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Development of the sympatho-vagal balance in the cardiovascular system in zebrafish (*Danio rerio*) characterized by power spectrum and classical signal analysis

Thorsten Schwerte*, Caroline Prem, Anita Mairösl and Bernd Pelster

Institute of Zoology and Limnology and Centre for Molecular Biosciences, University of Innsbruck, Innsbruck, Austria

*Author for correspondence (e-mail: thorsten.schwerte@uibk.ac.at)

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Summary

The development of sympatho-vagal control of cardiac activity was analyzed in zebrafish (*Danio rerio*) larvae from 2 to 15 days post fertilization (d.p.f.) by pharmacological studies as well as by assessing short term heart rate variability. Changes in heart rate in response to cholinergic and adrenergic receptor stimulation or inhibition were investigated using *in situ* preparations and digital video-microscopic techniques. The data revealed that the heart responded to adrenergic stimulation starting at 4 d.p.f. and to cholinergic stimulation starting at 5 d.p.f. Atropine application resulted in an increase in heart rate beyond 12 d.p.f., while the inhibitory effect of cholinergic stimulation ceased at this time of development. Adrenergic inhibition (propranolol) reduced heart rate for the first time at 5 d.p.f., but the reduction was only very small (3.8%). Between 5 and 12 d.p.f. propranolol application always resulted in a minor reduction in heart rate, but because the effect was so small it was not always significant. Because the presence of an adrenergic or cholinergic tone may influence the stability of heart rate, we analyzed short-term heart rate variability (HRV). The frequency band width of heart rate variability revealed that HRV increased between 4 d.p.f. and 15 d.p.f. From 13 to 15 d.p.f. atropine reduced the frequency band width of HRV, whereas the combination of atropine and propranolol effectively reduced the frequency band width between 11 and 15 d.p.f. Classical power spectrum analysis using electrocardiograms is not possible in tiny zebrafish larvae and juveniles. It was therefore performed using optical methods, recording cardiac movement and cardiotachograms calculated from these measurements. Whereas heart movements contained frequency components characterizing HRV, the cardiotachogram did not show typical frequency spectra as known from other species.

Key words: cardiovascular system, adrenaline, acetylcholine, sympatho-vagal balance, heart rate variability, zebrafish, *Danio rerio*.

Introduction

In adult vertebrates cardiac activity and thus blood flow to the organ systems are mainly determined by the metabolic demand of the tissues. In contrast to adult fish and amphibians, that are typically oxyregulators, their embryonic or early larval stages presumably are oxyconformers, showing a decreasing oxygen uptake at decreasing oxygen tension (Hastings and Burggren, 1995). In some zebrafish heart mutants, at least during the first 10 days of development, a sufficient oxygen supply is obtained simply by diffusion of oxygen through the skin, and therefore no beating heart is needed (Chen et al., 1997; own observations). Accordingly, cardiac activity is not yet coupled to the metabolic demand of tissues at this stage. This was also supported by the observation that disruption of the haemoglobin in early stage zebrafish (3–12 d.p.f.) has no significant effect on metabolism or on cardiac performance (Pelster and Burggren, 1996; Jacob et al., 2002; Schwerte et al., 2003). This prompts questions about when during development do cardiovascular control systems appear and which types (intrinsic, neuronal and humoral) of control are involved during different stages of development.

The use of non-invasive optical methods, like digital motion analysis, developed in our lab (Schwerte and Pelster, 2000; Schwerte and Fritsche, 2003), to analyze the heart appears especially promising to address the question of cardiovascular control in early larval stages. In previous studies we used these techniques to investigate cardiovascular and respiratory responsiveness of developing fish to different environmental conditions (Schönweger et al., 2000; Jacob et al., 2002; Schwerte et al., 2003; Turesson et al., in press). The results of these studies indicated that in zebrafish receptors sensing hypoxic conditions are already present in very early stages (3 days post fertilization; d.p.f.). Vascular reactivity to nitric oxide was shown in 5 d.p.f. animals (Fritsche et al., 2000).

Whereas these studies recorded changes in heart rate over time in response to the treatment, over the last two decades several studies have shown that there are fluctuations in the instantaneous heart rate on a beat-to-beat basis. Changes in heart rate on a beat-to-beat basis are called the heart rate variability signal (HRVS). This signal may include information about autonomic (neuronal or intrinsic) mechanisms controlling cardiac activity, especially if a power spectral analysis is performed, which allows the dissection of the oscillatory components hidden behind the heart rate variability signal (Altimiras, 1999). In contrast to the large number of studies analyzing heart rate variability (HRV) in adult mammals there is little data available about non-mammalians. Altimiras et al. (Altimiras et al., 1995) and Campbell et al. (Campbell et al., 2004) demonstrated the physiological significance of short-term heart rate modulation for adult teleost fishes, but to our knowledge nothing is known about HRV in fishes during early development. In adult animals the complexity of HRV is mainly based on the autonomic innervation of the sinoatrial node. Vagotomy, for example, significantly reduces HRV (Campbell et al., 2004). However, during embryonic and early larval development the autonomic control system might not yet be functional (Protas and Leontieva, 1992; Jacobsson and Fritsche, 1999; Pelster and Schwerte, 1999).

Accordingly, HRV initially might have a completely different background, and the adult pattern might become visible only after the vago-sympathetic control system is established. We hypothesize that changes in HRV will reflect the onset of cardiovascular regulation by the autonomic nervous system. The analysis of HRV in small larvae and juveniles of zebrafish is a difficult task. Classical methods such as electrocardiogram or pressure measurements are too invasive and would produce too many artefacts. In the present study we therefore developed a non-invasive method to assess variations in heart rate. We characterized changes in HRV during development in comparison to the onset of adrenergic and cholinergic cardiovascular regulation. To evaluate the method and to show that measured changes in HRV are connected to the autonomic nervous system we used classical pharmacological methods to inhibit autonomic nerve function. We hypothesized that nervous-system-borne HRV will disappear after blocking the autonomous nerve system.

Materials and methods

Animals

Zebrafish (*Danio rerio* Hamilton 1822) larvae were obtained from our own breeding colony. Because of their greater transparency, poorly pigmented mutants of the zebrafish (Albino, Brass) were used. Parent animals used to start the breeding colonies were either obtained from a local supplier or generously provided by Dr Frohnhöfer (Max-Planck Institute for Developmental Biology, Tübingen, Germany) and Ms Loos (University of Konstanz, Germany). Breeding colonies and larvae were kept in small aquaria at 28°C. Animals were fed after swimbladder filling (4–6 d.p.f.) with micro powder food (Zebrafish Management; www.zmsystems.demon.co.uk).

Animal experiments were performed according to animal ethics permission GZ 66.008/4-BrGT/2004 of the Austrian Bundesministerium für Bildung, Wissenschaft und Kultur.

The imaging system

An inverted microscope (Zeiss Axiovert 25 CF) was placed on a solid, heavy-weight steel plate to reduce vibration. The microscope was connected to a digital high speed digital camera (Basler 504k, Ahrensburg, Germany), which was connected to a personal computer equipped with a Datacube image acquisition board. Camera configuration tools VLL Toolbox and CCT+ software (Basler) were used. Recording of the sequences of the single digital pictures was managed by Video Savant 4.0. Recording frame rate was adjusted to 1000 frames s^{-1}. The region of interest was 240×60 pixels. Recordings were performed with a 40× objective (resulting pixel size: 0.58 μm^2).

Animal anaesthetization

All animals were anaesthetized with an initial concentration of 80 mg l^{-1} tricaine (MS-222) neutralized with phosphate buffer. Retention of the anaesthetization was performed using a concentration of 35 g l^{-1} tricaine (40 g l^{-1} in animals older than 10 d.p.f.). Larvae were transferred into the temperature-controlled incubation chamber of the microscope stage. The temperature was set to the incubation temperature of the eggs (28°C).

Analysis of heart rate variations

For analysis of the HRV, images of the beating heart were made with 1000 or 200 frames s^{-1} and stored on the frame grabber's on-board memory. A line was drawn beginning outside the heart tissue, crossing the central ventricle and atrium, ending in the sinus venosus. The luminance profile of this line was stored in a text file for all image frames of the whole image sequence. This text file was imported into a custom-made computer program (using LabView 7.1, National Instruments, Vienna, Austria). This program allowed for signal analysis of the luminance periodogram on each point along the drawn line. The changes in signal intensity in the luminance periodogram in most of the points reflect the periodic event of the beating heart (Fig. 1). Those points with the best signal-to-noise ratio were chosen for further analysis. This analysis consists of four steps. For a first estimation of the frequency content of the raw signal a power spectrum analysis was performed on the luminance periodogram. Secondly, the luminance periodogram was filtered by an equi-ripple low pass filter with a pass frequency of 0.1 Hz and a stop frequency of 10 Hz. On this filtered signal peak detection was performed. Peak detection was based on an algorithm that fits a quadratic polynomial to sequential groups of data points. The number of data points used in the fit was specified by width of typical peaks found in the acquired signals. For each peak, the quadratic fit was tested against the threshold level. The threshold was determined for each animal separately

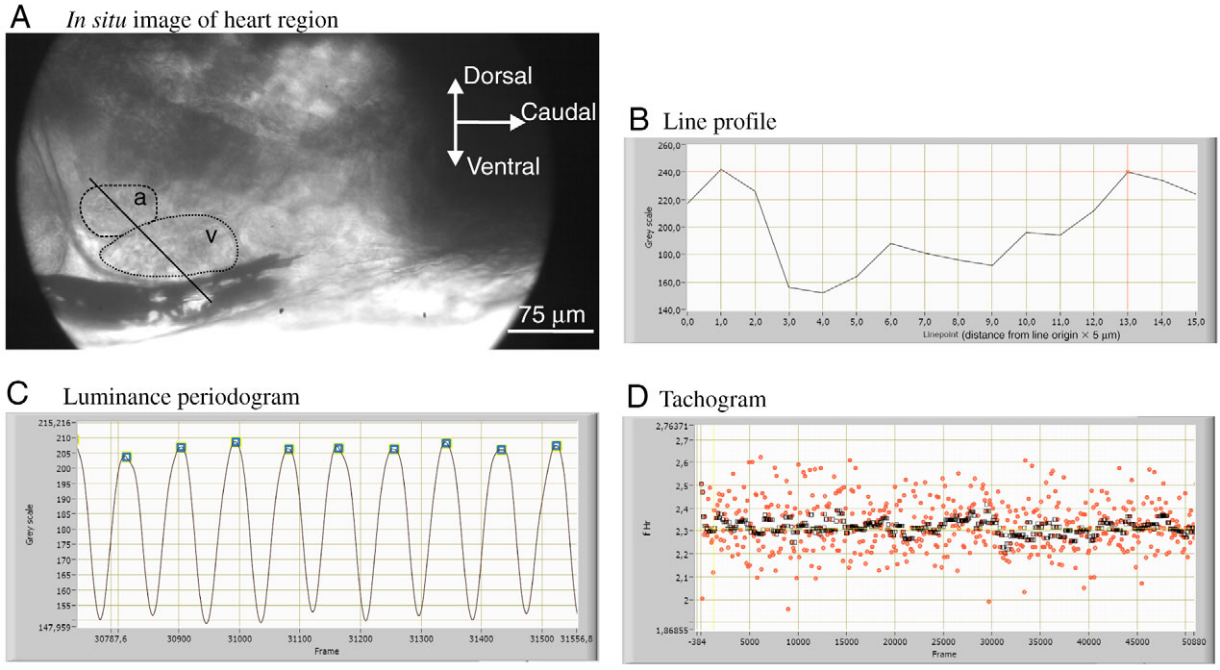

Fig. 1. The method of heart rate analysis using high speed video imaging. For analysis of the short-term heart rate variability (HRV) images (frames) of the beating heart were taken with 1000 or 200 frames s^{-1} and stored on the frame grabber's on-board memory. One sample image is shown in (A). Dotted lines indicate the perimeter of the atrium (a) and the ventricle (v). For each image a line was drawn beginning outside the heart tissue, cross sectioning the centre of ventricle and atrium and ending in the sinus venosus (solid line). Luminance profiles along these lines (see B), were stored for all frames. The intersection of the red lines marks the point on the line, where grey values through all frames are compiled to a luminance periodogram (C). The detected peaks (blue squares in C) were used for the calculation of inter-beat frequency (Hz) and plotted against frames in the tachogram (D). Red circles are the unfiltered raw data of every individual beat, and black rectangles represent the median filtered signal (median ranking=10). These data were taken for further analysis.

and was dependent on small differences in illumination and tissue orientation in the embedded animals. Peaks with heights lower than the threshold level (smaller peaks induced by movements of the atrium contraction) were ignored. Peaks were detected only after the procession of approximately half of the data points representing the width of the peak beyond the location of the peak. Peak-to-peak distances (reflecting beat-to-beat distances) were converted to a beat-to-beat frequency for each interval. These frequencies were plotted against time to a cardiotachogram. This tachogram was analysed by power spectrum analysis to evaluate frequency domains in the cardiotachogram.

Stroke volume was determined using digital image analysis (Schwerte and Pelster, 2000) and basically followed the method described by Hou and Burggren (Hou and Burggren, 1995). Video sequences of the ventricle were saved into computer memory. The perimeter of the ventricle image was outlined manually during end diastole and during end systole using a mouse or a graphic tablet. The perimeter was analyzed with a 'fit-to-ellipse' algorithm, which first calculated the centre of mass of the perimeter and subsequently the best fitting ellipse (Schwerte and Pelster, 2000). The major and minor axes of the ellipse were extracted and directly transferred into a Microsoft Excel worksheet for calculation of stroke volume using the formula for a prolate spheroid ($4/3\pi ab^2$) (Hou and Burggren, 1995). For analysis, five diastoles and systoles were analyzed, and mean stroke volume was calculated as the difference between diastolic and systolic ventricular volume. Cardiac output was calculated as the product of stroke volume×heart rate.

Experimental protocol for adrenergic and cholinergic pharmacology

The tricaine anaesthetized animals were allowed to settle down for 5 min, which was shown to be enough in earlier experiments. The tip of a pulled glass capillary (10 µm tip diameter) filled with test solution and connected to a WPI UltraMicroPump II (Berlin, Germany) was placed in the immediate proximity to the heart outside of the fish. After 5 min of control period, 1000 nl solution were released within 1 min. Changes in heart performance were analyzed for 20 min after ejection of the desired solution.

Drugs

Isoproterenol, propranolol, acetylcholine and atropine were obtained from Sigma Chemicals (Vienna, Austria).

Statistical analyses

Significant differences among treatments for these indicators were evaluated using Student's *t*-tests. Unless specified otherwise, a significance level of $P<0.05$ was used.

Heart rate variability

The frequency bandwidth of the cardiotachograms was measured by determining mean distance of maximum frequency values from the median. Thresholds over and under the median values were set to limits enclosing 98% of all interbeat frequencies closest to the median.

Power spectrum analysis

The power spectrum calculations were made with custom made software using LabView 7.1 (National Instruments), which includes all necessary mathematical tools. Luminance periodograms for detection of individual heart beats were obtained from 1000 frames s^{-1} acquired video data to fulfil the Nyquist criterion (a signal must be sampled at a rate at least twice the rate of its highest frequency component).

To obtain a better estimate of the power spectrum, two different methods were tested: the Welch method and the Blackman–Tukey method. The Welch method is characterized by averaged periodograms of overlapped, windowed segments of a time series. It improves the statistical likelihood of the estimated power spectrum. Using this approach, the signal is divided in segments short enough to be stationary and long enough not to limit the spectral resolution. The Blackman–Tukey Method is based on a Fourier transformation of the smoothed, truncated auto-covariance function. Power spectrum analysis was performed on luminance periodograms (Fig. 1C) and tachograms (Fig. 1D).

Results

Pharmacological characterization

Animals treated with acetylcholine (Fig. 2A) showed the first significant decrease in heart rate at 5 d.p.f. (maximum decrease 8.5%). This reaction was observable until 10 d.p.f. From 11 to 15 d.p.f. cholinergic stimulation did not result in a significant reduction in heart rate. Application of atropine (muscarinergic antagonist) led to significant increases in heart rate between 12 and 15 d.p.f. (maximum increase 21.2%). In younger animals atropine application resulted in no change of heart rate.

Animals treated with isoproterenol (β-adrenergic agonist) showed a significant increase in heart rate between 4 and 15 d.p.f. (maximum increase 21.3%). A significant effect of propranolol (β-adrenergic antagonist) on heart rate was first observed at 5 d.p.f. Propranolol application consistently induced a decrease in heart rate of between 2% and 8.5% at 5–15 d.p.f. Owing to the small magnitude of this effect it was only significant on 5, 7, 10, 13 and 15 d.p.f., while at all other stages it was not significant (Fig. 2B). There were no significant differences in stroke volume between experimental (isoproterenol, acetylcholine) and control groups (data not shown). Animals preincubated with the inhibitors propranolol or atropine prior to the application of the agonists isoproterenol or acetylcholine did not show any significant changes (data not shown).

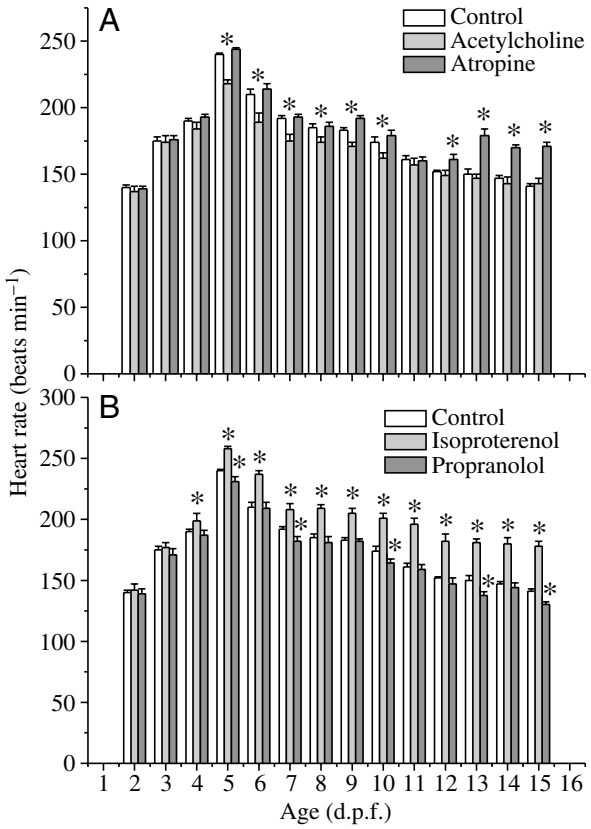

Fig. 2. Absolute changes in heart rate after application of 1 μl (A) acetylcholine, atropine, (B) isoproterenol or propranolol (final concentration 10^{-4} mol l^{-1}). Asterisks indicate statistically significant changes compared to control values (P<0.05). N=7 (per stage).

Heart rate variability (frequency bandwidth)

In general, heart rate increased with developmental age until 5 d.p.f., reaching a value of about 240 beats min^{-1}. After 5 d.p.f. mean heart rate decreased slowly down to a value of about 140 beats min^{-1} at 15 d.p.f. Power spectrum analysis of the unfiltered heart rate related luminance signal provided a dominant frequency component reflecting the mean heart rate and harmonics. This frequency domain was observed to produce an increasingly broader peak the older the animals became (Fig. 3A,B). Heart beating frequency was the dominant compound in luminance signals derived from all tissues around the heart in an area of several hundred micrometers from the heart.

The short term variations in heart rate showed a prominent scatter around the median values of the cardiotachogram (Fig. 4A,B). In older animals the frequency bandwidth was significantly wider than in younger ones (Fig. 5). Variability at 4 d.p.f. was below 0.25 Hz, while at 15 d.p.f. the variability amounted to 0.42 Hz. In 10–15 d.p.f. animals incubated with propranolol (β-adrenergic antagonist) in combination with atropine (muscarinergic antagonist) the frequency bandwidth was significantly lower compared to control animals (0.34 Hz compared with 0.42 Hz, respectively). Animals incubated with

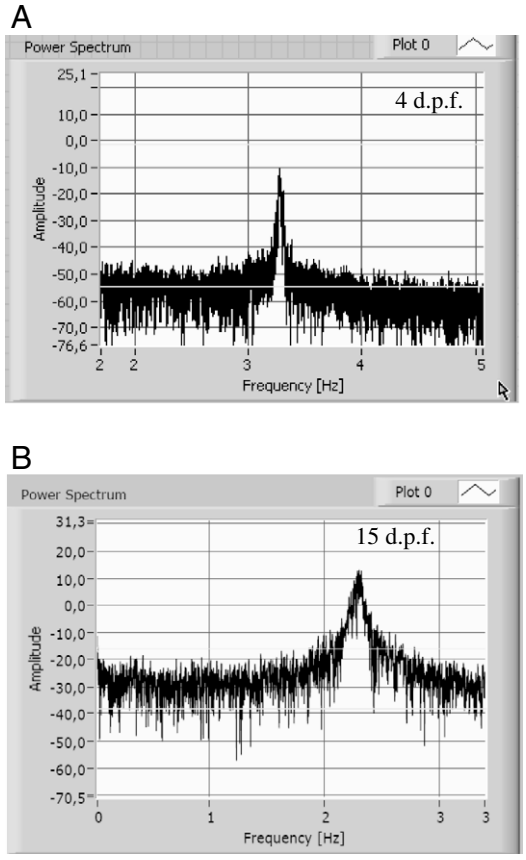

Fig. 3. Typical power spectra of unfiltered luminance change frequencies in *in situ* recordings of zebrafish hearts in (A) 4 d.p.f. and (B) 15 d.p.f. zebrafish.

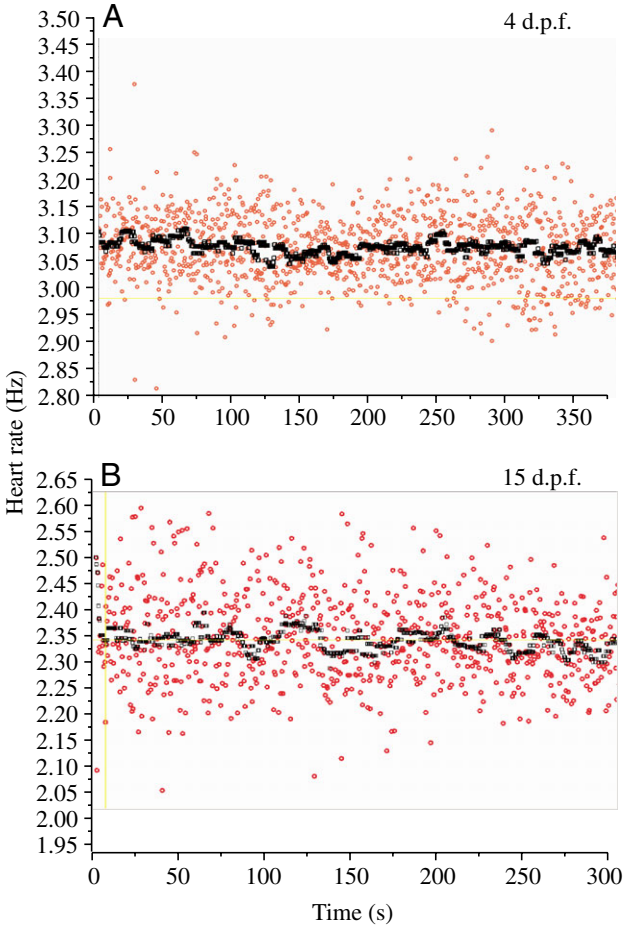

Fig. 4. Typical data for heart rate variability in (A) 4 d.p.f. and (B) 15 d.p.f. zebrafish. The red circles are beat-to-beat frequencies of individual heart beat. The black circles are median values of the individual beats (median ranking=10).

propranolol alone did not show a significant decrease in frequency bandwidth. In contrast, atropine application induced a significant decrease in frequency bandwidth between 13 and 15 d.p.f. (0.375 Hz and 0.425 Hz, respectively).

Heart rate related signal power spectrum analysis

The power spectrum analysis of the cardiotachogram of the low-pass filtered heart rate-related luminance signal did not reveal a stable or reproducible pattern of dominant frequencies (data not shown) at any of the stages of development examined.

Discussion

Critique of methods

The aim of this study was to analyze when during development a sympatho-vagal balance of the heart rate is established. To answer this question we chose a pharmacological approach in combination with non invasive signal analysis of heart rate variability. Measurement of cardiac performance in millimetre-sized animals such as zebrafish larvae and juveniles is a difficult task. To be able to detect differences in heart rate variability, it was absolutely necessary to immobilize the animals in order to avoid any signal related to body movements. This can only achieved with anaesthetized animals. For these experiments and for measurements of respiration frequency during development (Turesson et al., in press) we refined our anaesthetic protocol to keep any potential influence on autonomic processes as small as possible. By counting heart rate and ventilatory movements in anaesthetized and non anaesthetized animals a maintenance concentration of 35 mg l^{-1} (40 mg l^{-1} in stages older than 10 d.p.f.) MS222 was shown to have no influence on resting frequencies throughout all stages. A detailed study of the effect of MS222 on autonomous processes and the survival rate of zebrafish has also been carried out by Rombough (Rombough, 2002).

Pharmacological characterization

Cholinergic stimulation significantly decreased heart rate in developing zebrafish larvae starting at 5 d.p.f., whereas isoproterenol led to a significant increase in heart rate as early as 4 d.p.f. Statistically significant changes in heart rate were no longer seen in animals that were preincubated with either of the receptor-specific blocking agents propranolol or atropine

(Fig. 2A). We therefore conclude that functional cholinergic receptors are present at 5 d.p.f., but adrenergic receptors become functional by 4 d.p.f. Hsieh and Liao (Hsieh and Liao, 2002) cloned, sequenced and characterized a zebrafish M2 muscarinic acetylcholine receptor. They pharmacologically analyzed the role of this receptor and observed a response to carbachol at 3 d.p.f. Carbachol, at a high concentration (10^{-4} mol l^{-1}) injected directly into the circulation, induced a decrease in heart rate of about 80%, and this effect could be completely inhibited by atropine incubation. In the present study, a cholinergic response was observed only at 5 d.p.f., but we used the enzymatically degradable acetylcholine and peripheral incubation instead of injection of a stable agonist like carbachol. It therefore appears quite possible that the acetylcholine receptor actually did respond a little bit earlier. Jacobsson and Fritsche (Jacobsson and Fritsche, 1999) analyzed the early existence of adrenergic and cholinergic receptors in *Xenopus* larvae, and they observed that *Xenopus* larvae do respond to cholinergic stimulation much earlier than to adrenaline.

The positive response to an externally applied agonist demonstrates the presence of a functional receptor, especially if this response can be suppressed by a specific antagonist. It does not imply, however, that the receptor can also be reached by a signal from the autonomic nervous system. In fact, for birds and amphibians it has been reported that functional receptors are established in the heart long before the autonomic innervation is completed (Tazawa et al., 1992; Protas and Leontieva, 1992; Jacobsson and Fritsche, 1999). However, the increase in heart rate observed in 12 d.p.f. zebrafish larvae upon incubation with atropine clearly indicated the presence of vagal tone on the heart at this stage. Until 10 d.p.f., cholinergic stimulation decreased heart rate, but at 11 d.p.f. and later this response was no longer observed. One possible reason for this may be a cholinergic tone that is established at about 11 or 12 d.p.f. After establishing the tone, cholinergic stimulation was not longer as effective as before 11 d.p.f., because the heart was already under certain activity of the vagus. Inhibition of this tone by atropine application therefore resulted in an acceleration of the heart at 12 d.p.f. and in later stages. It is not very probable that this tonus could be responsible for the completely missing inhibitory cholinergic effect, because this would mean that the cholinergic tone would be at a maximum. Although we know from diffusion experiments with fluorescent dyes that molecules with molecular mass ranging up to 0.5 kDa diffuse well in the tissue (unpublished data) it cannot be excluded that the maturing skin of zebrafish becomes increasing less permeable with age. The inhibitory effect of atropine and of propranolol revealed that diffusion of these components was not impaired at this stage. Another possibility would be that increasing activity of acetylcholine esterase may have lowered the effective acetylcholine concentration. A quantitative assessment of the magnitude of the cholinergic tone, however, was beyond the scope of this study.

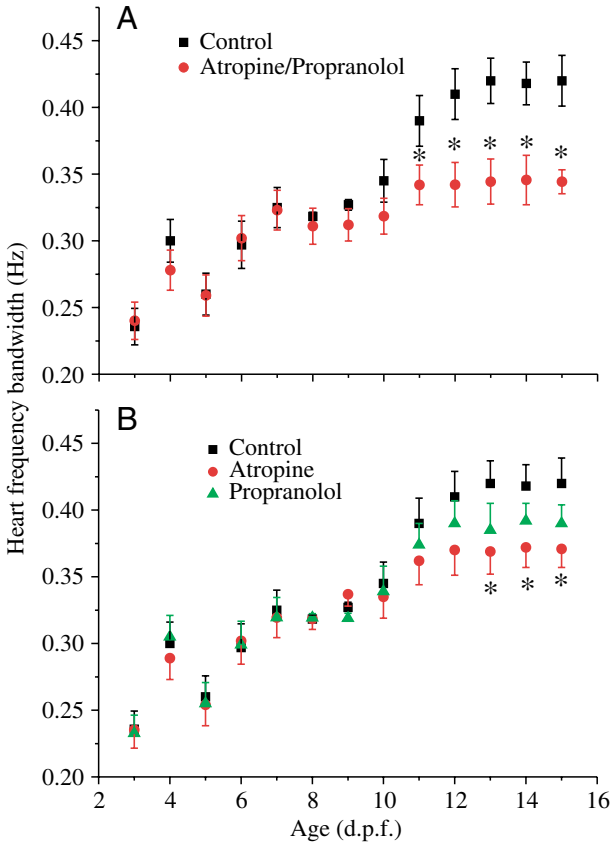

Fig. 5. Absolute changes in heart rate variability (HRV) frequency bandwidth, in control animals and in animals after incubation with (A) propranolol (β-adrenergic antagonist, 10^{-5} mol l^{-1}) and atropine (muscarinergic antagonist; 10^{-5} mol l^{-1}) or (B) with each agonist separately. Asterisks indicate statistically significant differences between incubated and control animals (P<0.05). N=7 (per stage).

In contrast to the adrenergic tone that perhaps may be due to the activity of the autonomic nervous system or to circulating hormones (Jacobsson and Fritsche, 1999) it appears unlikely that a cholinergic tonus is caused by circulating acetylcholine, because of the acetylcholine esterase activity. We therefore conclude that at 11 or 12 d.p.f. the heart comes mainly under the influence of the vagus, which is also the dominating influence in many adult fish (Axelsson et al., 1987; Taylor et al., 1999; Campbell et al., 2004).

The picture emerging from our data for the adrenergic response is not as clear as the picture obtained for the cholinergic response. Adrenergic receptor functionality was observed for stages between 4 and 15 d.p.f. Propranolol application (β-adrenergic antagonist) in turn led to a significant decrease in heart rate at 5 and 7 d.p.f., but the response was in the range of only 5–8%. This situation did not change until 15 d.p.f., so that at some developmental stages the decrease in heart rate induced by propranolol was just significant, on others it was not (Fig. 2B). Accordingly, it is difficult to say whether an adrenergic tone is established at this stage of development in zebrafish larvae. In addition, if present, its physiological significance remains questionable, because it would change heart rate by may be 5–10 beats min^{-1} at a level of 160–180 beats min^{-1}.

This is in contrast to results reported for *Xenopus* larvae, in which at least in some stages (Nieuwkoop and Faber (NF) stage 45–47) a strong adrenergic tonus appears to be present Jacobsson and Fritsche (Jacobsson and Fritsche, 1999). However, our results are in line with the results obtained from adult teleosts, in which the relative importance of the adrenergic innervation and of circulating hormones remains unclear (Taylor et al., 1999).

Stroke volume did not show any significant changes as a result of drug administration. Similarly, Jacobsson and Fritsche (Jacobsson and Fritsche, 1999) reported that epinephrine had no effect on stroke volume of *Xenopus* larvae (NF stage 33–53). Cholinergic stimulation, however, reduced stroke volume in *Xenopus* larvae in stage NF 45–53. From this we conclude that in zebrafish larvae cardiac output is predominantly determined by changes in heart rate. In adult fish during strenuous exercise and severe hypoxic conditions cardiac output appears to be adjusted by changes in stroke volume and in heart rate, whereas under resting conditions minor adaptations in cardiac output may be mostly due to adaptations in heart rate (Nilsson, 1983; Campbell et al., 2004).

Analysis of heart rate variability

Power spectrum analysis has been shown to be a powerful tool to display the frequency spectrum in beat-to-beat heart rate changes (Altimiras et al., 1995; Altimiras, 1999; Campbell et al., 2004). To our knowledge this is the first time that this technique has been used in fish larvae. One reason for this may be the difficult task to find a clearly defined reproducible parameter that can be measured to analyze cardiac cycle timing (Altimiras, 1999). The analysis of a pressure signal obtained by a micropressure converter (Pelster and Burggren, 1996) or the acquisition of an electrocardiogram (ECG) (Baker et al., 1997) are very time consuming and, more importantly, are quite invasive for these tiny animals, and therefore not ideal for the analysis of heart rate variability. Zebrafish hearts are very sensitive to mechanical manipulation in these early developmental stages. In adult short-horn sculpin, *Myxocephalus scorpius,* a recovery period of about 48 h was necessary to overcome the stress of handling and surgery, so that 'normal' and reproducible beat-to-beat spectra could be recorded (Campbell et al., 2004). Given the rapid developmental changes observed in zebrafish larvae such an experimental time course is not acceptable. Non-invasive optical methods therefore appeared to be the only option for our study.

Our custom-made program enabled us to extract periodic luminance changes in images from the heart. Measurements were taken along a line beginning outside heart tissue, which cross-sectioned the ventricle and atrium. Peaks in this luminance periodogram (Fig. 1C) were compiled to a tachogram. Power spectra of these tachograms did not show stable frequency spectra in our animals. Altimiras et al. (Altimiras et al., 1995) analyzed three different teleosts and observed that clear spectral patterns did not always exist, which was, at least in part, attributed to an erratic influence of respiratory movements. In 12 d.p.f. zebrafish, for example, ventilation rate is as low as 1–2 beats min^{-1} compared to a heart rate of about 160 beats min^{-1}. Thus, the interference of respiratory movements cannot be responsible for the lack of clear spectral patterns in these larvae. Although we avoided the stress of surgery, handling stress could not be avoided completely but was kept to a minimum. Given the long time course of recovery observed by Campbell et al. (Campbell et al., 2004) it therefore cannot be excluded that the lack of identifiable spectra may be in part related to handling stress.

The signals analyzed later are qualitatively different. As discussed by Altimiras (Altimiras, 1999) the ECG, used by Campbell et al. (Campbell et al., 2004), is the most precise method of obtaining a reproducible trigger of cardiac pacemaker performance. All other parameters linked to cardiac function are more or less indirect and therefore less appropriate to evaluate heart rate variations. The most common reasons for this are damping effects, which make the signal flatter, or the pacemaker-related peak broader. The consequence is that computational peak detection produces less accurate values than the extremely sharp peaks found in the ECG. The strongest argument, put forward by Altimiras (Altimiras, 1999), in other animals, is the effect of movements, except from cardiac movements that may influence the exact determination of the event of cardiac contraction. However, in our experiments this should affect experimental and control animals in the same way, and therefore does not cause any bias for the results of our study.

Nevertheless, analysis of the frequency bandwidth (see Figs 3A,B, 4A,B) revealed that heart rate variability increased with age. In the time frame of our experiments, which was mainly limited by the changes in transparency of the developing fish, the greatest variability was registered from 12 to 15 d.p.f. This was exactly the time, when vagal blocking with atropine led to a significant decrease in heart rate variability (Fig. 5A,B). Adrenergic blocking, in turn, did not affect heart rate variability, while the largest effect was observed when atropine and propranolol were administered together. These results confirm our conclusion from the pharmacological experiments that a vagal tone is established on the heart at 11–12 d.p.f., but the influence of the sympathetic trunk is of minor importance. Whereas heart rate variability was completely abolished after bilateral vagotomy in the study of Campbell et al. (2004), we still observed some variations after application of atropine and propranolol. This can probably be attributed to the fact that vagotomy is the method of choice to be absolutely sure that no parasympathetic stimulus reaches the heart. Pharmacological receptor blocking, especially when applied externally, does not fulfil this criterion, and therefore cannot block the vagal input completely.

Physiological implications

The developmental time around 12 d.p.f. is not only the start of sympato-vagal balancing of the heart rate but around this time regulatory integrity of cardiorespiratory functions are nearing completion. Rombough (Rombough, 2002)

demonstrated that gills start to function as oxygen uptake organs at 12 d.p.f., whereas they mainly serve ionoregulatory functions in earlier stages. The study of Jacob et al. (Jacob et al., 2002) revealed that 12–14 d.p.f. is the time during development when bulk diffusion is no longer sufficient to supply oxygen to the tissues, and convective oxygen transport via the circulatory system needs to take over oxygen transport functions. NMDA-receptor-mediated regulation of gill ventilation also starts working at 12 d.p.f. (Turesson et al., in press).

In summary, the results of the present study show that adrenergic and cholinergic receptors are present long before the autonomic innervation systems are working. Accordingly, humoral control is established at about hatching time or shortly thereafter. At 11–12 d.p.f. a cholinergic tonus is established, while the adrenergic tone appears to be weak. Changes in cardiac performance in response to a hypoxic stimulus observed at 3–5 d.p.f. (Jacob et al., 2002) therefore have to be attributed to humoral control. The analysis of heart rate variation was possible using high-speed image analysis. This technique, in combination with signal analysis, turned out to be an appropriate tool to investigate heart rate variations non-invasively.

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References

Altimiras, J. (1999). Understanding autonomic sympathovagal balance from short-term heart rate variations. Are we analyzing noise? *Comp. Biochem. Physiol.* **124A**, 447-460.

Altimiras, J., Aissaoui, A. and Tort, L. (1995). Is the short-term modulation of heart rate in teleost fish physiologically significant? Assessment by spectral analysis techniques. *Braz. J. Med. Biol. Res.* **28**, 1197-1206.

Axelsson, M., Ehrenstrom, F. and Nilsson, S. (1987). Cholinergic and adrenergic influence on the teleost heart in vivo. *J. Exp. Biol.* **46**, 179-186.

Baker, K., Warren, K. S., Yellen G. and Fishman, M. C. (1997). Defective ‘pacemaker’ current (Ih) in a zebrafish mutant with a slow heart rate. *Proc. Natl. Acad. Sci. USA* **94**, 4554-4559.

Campbell, H. A., Taylor, E. W. and Egginton, S. (2004). The use of power spectral analysis to determine cardiorespiratory control in the short-horned sculpin *Myoxocephalus scorpius*. *J. Exp. Biol.* **207**, 1969-1976.

Chen, J. N., van Eeden, F. J., Warren, K. S., Chin, A., Nusslein-Volhard, C., Haffter, P. and Fishman, M. C. (1997). Left-right pattern of cardiac BMP4 may drive asymmetry of the heart in zebrafish. *Development* **124**, 4373-4382.

Fritsche, R., Schwerte, T. and Pelster, B. (2000). Nitric oxide and vascular reactivity in developing zebrafish, danio rerio. *Am. J. Physiol.* **279**, R2200-R2207.

Hastings, D. and Burggren, W. (1995). Developmental changes in oxygen consumption regulation in larvae of the South African clawed frog *Xenopus laevis*. *J. Exp. Biol.* **198**, 2465-2475.

Hou, P. C. and Burggren, W. W. (1995). Cardiac output and peripheral resistance during larval development in the anuran amphibian *Xenopus laevis*. *Am. J. Physiol.* **269**, R1126-R1132.

Hsieh, D. J. and Liao, C. F. (2002). Zebrafish M2 muscarinic acetylcholine receptor: cloning, pharmacological characterization, expression patterns and roles in embryonic bradycardia. *Br. J. Pharmacol.* **137**, 782-792.

Jacob, E., Drexel, M., Schwerte, T. and Pelster, B. (2002). Influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *Am. J. Physiol.* **283**, R911-R917.

Jacobsson, A. and Fritsche, R. (1999). Development of adrenergic and cholinergic cardiac control in larvae of the African clawed frog *Xenopus laevis*. *Physiol. Biochem. Zool.* **72**, 328-338.

Nilsson, S. (1983). *Zoophysiology.* Berlin: Springer.

Pelster, B. and Burggren, W. W. (1996). Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebrafish (*Danio rerio*). *Circ. Res.* **79**, 358-362.

Pelster, B. and Schwerte, T. (1999). Analysis of functional development of the circulatory system in lower vertebrates by video imaging. *Comp. Biochem. Physiol.* **124A**, S67.

Protas, L. L. and Leontieva, G. R. (1992). Ontogeny of cholinergic and adrenergic mechanisms in the frog (*Rana temporaria*) heart. *Am. J. Physiol.* **262**, R150-R161.

Rombough, P. (2002). Gills are needed for ionoregulation before they are needed for O(2) uptake in developing zebrafish, *Danio rerio*. *J. Exp. Biol.* **205**, 1787-1794.

Schönweger, G., Schwerte, T. and Pelster, B. (2000). Temperature-dependent development of cardiac activity in unrestrained larvae of the minnow *Phoxinus phoxinus*. *Am. J. Physiol.* **279**, R1634-R1640.

Schwerte, T. and Pelster, B. (2000). Digital motion analysis as a tool for analysing the shape and performance of the circulatory system in transparent animals. *J. Exp. Biol.* **203**, 1659-1669.

Schwerte, T. and Fritsche, R. (2003). Understanding cardiovascular physiology in zebrafish and Xenopus larvae: the use of microtechniques. *Comp. Biochem. Physiol.* **135A**, 131-145.

Schwerte, T., Uberbacher, D. and Pelster, B. (2003). Non-invasive imaging of blood cell concentration and blood distribution in zebrafish *Danio rerio* incubated in hypoxic conditions in vivo. *J. Exp. Biol.* **206**, 1299-1307.

Taylor, E. W., Jordan, D. and Coote, J. H. (1999). Central control of the cardiovascular and respiratory systems and their interactions in vertebrates. *Physiol. Rev.* **79**, 855-916.

Tazawa, H., Hashimoto, Y. and Doi, K. (1992). Blood pressure and heart rate of the chick embryo (*Gallus domesticus*) within the egg: Responses to autonomic drugs. *Comp. Physiol.* **11**, 86-96.

Turesson, J., Schwerte, T. and Sundin, L. (in press). Late onset of NMDA receptor-mediated ventilatory control during early development in zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. A*.