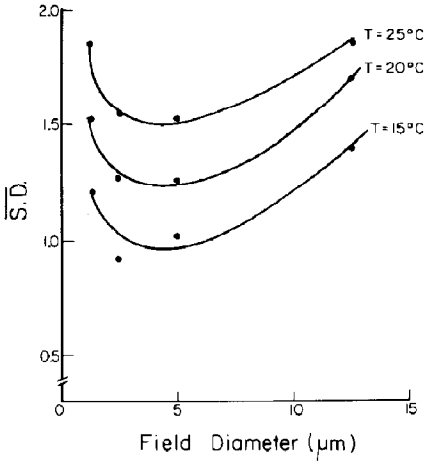

Fig. 7. The average linewidth of the spectral peak (S.D.) vs. field diameter at three different temperatures. Each point represents an average of values from 200 10-s spectra.

temperature and examined area, we suggest the following model:

Most cilia within a relatively small area are synchronized in a metachronal wave pattern, as was observed by Aiello and Sleigh [18], for short periods of time, in frog palate epithelium. However, part of the ciliary ensemble may beat with different frequencies from the average one. Over time, the deviant cilia are forced to beat with the average frequency and phase of the ensemble, according to Gray's principle of minimum interference [19], while other cilia within the ensemble may proceed to deviate, in a continuous dynamical process [15]. The size of the fraction of cilia deviating from the average frequency at any given time is a function of temperature. In order to show the validity of the suggested model we extended our previous model [14] to include this case too (see appendix). The points in figs. 3–5 represent experimental findings while the crosses are calculated values based on the suggested model. The experimental and calculated results are in close agreement.

3.3. The area under the measured spectrum

The absolute value of the area under the spectrum is a function of the intensity of the illuminating lamp, the optical geometry of the experimental setup, and the light scattering due to ciliary motion. If the two instrumental parameters are kept constant, the changes in the measured area represent changes in ciliary motion. When all the above conditions were kept constant, the area maintained a reasonable constant value.

Increasing the temperature from 12.5 to 27.5°C decreased the spectrum area by a factor of approx. 5, quite a drastic effect, at all field diameters. A typical curve is given in fig. 8 for a field diameter of 1.25 μm representing a relatively small number of cilia. Under such experimental conditions the change in spectral area represents actual change in ciliary amplitude.

Therefore, according to our results, the actual amplitude of a cilium is temperature dependent, decreasing with increasing temperature. This seems quite reasonable. When the frequency is low (low temperature) the cilium has an opportunity to

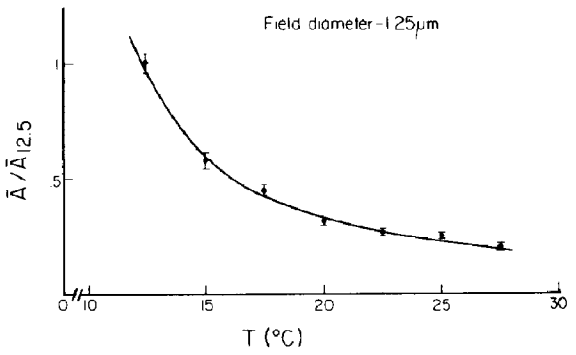

Fig. 8. Average area under the spectrum ($\bar{A}$) vs. temperture at a constant field diameter ($d = 1.25$ μm). The values are normalized with respect to the value of $\bar{A}$ at 12.5°C. Each point represents an average of values from 200 10-s spectra.

swing over a large arc resulting in high amplitude, while when the frequency is high (high temperature), the beating is over a small arc with subsequently lower amplitude, as was previously shown [18,20]. *

This dependence can be explained by the following model. Assume that the cilium behaves like a rigid rod during its effective stroke, thus its kinetic energy is:

$$E_k = \tfrac{1}{2} I \bar{f}^2 \tag{4}$$

where I is the moment of intertia, and can be represented by:

$$I = \int_0^R \rho R^2 \mathrm{d}R = \tfrac{1}{3}\rho R^3 \tag{5}$$

where R is the actual cilium length involved in the beating process and ρ is the average density of the cilium.

Introducing eq. 5 into eq. 4 gives:

$$R = \left(\frac{6E}{\rho}\right)^{1/3} \bar{f}^{-2/3} \tag{6}$$

The spectrum area is proportional to the square of the signal amplitude which, in turn, is proportional to the actual cilium amplitude, therefore:

$$\bar{A} = k'R^2 = k\bar{f}^{-4/3} \qquad \left[k = k'\left(\frac{6E}{\rho}\right)^{2/3}\right] \tag{7}$$

The same model can be applied to an ensemble of cilia provided that they beat with the same phase, or if the phase difference between them is relatively small. Such conditions apply when the examined field diameter is relatively small compared to the wavelength of the metachronal wave. This requirement is definitely fulfilled for a field diameter of 1.25 μm. When the number of cilia is increased from one cilium to N cilia (beating at the same phase) the proportionality constant (k) in eq. 7 will be altered, without, however, any additional changes being introduced into the equation.

Representing our experimental results according to eq. 7, for small examined areas (1.25 and 2.5 μm field diameter) resulted in a straight line (fig. 9). It is reasonable to assume that the kinetic energy (E) is linearly dependent on absolute temperature. However, since the relative changes in temperature are small ($\Delta T = 15$°C while $T =$

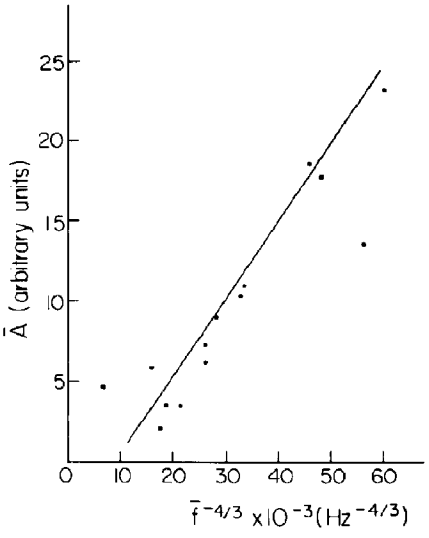

Fig. 9. Average area under the spectrum ($\bar{A}$) vs. $\bar{f}^{-4/3}$ at small field diameters ($d = 1.25$ and 2.5 μm). Each point represents an average of values from 200 10-s spectra.

* These two papers which agree with our findings deal with different ciliary systems and therefore a comparison with them has to be undertaken with caution. However, as far as we know, there are to date no other relevant data with which to compare.

285°C representing a change of 5% only) the expected change in kinetic energy is within our experimental accuracy. Therefore, we may take the kinetic energy as virtually constant. The correspondence between the experimental results and the suggested model strengthens our basic assumption that the change in the spectrum area reflects changes in the actual cilium amplitude under the appropriate conditions (small areas).

4. Conclusion

We have shown that the three main parameters which define the spectrum peak have physiological meaning, and can be used to describe more accurately the process of ciliary beating.

All three parameters are exponentially dependent on temperature: $\bar{f}$ and $\overline{\text{S.D.}}$ decreasing with decrease in temperature, while $\bar{A}$ increases with decrease in temperature.

Mathematical models are suggested which confirm our basic assumptions.

Appendix

In order to demonstrate the feasibility of the suggested model we have extended our previously simulating model [14] to include also effects of temperature and the number of cilia measured as will be explained below. If a sine function is chosen to describe the optical signal obtained by the beating of a cilium, then, at a given moment the signal $g(t)$ is given by:

$$g(t) = a \sin[f(t) \cdot t] \tag{8}$$

where a is the amplitude and f the frequency.

According to our model, f is time dependent and its value capable of changing over relatively small intervals of time. Whether the cilium will beat with frequency $\bar{f}$ or another frequency is determined by $1 - P(T)$, the probability of the cilium changing its frequency from the average one. To determine if the cilium's frequency will be the average or not we sample a random number between 0 and 1 (in a uniform distribution) for every period of time ΔT. If the number sampled is less than P (for a given temperature) then the cilium's frequency will be the average one. If the number sampled is greater than P then we sample a new frequency for the cilium (in the manner described below) and its beats with the new frequency for the next Δt seconds.

The time interval Δt, is defined as the average time period of ciliary movement ($1/\bar{f}$, and is temperature dependent. The new frequency is sampled randomly around $\bar{f}$, in a normal distribution with standard deviation $\pm\sigma$, as previously described [14].

If all the cilia at the measured field beat with the same amplitude, then the absolute value of the amplitude does not affect the $\overline{\text{S.D.}}$ obtained. Therefore, for simplicity 'a' was taken as a constant. The standard deviation (σ) influences the spectrum as was previously shown by us [14], and may be temperature dependent. However, it is not the only parameter influencing the spectrum. Increasing σ resulted in broadening the measured spectrum or multipeak spectrum; the same effect is achieved by decreasing $P(T)$. Therefore, in order to decrease the number of free parameters, we have chosen a constant value for σ independent of temperature: $\sigma = 1.5$. Any other choice of σ between 3 and 1 (the range of σ values which may fit our experimental results) will change the absolute values of P, without any additional changes.

Using the above-mentioned assumptions, eq. 8 and frequencies $f(t)$ chosen according to the description above, we create simulated optical signals $g(t)$ while we then treat them by the same procedures used for experimental signals, to obtain simulated $\overline{\text{S.D.}}$ values. The correspondence between the calculated $\overline{\text{S.D.}}$ and the experimental ones is achieved by the only remaining free parameter, $P(T)$. It was found that the best fit of calculated to experimental results for an examined area of 1.22 μm^2 (fig. 3) was achieved using the set of P values given in table 1. Further, it is reasonable to assume that the P and σ values are an intrinsic property, not dependent on the size of the observed field. When we applied this assumption to the case of a field diameter of 2.5 μm, taking into account the proportional increase of the number of cilia measured and keeping all

Table 1

Values of the temperature-dependent parameters of the model used in simulations

T (K)	$\bar{f}$ (Hz)	Δt (s)	P
12.5	7	0.143	0.90
15.0	9	0.110	0.94
17.5	10	0.100	0.93
20.0	12	0.083	0.90
22.5	14	0.071	0.52
25.0	17	0.059	0.44
27.5	23	0.043	0.30

other parameters as in the case of the 1.25 μm field diameter, a good fit to the experimental results was obtained (fig. 4). This indicates that these parameters are indeed an intrinsic property of the cilium and that the ergodic hypothesis is applicable to this system.

Increasing the field diameter from 2.5 to 5 μm introduces a non-negligible phase difference of 180° between cilia at opposite edges of the examined area [15,16]. In order to simulate this situation in a simple manner, we first divide the 5 μm field into two halves. We then define an average phase difference, φ, as the difference in average phase between all cilia beating at the main field frequency, $\bar{f}$, in one half of the field and all cilia beating with $\bar{f}$ in the second half. All other parameters were the same as for the one cilium case. It was found that $\varphi = 120°$ gave the best results (fig. 5), and was independent of temperature. This was in good agreement with the observed metachronal wavelength [16].

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