Mechano- and electrophysiological studies on cochlear hair cells and lateral line cupulae

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Chapter 3

Superficial neuromast mechanics in the zebrafish
(Danio rerio)
ABSTRACT

The sub-micrometer mechanical behaviour of cupulae of the zebrafish (Danio rerio) superficial lateral line system in response to vibratory fluid jet stimuli was measured using a microscopically guided laser interferometer. Cupular displacement as a function of fluid velocity measured at tens of micrometers above the sensory epithelium is almost constant at low frequencies (<20 Hz), reaching mechatro-sensitivities in the range of 1-5 nanometer cupular displacement per micrometer/second fluid velocity. The base region of the cupula driving the sensory hair bundles is estimated to possess only a fraction (20-30%) of this mechatrosensitivity. The cupular mechatrosensitivity as a function of stimulus frequency of the driving fluid velocity gradually decreases at slopes of approximately 10 dB/dec from low frequencies up to about 70 Hz. At higher frequencies cupular displacement declines with 20 dB per decade increase of frequency of fluid velocity. Measurements at different heights show that the cupula bends during stimulation. A model, previously developed to describe hydrodynamic cupular excitation of lateral line canal cupulae, can only partially account for the observed cupular dynamics in superficial neuromasts. An improved description was obtained by taking into account the frequency dependent fluid boundary layer along the fish skin. The results suggest that the stiffness coupling to the hair cells of the sensory epithelium is governed by the compliance of the cupula. Detection properties of zebrafish superficial cupulae are compared with those of canal neuromasts of the ruffe (Gymnocephalus cernuus), indicating that canal neuromasts possess an overall superior signal-to-noise ratio.
INTRODUCTION

The mechanosensory lateral line system in aquatic vertebrates is a sensory system for the detection of local low-frequency water displacements. It is involved in several biological functions, such as the detection of prey (Dijkgraaf, 1963; Bleckmann, 1980; Montgomery and MacDonald, 1987; Enger et al., 1989; Janssen et al., 1994) and stationary objects (Weissert and von Campenhausen, 1981; von Campenhausen et al., 1981; Hassan et al., 1992) as well as schooling (Pitcher et al., 1976; Partridge and Pitcher, 1980). The functional units of the lateral line system are the neuromasts. Each neuromast consists of a sensory epithelium, the macula, which contains sensory hair cells with supporting cells in between. The hair cells, which are the actual mechanosensory cells in the neuromast, have a highly specialised organelle positioned on their apical side called the hair bundle. The hair bundle is usually composed of several tens of actin-filled membrane extrusions, the stereocilia, which are graded in height and arranged in a staircase-like manner. Each hair bundle also contains a single kinocilium, which is located on the side of the tallest stereocilia. A cross section through a bundle shows a hexagonal arrangement of the stereocilia, while the kinocilium is clearly recognisable by its 9+2 microtubular structure (Flock et al., 1962). Deflection of the bundle in the direction of the tallest stereocilia and kinocilium favours the opening of the mechanically-gated channels (transducer channels) and thus increases the flux of cations into the cell (Hudspeth, 1989). Deflection in the opposite direction leads to closure of the transducer channels.

Within one neuromast, hair cells are found with opposing bundle polarity (Flock et al., 1962), which are mainly paired due to their development in the macula (Rouse and Pickles, 1991). The hair bundles within one macula protrude into an overlying structurally-organised driving element called cupula, which mechanically couples the fluid flow to the hair bundles. A distinction is made between neuromasts located in the fish’s epidermis with their cupula protruding in the water surrounding the fish, called superficial neuromasts (SN), and canal neuromasts (CN), which are recessed in the scales or in bony canals (Coombs et al., 1987). SNs are found in almost all species of fish and in amphibians and CNs neuromasts exclusively in fish.

The SNs and CNs not only differ in their location on the animal, they also show distinct morphological differences, like the number of hair cells, the size and shape of their cupula (Coombs et al., 1987; Janssen et al., 1987; Münz, 1989) and the number of afferent nerve fibres innervating a neuromast (Münz, 1989). Both
types of neuromast also respond to different kinds of stimuli. The combination of canal and cupula in the CN-system acts as a water acceleration detector (van Netten, submitted) as shown by measurements on CN cupular motion (van Netten, 1991; van Netten and Kroese, 1987) and afferent fibre recordings (Kroese and Schellart, 1992), whereas the SN-system can be considered a detector of water velocity (Kroese et al., 1978; Kroese and Schellart, 1987; Kroese and Schellart, 1992; Engelmann et al., 2000; Kroese et al., 1980).

In the present study we performed detailed measurements of cupular motion in the zebrafish, which in early development contains only SNs. They are distributed over several locations on the head, where they form the anterior lateral line system, and along a line on the side of the body and tail, forming the posterior lateral line system. The lateral branch of the posterior lateral line contains seven to eight neuromasts along the head to tail midbody line (Metcalfe, 1985). On average each neuromast contains about ten hair cells with pear-shaped cell bodies that are arranged in a rosette-like formation. The apical surfaces of the hair cells, which carry the hair bundles, are slightly recessed in a small pit formed by two crescent-shaped epidermal cells (Fig. 1). The hair bundles, each with a long kinocilium, have two opposing polarities (Williams and Holder, 2000; Whitfield, 2003) and protrude into a column-shaped cupula.

The zebrafish hair cells are functionally active at day 5 after fertilisation (Metcalfe et al., 1985). This has also been shown by measurements of the microphonic potentials, extracellular potential changes due to transducer channel activation (Nicolson et al., 1998). The sensory-neuron growth cones co-migrate with the primordium (Metcalfe, 1989) so that by this time the hair cells are also innervated by afferent sensory neurones (Alexandre and Ghysen, 1999).

Conclusions regarding the physiological response properties of SNs are all based on action potential recordings of the lateral line nerve. However, no direct micromechanical measurements of the superficial neuromast cupula have been described so far. Based on the relative short length of the cupula (< 50 µm), its hydrodynamic response is likely to be heavily affected by the boundary layer of the fish surface (Kalmijn, 1987).

Here we present the first measurements of the mechanical response of superficial neuromast cupula in the tail region of the developing zebrafish. To describe the underlying mechanics of the cupular response of the SN, the data are fitted by a model originally developed to describe the mechanical behaviour of CNs extended with the hydrodynamic properties of the fluid motion in the...
boundary layer. In SNs the elastic coupling of the cupular mid-regions to the sensory epithelium, which in CNs is accounted for by the hair bundle stiffness, is found to be several orders of magnitude smaller than the expected hair bundle stiffness, and is therefore most likely dominated by the compliant cupula. The results obtained on the superficial lateral line system are compared with previous results of the canal lateral line system with respect to sensitivity and signal-to-noise ratio of hydrodynamic stimulus processing.

Figure 1: Transmission electron micrograph showing a cross-section through three hair cells (HC) of a zebrafish neuromast. The hair cells are surrounded by cytoplasmic extensions of the supporting cells (SC) positioned underneath the hair cell body. Note the staircase like arrangement of the stereocilia (arrow) and opposing hair bundle polarity. Sections of kinocilia (K) are visible. Also the pit formed by the two epidermal cells (EC) can be clearly seen. Scale bar: 10 µm. Photomicrograph: Laboratory of Cell Biology and Electron microscopy, University of Groningen.
CHAPTER 3

METHODS

Wild-type zebrafish (*Danio rerio*), kept at 26° C and subjected to a 13-11 hour light-dark cycle were used to breed young zebrafish. Cupular mechanics was measured in zebrafish ranging from day 5 (PF5) at which the organ is functional, to day 14 (PF14) after fertilisation. The time of spawning was not exactly monitored, and has an uncertainty in the order of hours. Developmental stages will, therefore, be referred to in days after fertilisation, with PF0 being the day of fertilisation. After natural spawning the eggs were collected from fish tanks with a nylon grid that permits the eggs to pass. The eggs were rinsed with 10% Modified Barth’s Solution (10% MBS) containing in mM: 8.8 NaCl, 1 KCl, 2.4 NaHCO₃, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 1 HEPES, pH 7.4. Eggs were transferred in a petridish with 10% MBS with additional methylene blue (0.2 mg/100 ml) that was used to discriminate between fertilised and non-fertilised eggs. Blue non-fertilised eggs were taken out at PF1 and at this time the solution was replaced with 10% MBS without methylene blue.

Individual zebrafish larvae were anaesthetised using 10% MBS containing 0.02% MS222 (Tricaine, Sigma) before being transferred to the experimental bath containing the same solution. The fish were mechanically fixed in a bath by stabilising their head into a mould made from Sylgard 164 (Dow Corning), which adhered to the glass bottom of the recording chamber. A thin strip of parafilm, stretched across a stainless steel ring, was used to press down the tail close to the measurement position to mechanically stabilise the preparation. Care was taken not to obstruct the blood flow to the tail region. Stable blood flow up to the tip of the tail was used as a criterion to judge the condition of the fish during an experiment. Other criteria to monitor the quality of the preparation were the shape of the hair cells and the visibility of the hair bundles.

All animal procedures were in accordance with regulations of the Dutch Act on Laboratory Animals, and the institutional animal care and use committee (RuG-DEC).

Stimulus

Mechanical stimuli were applied using a piezoelectrically driven apparatus producing a fluid jet from a glass pipette. Pipettes were pulled on a Sutter P97 electrode puller (Sutter Instruments, CA, USA) and had tip diameters ranging from 30 to 50 µm, resulting in a fluid displacement-stimulating device up to several tens of hertz (see chapter 2). The fluid jet (10% MBS with 0.02% MS222) was directed along the anterior-posterior axis at an angle of 20° with the horizontal plane and at
a distance of 100 to 200 µm from the cupula. The position of the fluid jet was adjusted so that it was aimed at the centre of the cupula. Sinusoidal signals were generated by a 16-bit DA converter (Ariel, DSP-16) filtered by an 8-pole Butterworth filter and subsequently attenuated to the desired amplitude before application to the stimulus device.

**Cupular motion detection**

Cupular motion in response to mechanical stimulation was detected using a heterodyne laser interferometer (van Netten, 1988; Chapter 2, Fig. 2), which was coupled to a transmitted-light microscope (Axiotron, Zeiss, Germany), modified into a fixed stage configuration and equipped with Nomarski differential interference contrast optics. Polystyrene microspheres (PolySciences) were attached to the poorly reflecting cupula, to locally increase the reflectivity well above the level required for proper operation of the laser interferometer. The microspheres had a diameter of 1.053 µm and a density close to that of water (1050 kg/m³) and were applied via a gravity-driven fluid flow (10% MBS with 0.02% MS222) emerging from a glass micropipette (Ø 5 µm) placed close to the cupula. A steady stream of fluid carrying the microspheres was blown against the cupula at a flow rate that hardly displaced the cupula, until a microsphere became attached to the cupula. In many cases a sphere did not strongly adhere and was visibly moving due to Brownian motion, often drifting off after tens of seconds. A well-attached sphere did not show microscopically detectable signs of Brownian motion. Spontaneous release of a sphere was often preceded by a sudden increase in the noise of the laser interferometer output signal. The noise of the motion detected with the laser interferometer was thus used as a parameter to judge the quality of attachment. Spheres were applied on the side of the cupula opposite to the mechanical fluid jet stimulus. After attachment of a sphere the application pipette was moved several hundred micrometer away from the cupula. Laser light reflected by the sphere, containing information about the sphere velocity, was detected by a photomultiplier (model H6780-02, Hamamatsu, Japan). A modified demodulator (Polytec, Germany) was used to demodulate the photo-multiplier output and produced a voltage output which was linearly proportional to the velocity of the sphere within the range of 10^{-4} to 10^{3} µm/s (van Netten, 1988).

Frequency responses of the cupula were obtained by applying sinusoidal fluid jet stimuli at frequencies ranging from 5 to 1000 Hz to the cupula. The demodulated signals are a calibrated measure of cupular velocity and were filtered at 8 times the stimulus frequency (8-pole Butterworth) and subsequently amplified.
In response to each stimulus a number of consecutive stretches of cupular velocity, each 16 stimulus periods in duration, were sampled at 32 times the stimulus frequency and on-line averaged by the data acquisition board (Ariel, DSP-16). The averaged responses at each frequency were saved to disc and the Fast Fourier Transform (FFT) was used to calculate the amplitude and phase of the response component at each stimulus frequency. The frequency responses obtained this way are shown as sensitivity curves with respect to excitatory fluid velocity. The amplitude of the sensitivity is consequently expressed as cupular displacement in nanometer per excitatory fluid velocity expressed in micrometer per second (nm/(µm/s)). Note that this sensitivity unit equals milliseconds and that its actual value at low frequencies is a measure of the time-constant of the cupular impulse response (van Netten, submitted).

**Fluid jet calibration**

The cupular motion data were corrected for the fluid jet frequency response as described in Chapter 2. In short, the frequency response of a fluid motion sense probe (a sphere attached to a very compliant glass fibre) was recorded in response to stimulation with a piezoelectric-driven stimulus sphere with a known frequency response. Subsequently the same sense probe was positioned approximately 200 µm above the cupula so that it could be used to measure the frequency response of a fluid jet directly after its application to measure the frequency response of the cupula. The frequency-dependent characteristics of the fluid jet, so obtained, were used to correct the measured cupula displacement. The measurements as a function of frequency are depicted as mechanosensitivity curves, defined as cupular displacement per fluid velocity in units of nm/(µm/s).

**Cupula and boundary layer model**

A mathematical model for cupular mechanics previously developed for lateral line canal neuromasts (van Netten, 1991), in combination with the hydrodynamics of laminar frequency dependent boundary layer above a infinite plate (e.g. Lamb, 1932), was used to describe the cupular frequency responses. In the model, the cupula is represented as a half sphere sliding over a frictionless plate. The cupula is assumed to be elastically coupled to the reference frame (van Netten, 1991). The sensitivity of the cupula, \( S_c(f) \), defined as cupular displacement, \( X_0(f) \), per excitatory fluid velocity flow past the cupula with amplitude \( V \) and frequency \( f \), is then described by (van Netten, submitted):
\[ S_c(f) = \frac{X_0(f)}{V} = \frac{1 + \frac{1}{2} \sqrt{2}(i+1) \left( \frac{f}{f_t} \right)^2 + \frac{1}{3} i \frac{f}{f_t}}{N_r + i \left( \frac{f}{f_t} \right) + \frac{1}{2} \sqrt{2}(i-1) \left( \frac{f}{f_t} \right)^{3/2} - \frac{1}{3} \left( \frac{f}{f_t} \right)^2} \]  

(1)

with

\[ f_t = \frac{\mu}{2 \pi \rho a^2} \]  

(2)

and

\[ N_r = \frac{S a \rho}{6 \pi \mu^2} \]  

(3)

where \( \rho \) is the fluid density, \( \mu \) is the dynamic viscosity, \( a \) is the radius of the cross-section of the cupula at the height above the epithelium at which the measurements were taken, and \( S \) is the stiffness of the elastic coupling to the frame of reference. The parameter \( f_t \) is a transition frequency, separating the viscous and inertial regimes in the frequency domain. It effectively scales the frequency axis. The parameter \( N_r \) is the resonance number (van Netten, submitted). For \( N_r < 1 \), the -3dB cut-off frequency, \( f_c \), defining the upper bound of velocity detection is given by \( f_c = f_t N_r \) (van Netten, submitted).

To include effects of the frequency dependent properties of the fluid boundary layer next to the fish’ skin, we employed a solution originally due to Stokes (1851). It describes the fluid velocity attenuation in the boundary layer, \( A(f, z) \), defined as the fluid velocity, \( V(f, z) \) at frequency \( f \) parallel to and at a height \( z \) above the fish surface, divided by the fluid velocity, \( V_0 \), far outside the boundary layer (e.g. Lamb, 1932):

\[ A(f, z) = \frac{V(f, z)}{V_0} = \left[ 1 - \exp \left( -z \left( \frac{1+i}{\delta(f)} \right) \right) \right] \]  

(4)

where

\[ \delta(f) = \sqrt{\frac{\mu}{\rho f}} \]  

(5)

defines the frequency dependent boundary layer thickness or penetration depth within which the amplitude and phase of the fluid flow change most significantly (Batchelor, 1967).
Fits to the averaged measured data were made using two approaches. The first consisted of solely the cupular model (i.e. using \( S_c(f) \) in Eq. 1, see Fig. 3B) while taking \( V \) as the calibrated fluid-jet velocity whilst \( N_r \) was adjusted by eye. The transition frequency \( f_t \) was kept fixed at 6366 Hz, as a result of inserting the cross-sectional radius of the cupula, \( a = 5 \) µm, and taking \( \rho = 1000 \) kg/m\(^3\) and \( \mu = 10^{-3} \) kg/(m·s) in Eq. 2. The stiffness, \( S_c \), could then be determined using the obtained values for \( N_r \) and using Eq. 3.

Alternatively, as shown in Fig. 3C, the cupular and boundary layer model were combined (Eqs. 1 and 4), defining the overall sensitivity, \( S_{cbl} \), per fluid velocity outside the boundary layer by:

\[
S_{cbl}(f, z_0) = A(f, z_0) \cdot S_c(f)
\]  

Fits to the data made by using Eq. 6 thus include the attenuation imposed by the boundary layer on the fluid velocity, \( V_0 \), as emerging from the calibrated fluid jet apparatus, yielding values for the resonance number \( N_r \) thus leading to an estimate of the stiffness coupling, \( S_c \), and the parameter defining the height of the cupula, \( z_0 \), at which the measurement was taken.

**Microphonic potentials**

Microphonic potentials were measured using an Axopatch 200B patch clamp amplifier (Axon Instruments Inc., CA, USA) in the current clamp mode (\( I = 0 \)). Soda glass pipettes (tip diameter \( \approx 1 \) µm) were filled with 10% MBS, resulting in electrical resistances in the order of 20-40 MΩ. They were positioned just above the epithelial layer, close to the cupula without touching it.

Signals for fluid jet stimulation of the cupula used during recording of microphonic potentials were generated using the full amplitude range of a 12 bit CED 1401Plus data acquisition board in combination with the Signal software package (CED, Cambridge, UK). The recorded potentials were subsequently filtered and attenuated to the desired amplitude (~2 µm at the tips of the kinocilia) based on visual inspection of cupular motion. The sine wave stimuli consisted of 20 periods at 8 or 16 Hz. The response during the last 16 periods was filtered (eight-pole Bessel filter) at 16 times the stimulus frequency and recorded at 64 samples per stimulus frequency period. The traces were further low-pass filtered (-3 dB at 40 Hz) off-line with the finite impulse response filter implemented in the Signal software to remove frequencies above the expected dominating frequency at twice the stimulus frequency.
RESULTS
Mechanical and electrophysiological data were obtained from the three terminal neuromasts of the lateral branch of the posterior lateral line, located at a few hundred micrometers from the tip of the tail. At this location the tail region is thin allowing for high quality visualisation using Nomarski differential interference contrast optics. This technique enables to discern individual hair bundles and kinocilia of the terminal neuromasts. Usually three terminal neuromasts were present, but occasionally there were only two. In some cases visualisation of the hair bundles was hampered by pigmentation, which also precluded interferometric measurements. The height at which the microsphere attached to the cupula relative to the epithelial layer was determined using the calibrated focussing unit of the microscope. The length of the tallest kinocilium and the length of the hair bundles were determined similarly. The average number of hair bundles per (terminal) neuromast was 10.4 ± 1.7 (n = 51, PF5-10), and the bundle’s average height was 5.2 ± 0.6 µm (n = 47, PF5-10). The average height of the tallest kinocilium per neuromast was 29.7 ± 2.9 µm (n = 52, PF5-10). The average height of the cupulae was about 45 µm.

Microphonic potentials
To verify whether the hair cells of a neuromast already had operational mechano-transducer channels on day 5 and to check whether the transducer machinery was still intact after the procedure of mechanically fixing the fish, microphonic potentials were measured from the terminal neuromasts. Fig. 2A shows microphonic potentials in response to a fluid jet stimulus that induced vibrational displacements of the cupula at 16 Hz. The voltage signal driving the piezoelectric disc of the fluid jet apparatus is shown on top. The microphonic potential contains a clear component at twice the frequency of stimulation (32 Hz), resulting from the two populations of hair cells within one neuromast, which are morphologically polarised (Kuiper, 1956; Flock et al., 1962). The harmonic distortion in the stimulus can be shown to be small, inducing a second harmonic component about 40 dB smaller than the fundamental frequency (see chapter 2). It does, therefore, not contribute significantly to the measured response. A second example of a microphonic potential is given in Fig. 2B. Besides the expected component at twice the stimulus frequency (2 × 8 = 16 Hz), the measured microphonic potential also contains strong components at the stimulus frequency as well as at higher harmonic frequencies, and furthermore a DC component that can also be clearly seen in the spectrum obtained via an FFT (Fig. 2C). To investigate to what extent the
measured response at the fundamental frequency originated from cross-talk of the piezoelectric driver voltage, the potential changes were also measured at approximately 50 µm above the hair cells (Fig. 2D). At this distance a 6.4 µV component at the stimulus frequency remains, which can only explain part of the component at the stimulus frequency (14 µV) measured close to the hair cells.

Cupular mechanics

Frequency responses of mechno-sensitivity of four SNs were determined along with the necessary sense probe measurements to correct for the fluid jet’s frequency-dependent characteristics. To make sure that the fish did not move as a
result of fluid jet stimulation, also the displacement of the epithelium close to the cupula was measured. It was found to hardly exceed the noise levels (data not shown) so that no corrections needed to be made.

Fig. 3A shows amplitude and phase of the sensitivity as a function of the frequency of the fluid jet velocity measured from four neuromasts. The responses show similar characteristics, both in the amplitude response as well as the phase response. At low frequencies (< 20 Hz) the amplitudes appear to be fairly constant, implying constant velocity sensitivity, varying in range between 1 to 5 nm/(µm/s). The phase at low frequencies varies between -20° and -40°, and gradually changes towards lower values with increasing frequency. The cut-off frequencies ($f_{3\text{dB}}$) determined by the intersection of the 0 dB/dec and -20 dB/dec asymptote are in the range of 40 to 134 Hz, with a mean of 71 Hz. In Fig. 3B the averaged result of Fig. 3A is plotted, together with a model fit (solid lines) based solely on the cupular

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**Figure 3: Cupular frequency response.** (A) The individual sensitivity responses of four zebrafish superficial neuromasts expressed in nm/(µm/s) with the phase given with respect to the fluid velocity. (B) Average sensitivity of the four responses in A (triangles). The solid lines show the fit to the averaged response using the cupular dynamic model (Eq. 1). Parameters: $N_r = 0.011$, $f_t = 6366$ Hz. (C) Same averaged responses now fitted by the boundary layer model (Eq. 6), with $N_r = 0.0017$, $f_t = 6366$ Hz and $z_0 = 34$ µm.
dynamic model (Eq. 1), without taking the boundary layer above the skin into account. The value used for the parameter $f_t$ is based on the estimated cupular cross-sectional radius of approximately 5 µm, yielding $f_t = 6366$ Hz (Eq. 2). The data were then best fitted when taking the resonance number, $N_r$, equal to 0.011, defining an overall cupular cut-off frequency of velocity detection of $f_c = f_t N_r = 70$ Hz. However, both the amplitude and phase of the model can be seen to deviate significantly from the measured data.

Fits of the combined model ($S_{cbl}$, Eq. 6) to the averaged data are depicted in Fig. 3C (solid lines). It appears that combining cupular dynamics with the hydrodynamics of the boundary layer explains the measured amplitude and phase more adequately than the cupular dynamic model. The fitted amplitude clearly shows a midrange of frequencies (~10-100 Hz) at which the sensitivity declines with about 10 dB/dec. The phase is also more satisfactorily described, although the model obviously fails to do so at low frequencies. Again, using $f_t = 6366$ Hz, as prescribed by the average cupular cross-sectional radius of approximately 5 µm (Eq. 2), yields a resonance number $N_r$ equal to 0.0017, approximately a factor 6.5 smaller than the fitted value without the boundary layer model. This resonance number implies a stiffness coupling of $6.4 \, \mu$N/m, and yields a cupular associated cut-off frequency ($f_c = f_t N_r$) of about 11 Hz. The combined model fit to the averaged data predicts a maximum sensitivity close to this cupular cut-off frequency. The measured data do not permit a firm conclusion on the question whether or not a maximum is reached for individual neuromasts in this frequency range. The best fit for the height $z_0$ was 34 µm, slightly higher than, but comparable to the range of cupular heights measured from (19-28 µm).

To obtain information on the motion of the cupula along its height, which senses different regions of the boundary layer motion, microspheres were attached at different levels on the same cupula. Fig. 4 shows the measured displacement of the cupula in response to a 44 Hz stimulus at cupular levels of 8, 19 and 34 µm above the apical membrane of the hair cells. The two lowest microspheres (8 and 19 µm) were positioned well below the tallest kinocilium (28 µm), whereas the microsphere at 34 µm was placed above the kinocilia. Eight time points (45 degrees apart in phase) during one period are shown. Lines interconnect the cupular displacements at different levels, at the same phase of the stimulus. At a height of 8 and 19 µm, the cupula moves in phase, but it leads the cupular motion at a level of 34 µm by about 20°. Fig. 4B shows the more detailed displacement-time relationships at the same three levels. The time difference (~1.26 ms)
associated to the phase lead of the lower locations (8 and 19 µm) with respect to the higher level (34 µm) can be clearly seen.

The displacement amplitude at 8 and 19 µm height is smaller than expected from a stiff cupula pivoting around its base as judged from upper cupular region (34 µm). This means that the cupula bends as a result of the fluid flow applied. Projecting the interconnections between the spheres at 19 and 8 µm down to the level of the hair bundle tips (5 µm, dotted lines) results in a bundle displacement component of approximately 57 nm, which is about 20-30% of the displacement measured at half way up the cupular top.
DISCUSSION

This chapter describes the first data of mechanical cupular response properties of SNs in the zebrafish and shows the potential of the zebrafish larvae preparation as a model to study SNs \textit{in vivo}. The high quality visualisation that can be achieved in this preparation enables micromanipulations under visual control. This facilitates the positioning of reflective particles on the cupula, which enables accurate sub-micrometer measurements of cupular displacement at distinct locations with spatial resolution down to the micrometer level, using a suitably focussed laser interferometer. The preparation can also be used to record extracellular electrical responses related to transducer channel activity, as has been previously shown by Nicolson \textit{et al.} (1998). Proper visualisation is essential to accurately place the recording electrode close to the hair cells, because the detected signal decreases in amplitude with a space constant of about 5 µm. Recording from individual hair cells directly using either intracellular recording techniques or the whole cell patch clamp technique has so far proven to be too difficult to be successful due to the epithelial cells on top of the hair cells (personal observation; Nicolson \textit{et al.}, 1998).

Microphonic potential

Microphonic potentials measured in the zebrafish preparation confirm the presence of an operational transducer machinery at PF5. For proper mechanoelectrical transduction an intact gating spring apparatus is required, which contributes a significant fraction of a hair bundle's stiffness in other hair cell organs with relatively long kinocilia (Hudspeth, 1989; van Netten and Kros, 2000). The microphonic response at twice the mechanical stimulus frequency (Kuiper, 1956; Flock \textit{et al.}, 1962) functionally confirms the existence of two morphologically polarised populations of hair cells in lateral line neuromasts (Rouse and Pickles, 1991), which has previously been shown in zebrafish using phalloidin labelling (staining the actin in the stereocilia; Williams and Holder, 2000) or double labelling with phalloidin and anti-acetylated tubulin antibody (staining the kinocilia; Whitfield, 2003).

Interpretation of the microphonic response in terms of transducer activity is not straightforward. The response often contains a relatively large component at the stimulus frequency (Fig. 2C-D). This component can only be partially explained by direct electrical pick-up of the input signal to the piezoelectric element in the fluid jet apparatus by the measurement electrode (Fig. 2D). The remaining component at the stimulus frequency could arise from the response being picked up from an unequal number of hair cells out of the two morphologically polarised populations.
within the neuromast. As a result, the response of one group of hair cells may dominate, so that after summation of the responses of both groups a component at the stimulus frequency remains. A second possibility is a DC component in the fluid jet velocity. This causes one half of the hair cells to have their operational position shifted to a higher open probability resulting in a larger amplitude response, whereas the other half has its operational point pushed towards the closed state resulting in a decreased response to the mechanical stimulus (Fig. 5). Summation of both these responses also qualitatively leads to responses like the one shown in Fig. 2.

Figure 5: Microphonic potential resulting from a stimulus with DC displacement of the cupula. (A) Transducer current (normalised) as a function of cupula displacement is described as a first order Boltzmann distribution for both morphologically polarised hair cell populations (I and II). Notice the approximate 10% open probability at $X_0$, the equilibrium position of the cupula. The vibrational displacement stimulus, which contains a displacement offset, $X_{DC}$, is depicted above the current displacement curves. The stimulus translates into a transducer activity of both hair cell populations via the current displacement curves (dots mark the extreme displacements on the $I$-$X$ curves). (B) Transducer current activity in both populations as a function of time (three stimulus periods). The response of population I is clearly smaller because its bundles are pushed more towards the closed state by $X_{DC}$. Besides bigger, the response of population II is more symmetrical because it results from the middle part of the current-displacement curve. (C) The additive response of both hair cell populations, qualitatively corresponding to the microphonic response. The microphonic response shows a component at the stimulus frequency (3 periods) and a component at twice the frequency comparable to Fig. 1A.
Chapter 3

Cupular mechanics

The presented velocity-sensitivities of the zebrafish superficial lateral line cupulae as a function of the frequency show fairly constant amplitudes at the lowest frequencies measured (e.g. < 20 Hz; Fig. 3A). These SNs can thus be considered as detectors of fluid velocity in this low frequency range. The related low frequency sensitivity amounts to a few nanometer per micrometer/second fluid velocity. The average cut-off frequency was found to be about 70 Hz. The averaged measured mechano-sensitivity curves (Figs. 3B and 3C), as well as the individual curves (Fig. 3A), however, show a modest decrease around the cut-off frequencies of about 10 dB/dec. Beyond the cut-off frequency the sensitivity declines with an average slope of about -20 dB/dec, indicative of inertial fluid forces dominating the excitation of the cupula. The phase response clearly shows a phase rotation around the cut-off frequencies. The phase at low frequencies, however, does not match the zero phase expected for a pure velocity detector. Also beyond the cut-off frequency the measured phase does not approach -90°, which is expected for fluid-inertia controlled cupular excitation. The conclusion therefore is that although the superficial neuromasts investigated show features of a low frequency velocity detector, in line with electrophysiological measurements on other types of superficial neuromasts (Görner, 1963; Kroese et al., 1978; Kroese et al., 1980; Kroese and Schellart, 1992), several aspects of the frequency dependence point to a more complex behaviour than that of a low-pass first-order system.

First-order low-pass behaviour is expected from a cupula model (Eq. 1) with a resonance number \( N_r \) smaller than 1, resulting from an elastically loosely coupled cupula to the underlying epithelium (van Netten, 1991; van Netten, submitted). This model was originally developed to describe the mechanical behaviour of a CN cupula and assumes it to be shaped as a rigid half sphere, sliding over a frictionless plate. It takes into account both viscous and inertial forces arising from the fluid flow past the cupula. The model, when applied to the averaged data on zebrafish superficial cupular dynamics (Fig. 3B), describes the amplitude of cupular sensitivity to some extent, but it does not capture the more detailed behaviour, such as the slow decline (10 dB/dec) with frequency in the range of 10 to 100 Hz. More obvious is the poor prediction of the measured phase. Rather than decreasing from about 0° to -90° with increasing frequency, as expected from a first-order low-pass filter (van Netten, submitted) measured phase data are limited to a range around -45°.

The fitted value of the resonance number \( N_r = 0.011 \); see Eq. 3), together with the estimated cupular cross-sectional radius, results in a stiffness coupling of the
cupula of approximately $4 \times 10^{-5}$ N/m. This is very low compared to the value obtained for the canal cupula of ruffe ($10^{-1}$ N/m; van Netten, 1987), which can only in part be explained by the roughly 100 times smaller number of hair bundles per neuromast in the zebrafish. If the stiffness is predominately determined by the combined stiffness of the hair bundles this would suggest that the stiffness contribution per hair bundle in the zebrafish is about 100 times lower than in the ruffe and other hair cell organs (e.g. Markin and Hudspeth, 1995; van Netten, 1997; van Netten and Kros, 2000). A partial explanation of the approximately 100 times lower stiffness per bundle may be the effect of the relatively long kinocilium that projects several tens of micrometers into the cupular body. In CNs relatively short kinocilia (7-10 µm) but similar hair bundle lengths (~4-5 µm) have been observed (van Netten, unpublished). The most likely cause for the small value found for the cupular stiffness is the model assumption of a rigid (i.e. very stiff) half sphere. The zebrafish cupula has the shape of a column with an average diameter of about 10 µm and a length of approximately 45 µm. As observed (Fig. 4), the superficial neuromast cupula does not primarily slide but shows a predominant pivoting and also bending component in its displacement response. The relative low stiffness value found at a level of tens of micrometer above the cupular base therefore most likely reflects the flexibility of the cupula, the compliance of which dominates the stiffness coupling to the underlying sensory epithelium. An estimate of the effective displacement at the level of the hair bundles, by extrapolating the cupular shape from the two lower levels measured (Fig. 4A; 8 and 19 µm), shows the extent of motion at the hair bundle level to be 20 to 30% of the motion at a 25 µm level. This corresponds to a mechano-sensitivity at the level of the sensory hair bundle tips, of 0.25 to 1.25 nm/(µm/s) as transferred by the cupula from the fluid outside the boundary layer.

**Effect of the boundary layer on cupular excitation of superficial neuromasts**

The fluid driving the different parts of SN cupulae can be expected to be part of the frequency-dependent boundary layer formed along the fish skin. The frequency dependent boundary layer thickness, $\delta(f)$ (Eq. 5), will be larger than the height of the cupula (about 45 µm) at frequencies smaller than approximately 300 Hz, i.e. the assumed physiological range.

We have therefore combined the hydrodynamic model of the cupula (Eq. 1) with the hydrodynamic characteristics of an oscillatory boundary layer (Eq. 4). The combined description (Eq. 6) captures many details of the measurements on frequency dependence of the cupular mechano-sensitivity (Fig. 3C). This
substantiates the role played by the boundary layer associated with the fish’ skin in shaping the stimulus to a superficial neuromast, similar to the filtering effect of a canal in the case of canal neuromasts (van Netten, submitted; Kalmijn, 1987).

At low frequencies (< 10 Hz) the model predicts a reduced mechano-sensitivity that is not firmly supported by the measurements on individual measurements, but neither contradicted (Fig. 3A). Clearer, however, is the discrepancy of the combined model with the phase measured at low frequencies. The explanation for this discrepancy may be related to modelling a small part of the cupula at a specific height ($z_0$) by a (half)sphere. Modelling the cupula as a flexible beam or cylinder, excited differentially along its height, can therefore be expected to improve the description. Another factor that may influence the observed discrepancy between data and model is the fluid stimulus presented. In contrast to natural stimuli, the targeted stimulus produced by the fluid jet pipette may have less interaction with the fish’ skin, so that the boundary layer does not fully develop. This might also be a source of variance, because the positioning of the jet pipette varies with each experiment, causing differences in the fluid-skin interaction and thus the degree of development of the boundary layer. To get a better understanding of the mechanical input to the cupula, the interaction of the fluid jet with the fish’s skin has to be studied in more detail.

**Time constant of cupular impulse response**

Although the combined model description is limited at low frequencies, it nevertheless allows for a prediction of the timing of cupular displacement in response to a fluid velocity pulse (cf. Ćurčić-Blake and van Netten, 2005). Based on the mechano-sensitivities that are in the range of a few nm/(μm/s), which is equivalent to a few ms, time-constants of a few milliseconds can be expected for the cupular impulse response (van Netten, submitted). This is confirmed by the impulse response curves as calculated from the two models employed to describe the sensitivity curves, as shown in Fig. 6. The impulse response calculated from the cupular model without taking the fish boundary layer into account can be well fitted with a single time constant of about 2.2 ms. The model including the boundary layer produces a faster initial decay with a time constant of 0.6 ms, but it also exhibits a slower component of approximately 4 ms with a similar contribution as the fast component. These calculations indicate that the hydrodynamic excitation of superficial neuromasts in young zebrafish has similar decay-characteristics as found in the supraorbital lateral line canal of the ruffe, but it does not show oscillatory behaviour due to resonance as in canal neuromasts (Ćurčić-Blake and
van Netten, 2005). Significant different detection properties are discussed in the next paragraph.

**Comparison between SN and CN**
The SN dynamics observed shows low-pass filtering characteristics in response to fluid velocity, as evidenced by a fairly constant sensitivity at low frequencies, and a -20 dB/dec decrease beyond the cut-off frequency at about 70 Hz. This value of the effective bandwidth is about a factor of two higher than the corresponding value for the clawed toad (*Xenopus laevis*), 30 Hz (Kroese *et al.*, 1978; Kroese, 1979), and the SN of the trout (*Oncorhynchus mykiss*), 36 ± 13 Hz (Kroese and Schellart, 1992), obtained with electrophysiological methods. We do not have data on adult zebrafish neuromasts, but it is possible that SN frequency characteristics change during development.

The SN responses found can also be compared to the dynamics of CNs. Fig. 7 shows a comparison of a model fit of typically measured cupular responses of a CN from the ruffe (*Gymnocephalus cernuus*) (van Netten, 1991) and the model fit of the cupula response of the zebrafish SN (taken from Fig. 3C), both plotted as a function of frequency of fluid displacement. The CN also shows a velocity sensitive frequency response up to about 100 Hz. At low frequencies the SN appears to be more sensitive than the CN, whereas the CN is more sensitive around its resonance frequency at approximately 120 Hz.

Although the sensitivities, as displayed in Fig. 7A, seem to be advantageous for superficial lateral line systems, an important difference related to the noise in
both systems has to be taken in account. A significant difference exists in the Brownian noise that will excite both types of cupulae. The Brownian motion related displacement noise, $X_{\text{rms}} = \sqrt{kT/S}$, is inversely proportional to the square root of the stiffness, $S$, where $k$ is Boltzmann’s constant and $T$ the absolute temperature (Landau and Lifshitz, 1980). For the typical CN in Fig. 7A this results in a $X_{\text{rms}}$ of 0.65 nm, whereas the SN with its much lower stiffness will have a $X_{\text{rms}}$ of approximately 25 nm. The displacement noise of the CN is thus more than one order of magnitude smaller than that of the SN. The CN has about one hundred times more hair cells adding to the increased stiffness, effectively leading to the lower sensitivity at low frequencies (< 65 Hz). If this Brownian input noise is the largest noise source, it will define the detection threshold of the system at this stage of the signal processing. Another important peripheral noise source is the transducer apparatus of the hair cells (chapters 5 and 6), which contributes per hair cell an effective inaccuracy in the displacement detection of several nanometers. Assuming that the neuromast’s electrical output will be averaged over all available hair cells, this inaccuracy can be decreased by the square root of the number of hair cells. In terms of noise, the CN therefore benefits at two fronts from having a larger number of hair cells per neuromast. Firstly, it increases the sliding stiffness and therefore decreases the Brownian displacement noise. Secondly, if the response of all hair cells is averaged, the accuracy of displacement detection will be improved. Assuming that the transducer accuracy of a single hair cell is comparable to the 6.5 nm determined in cochlear outer hair cells (see chapter 6), an improvement by a factor of $\sqrt{10}$ (10 hair cells) to about 2.1 nm can be predicted for the SN, and a factor of $\sqrt{1000}$ for the CN (approximately 1000 hair cells) leading to about 0.21 nm. Both values for the accuracy of displacement detection of the transducer apparatus are below the displacement induced by the Brownian noise of their respective cupula. This will thus be the dominant source of noise to the mechanical detection system. Fig. 6B shows the signal-to-noise ratio of both cupular responses curves as a function of frequency for a 10 µm/s fluid velocity stimulus. They were obtained by dividing the frequency responses in Fig. 6A by the total noise consisting of the quadratic sum of $X_{\text{rms}}$ and the inaccuracy of the transducer apparatus. The CN clearly has an overall better signal-to-noise ratio, certainly around its most sensitive frequency of 120 Hz.

The present study shows that zebrafish larvae are a potential model to study the cupular hydromechanics of SNs. It would be interesting to investigate how the SNs of these one-week-old fish larvae compare to the superficial neuromasts in the
adult zebrafish and/or SNs in other species. The SN cupulae of the young zebrafish larvae are shown to act as velocity detectors for frequencies up to several tens of hertz. Their effective stimulus input, however, is largely affected by the hydrodynamics in the boundary layer. It would be valuable to the understanding of these sensory units to obtain detailed measurements of the hydrodynamic flow field produced by a fluid jet stimulus at the fish’ surface around the cupula. Furthermore, the column shape of the cupula and its compliance should be implemented in an extended model, to better describe the transfer of external fluid velocities to actual displacements at the level of the hair bundle, which then in turn can be related to mechano-electrical transfer.

Figure 7: Comparison of SN and CN cupula (A) Modelled cupula sensitivity of the zebrafish SN incorporating the stimulus attenuation in the boundary layer (solid line, parameters: $N_r = 0.0017$, $f_t = 6366$ Hz, $z_0 = 34$ µm) and modelled cupula sensitivity of a typical ruffe CN (dashed line, parameters: $N_r = 70$, $f_t = 10.3$ Hz). (B) Signal-to-noise ratio as a function of frequency for a 10 µm/s velocity stimulus. Dotted horizontal line indicates a signal-to-noise ratio of one. Note the overall superior signal-to-noise ratio for the CN compared to the SN.