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Cardiac performance in the zebrafish *breakdance* mutant

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Summary

In the Tübingen screen a *breakdance* mutant of zebrafish (*bre*) was described as an arrhythmia, in which the ventricle beats only with every second atrial contraction (2:1 rhythm). Surprisingly, a careful analysis of the effect of the *breakdance* mutation on cardiac performance of the zebrafish during development between 3 d.p.f. and 14 d.p.f revealed that homozygous *bre* mutants did not always show the 2:1 rhythm. Cardiac activity was continuously recorded for a period of 20 min in each larva, and during this period we observed that heart rate randomly switched between the 2:1 rhythm and a 1:1 rhythm. Furthermore, at 28°C and at 31°C the expression of the 2:1 rhythm decreased with development. At 31°C this was in part due to a significantly reduced survival rate of mutants beyond 4 d.p.f. Besides development, temperature had a marked effect on the expression of the 2:1 rhythm, and during the first days of development the expression of the 2:1 rhythm was significantly higher at elevated incubation temperatures. By contrast, in the 2:1 beating heart ventricular contraction rate was about 80 beats min^{-1} throughout development irrespective of the temperature, and even in the 1:1 rhythm mutants showed a significant bradycardia at all three temperatures (25°C, 28°C or 31°C). Compared to wild-type animals, cardiac output was significantly lower in *bre* mutants. Pressure traces recorded in the ventricle of mutants revealed a prolonged relaxation phase, indicating that the second pacemaker current could not be conveyed to the ventricle (AV-block). This phenotype is comparable to the human Long QT Syndrome, an arrhythmia caused by a modification of an ion channel involved in cardiac repolarization. The bradycardia and the modified temperature sensitivity of heart rate suggested that the activity of the pacemaker cells was also affected by this mutation.

Key words: *Breakdance* mutation, cardiac activity, temperature, development, cardiac arrhythmia, zebrafish, *Danio rerio*.

Introduction

The zebrafish *Danio rerio* is one of a few model organisms used to study vertebrate development. It has attracted attention during the completion of large scale-screens for mutations affecting numerous aspects of embryonic development (Haffter et al., 1996; Driever et al., 1996). Several of the identified mutations disrupt heart development and cardiac function (Chen et al., 1996; Alexander and Stainier, 1999; Alexander et al., 1998). In mammals an impaired heart formation typically is lethal in very early developmental stages. In the small and transparent zebrafish larvae, however, the circulatory system is not necessary for oxygen or nutrient transport during the first days of development, so that even mutants with a silent heart survive for at least 5 days after fertilization (d.p.f.; Chen and Fishman, 1997; Sehnert et al., 2002). In fact, several studies focusing on the contribution of the circulatory system to oxygen supply to tissues revealed that aerobic metabolism is fueled by bulk diffusion of oxygen through the skin, and does not require cardiac activity until about 12 or 14 d.p.f. (Jacob et al., 2002; Rombough, 2002; Pelster and Burggren, 1996). Accordingly, embryos with mutations affecting the formation of the heart often survive for at least a couple of days, enabling physiological experimentation in order to analyze the pathophysiology of the mutation. The importance of the zebrafish model with respect to cardiac physiology has recently been underlined by a study in which drugs that cause repolarization abnormalities in humans were shown consistently to cause bradycardia and AV block in the zebrafish (Milan et al., 2003). Some of the mutations affecting the heart have been attributed to a modification of an ion channel, like the mutant *slow mo*, where a defective 'pacemaker' current has been found (Baker et al., 1997), and a modified L-type Ca^{2+} channel has been identified as responsible for the *island beat* mutation in the zebrafish (Rottbauer et al., 2001). More than 50 mutations that affect the development and the function of the cardiovascular system of zebrafish have been identified in the large-scale screen, but the molecular basis and the physiology of most of these mutations remain unknown.

One of these mutations identified in the Tübingen screen is the *breakdance*-mutation (*bre*^{tb218}; Chen et al., 1996), which has been described as a mutation with an arrhythmic heart beat, i.e. the atrium contracts twice while the ventricle beats only

once (Chen et al., 1996; Langheinrich et al., 2003). Similar arrhythmias have also been described for humans and linked to the human ether-a-go-go-related gene (HERG). This gene encodes a channel responsible for the rapidly activating delayed rectifier K^+ current I_{Kr} and is important for the repolarization phase of the cardiac action potential (Vandenberg et al., 2002; Piippo et al., 2000; Sanguinetti et al., 1995). Langheinrich et al. cloned and sequenced the zebrafish homolog of this gene (*Zerg*) and identified an amino acid replacement in *bre* mutants (Ile59Ser; Langheinrich et al., 2003). Furthermore, impairing the expression of this gene by using morpholinos induced a 2:1 rhythm of the heart, an observation which supported the conclusion that the *bre* mutation is a mutation in this K^+ channel. This study, however, did not provide any evidence that the relaxation period of the ventricle is indeed prolonged in the mutant.

Unfortunately, the only available physiological information about this mutant is that atrium and ventricle contract with a 2:1 rhythm. Particularly because the genetic basis of this mutation has been elucidated, it appears important to know what consequences a defect in this channel would have, for instance on cardiac output and blood pressure? Is there an indication for a prolonged relaxation period in the ventricle, as to be expected if this potassium channel (I_{Kr}) is affected? Is only the ventricle or the AV-node affected by this mutation or is there also a modification in the activity of the pacemaker cells? Furthermore, temperature has a significant influence on development and on cardiac activity in wild-type animals. Hence, it would be interesting to know if cardiac activity in these mutants is affected by temperature in a similar way. Answers to these questions will provide valuable insight into the pathophysiology of this cardiac arrhythmia and will provide further insight into the possible defects underlying this mutation.

Materials and methods

Laboratory animals

Homozygous *breakdance* mutants (*bre*) of zebrafish *Danio rerio* Hamilton 1822 were used for our experiments. Eight parent animals, obtained from the Max-Planck Institute of Developmental Biology in Tübingen (Artemis Pharmaceuticals GmbH, Tübingen, Germany), were kept in an aquarium of 50 liters at a temperature of about 25°C. Eggs from these homozygous mutants were collected soon after spawning and transferred into small beakers, kept in a temperature controlled water bath. Micropressure measurements were performed with animals obtained from mating heterozygous *breakdance* parents ('Tübingen long fin' (TL) × *bre*, ID fish zebrafish stockcenter, Tübingen). For the experiments larvae were raised until 14 d.p.f. at a temperature of either 25°C or 28°C, and until 9 d.p.f. at 31°C. Wild-type animals (wdt) were taken from our own breeding colony. Wild-type animals were 'Tübingen long fin', so that mutants and wdt animals should have the same genetic background.

Mortality recording

Larvae of *breakdance* and wild-type zebrafish were raised at 25°C, 28°C and 31°C. For both groups (*bre* and wdt) 10 independent batches with about 100 individuals in each batch were observed. Larvae usually hatch at 3 d.p.f., and each subsequent day the dead larvae were sorted out and the fraction of surviving larvae was calculated.

Experimental protocol and recording of cardiac activity

Cardiac activity was recorded between 3 d.p.f. and 14 d.p.f. Development was faster at 31°C, so activity was recorded in this experimental group until 9 d.p.f. in order to compare identical developmental stages. For measurements larvae were removed from the incubation tank, anesthetized with 0.1 g l^{-1} tricaine (MS-222, pH 7.0) and embedded in 2.5% agarose (dissolved in 0.1 g l^{-1} tricaine). To record cardiac activity the embedded larvae were transferred to the incubation chamber in the temperature-controlled microscope stage. The temperature was set to either 25°C, 28°C or 31°C. Increased mortality of the homozygous mutants towards the end of the experiments meant that often only a few animals survived until 14 d.p.f., so that the *N*-value had to be reduced for older larvae. Cardiac activity was continuously recorded either for 1 min (snapshot observation) or for 20 min. The 1 min period was chosen to measure the incidence of the expression of the 2:1 rhythm in a large number of animals. This value reflects the probability with which a mutant animal selected out of a batch shows a 1:1 or a 2:1 rhythm during the first minute of observation. After confirming that homozygous mutants do not show the 2:1 rhythm all the time, the 20 min period was chosen to measure heart rate and to characterize individual variability in cardiac activity.

Video recording system

An inverted microscope (Leica, Vienna, Austria) was connected to a CCD camera (Hamamatsu, Herrsching, Germany), which was plugged into the luminance input of an SVHS video recorder (Sony, Vienna, Austria). The video cassette recorder was remote-controlled *via* the RS232 serial communication port. The recorded images were digitized by a monochrome frame grabber card (Imagenation PX-610, Beaverton, OR, USA) with a personal computer (PIII 450 MHz).

Determination of heart rate, stroke volume and cardiac output

The rhythm of contraction (2:1 or 1:1) was classified during the beginning of the observation period. Only animals showing a stable rhythm, either 2:1 or 1:1, were used for determination of heart rate, and animals that started switching during the measurements were excluded from data analysis. Heart rate (beats min^{-1}) was determined by measuring the time interval necessary for 30 heart beats. The average value obtained from triplicate measurements was extrapolated to get the number of beats min^{-1} for each individual fish. End-diastolic and end-systolic volumes of the ventricle were surveyed by outlining

the perimeter of the ventricle image using a mouse or a graphic tablet. The perimeter was analyzed with a 'fit-to-ellipse' algorithm, which first calculated its center of mass and subsequently the best-fitting ellipse (Jacob et al., 2002). The major and minor axes were extracted and directly transferred into a Microsoft Excel worksheet for calculation of stroke volume using the formula for a prolate spheroid ($4/3*a*b^2$) (Hou and Burggren, 1995). For analysis three diastoles and systoles were surveyed, and mean stroke volume was calculated as the difference between diastolic and systolic ventricular volumes. Cardiac output was calculated from heart rate and stroke volume.

Measurement of blood flow

Blood flow in the sinus venous was calculated from the velocity of the erythrocytes, determined by digital analysis of the video recordings, and blood vessel diameter. Details of this method have been described by Schwerte and Pelster (2000).

Measurement of blood pressure

Blood pressure in intact slightly anesthetized larvae was measured according to Pelster and Burggren (1996) using a servo-null micropressure system model 900A (World Precision Instruments, Berlin, Germany). Larvae were anesthetized using 0.05 g l^{-1} MS222. A glass electrode (tip diameter 5 μm) held in a micromanipulator was inserted through the larval body wall into the lumen of either the ventricle or truncus arteriosus to record central blood pressures. The output from the servo-null system was recorded with a PC using the software package LabView. Mean arterial pressure represents the arithmetic mean of the pressure curve (LabView).

Statistical analysis

The acquired data were statistically analyzed by using a *t*-test (software package STATISTICA). Significance was accepted when $P<0.05$ and marked with an asterisk or listed in Table 2. Data are presented as means ± S.E.M.

Results

Careful analysis of cardiac activity of homozygous *bre* zebrafish larvae, obtained by breeding homozygous *bre* parent animals, revealed that the heart of homozygous *bre*-mutants did not always contract in the 2:1 rhythm. Fig. 1A shows a typical example of a 7 d.p.f. animal at 28°C. This larva started with a 2:1 rhythm, but then, after several minutes of continuous 2:1 rhythm, the heart rate switched without any regular pattern between the 2:1 rhythm, where the ventricle contracted only after every second atrial contraction, and a 1:1 rhythm where each atrial contraction was followed by a ventricular contraction. In Fig. 1B, 100 s of the 20 min observation period is shown at an expanded time scale to demonstrate the switching in detail: in this time window the heart rate switched 15 times from the 2:1 to the 1:1 rhythm and back to the 2:1 rhythm. Towards the end of the 20 min observation period, several minutes with a stable 1:1 rhythm were observed, before the animal again switched back to the 2:1 rhythm for a few beats (Fig. 1A). The switching was observed at all developmental stages, and it occurred at irregular time intervals. This demonstrates that the 2:1 rhythm was not induced by the handling of the larvae during the experimental period. The frequency of switching ranged from once to 127 times during the 20 min observation period. Occasionally a switch for only 2 or 3 beats was observed (Fig. 1B).

Following the observation that the 2:1 rhythm was not expressed continuously in the larvae, we attempted to find out whether the expression of the 2:1 rhythm was related to development and/or temperature. A large number of larvae were screened for expression of the 2:1 rhythm at the onset of the observation period. The fraction of animals showing the 2:1 rhythm during the first minute at the beginning of the observation period changed with development and with

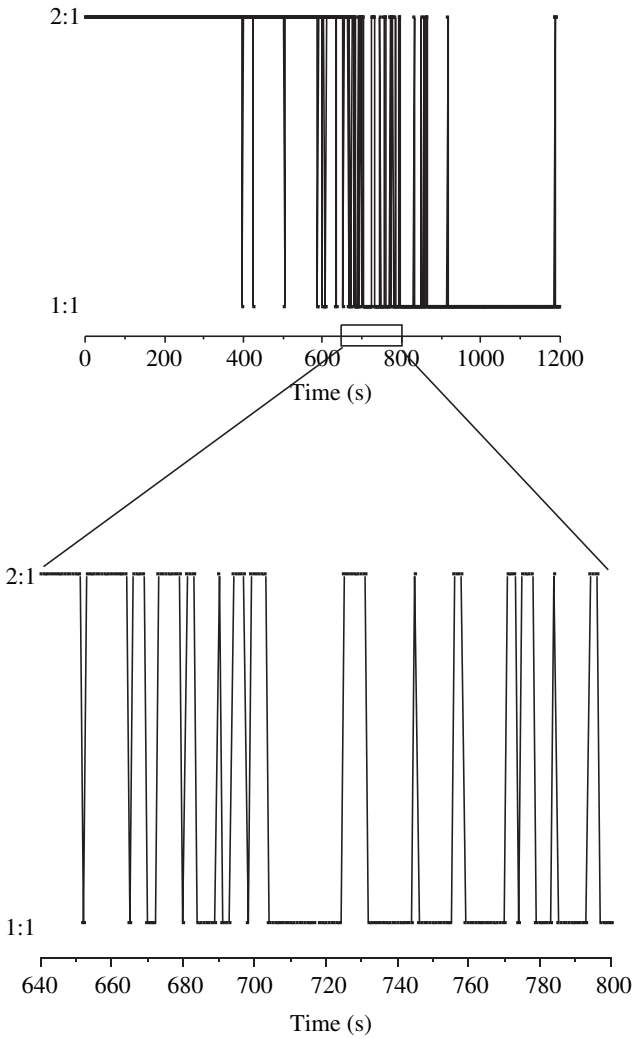

Fig. 1. (A) Changes in contraction rhythm between a 2:1 and a 1:1 rhythm over a 20 min period in a single homozygous *bre* mutant zebrafish (7 d.p.f.) raised at 28°C. (B) A time window of 100 s at an expanded time scale to illustrate beat-to-beat activity. Any change in the rhythm of contraction was recorded and dots represent 1 s intervals.

incubation temperature (Fig. 2). At 3 d.p.f., only 35.0% of the animals raised at 25°C showed a 2:1 rhythm, while 52.5% of the larvae raised at 28°C showed the 2:1 rhythm at 3 d.p.f., and 75% of the animals raised at 31°C, respectively. Thus, at 3 d.p.f. the expression of the 2:1 rhythm in the mutants increased with increasing temperature, and the lowest fraction of animals with the 2:1 rhythm was observed at 25°C. With proceeding development, in the 28°C and 31°C groups the fraction of animals showing the arrhythmia significantly decreased. At 14 d.p.f. the fraction with a 2:1 rhythm amounted to 12.5% at 28°C, while at 31°C the fraction of animals with the 2:1 rhythm had decreased to 10% at 9 d.p.f. (Fig. 2). In animals raised at 25°C the number of animals showing the arrhythmia was very low at 4 d.p.f., and then increased to 21.67% at 7 d.p.f. Thereafter the fraction decreased again. In this series of experiments we attempted to reanalyze the same animals over several days. Given the high mortality at 31°C the same animals could not be observed throughout the experimental series, but it could be observed that some individuals expressing a more or less stable 2:1 rhythm had switched to the 1:1 rhythm on the next day and *vice versa*. Thus, the expression of the 2:1 rhythm decreased with development and was not sorted out by the dying of the animals showing the 2:1 rhythm.

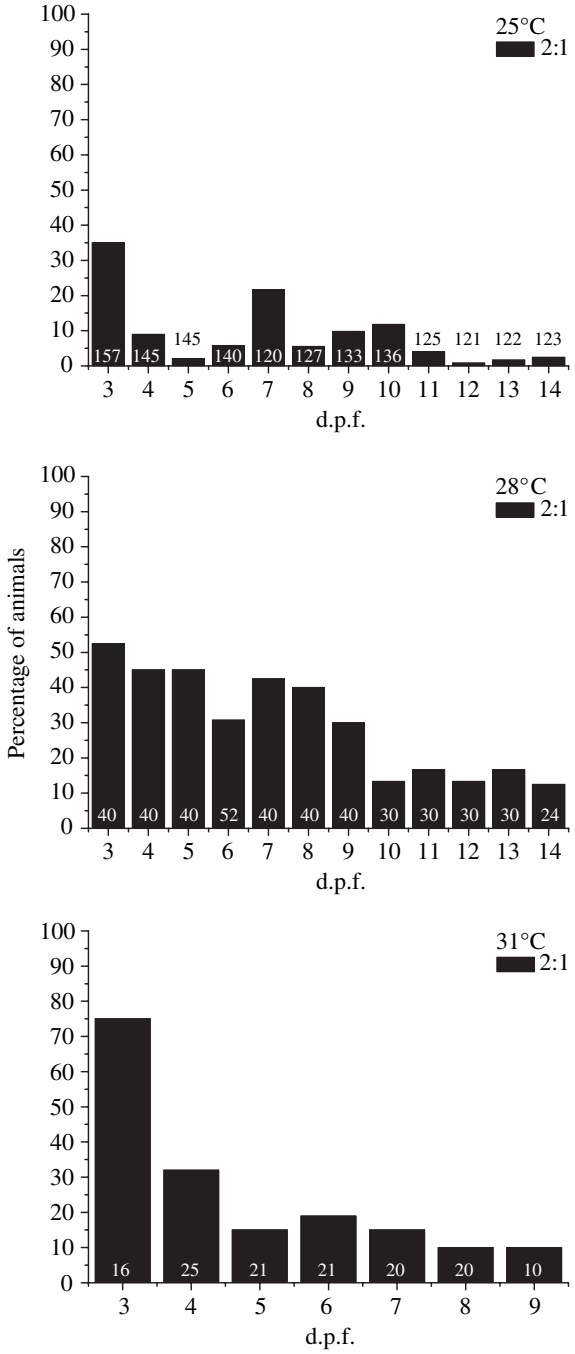

Fig. 2. Percentage of animals raised at three different temperatures (25°C, 28°C and 31°C) showing the 2:1 rhythm during the first 1 min of recording. The fraction of animals expressing the arrhythmia in homozygous *bre* mutants increased with increasing temperature. The number of analyzed animals is listed within or above each column.

These experiments nevertheless revealed a higher mortality of mutant animals compared to wild-type larvae, especially at higher temperatures and at later developmental stages (Fig. 3). The highest survival rate of mutant animals was observed at 25°C, where *bre* larvae showed nearly the same survival rate as wdt animals until 11 d.p.f. Beyond 11 d.p.f. the survival rate of *bre* mutants was reduced compared to wild-type animals. In *bre* larvae raised at 28°C a steady decrease in the survival rate was observed from 9 d.p.f. on, reaching its minimum at 12–14 d.p.f. Survival of wild-type larvae also decreased from 11 d.p.f. on, but the mortality was significantly lower than in mutant animals. 31°C proved to be a very extreme situation. Deformations of the heart and impaired heart performance commonly occurred in both groups. Thus, survival had already started to decrease at 4 d.p.f., and the survival of *bre* larvae was significantly lower than that of wild-type larvae. At 5 d.p.f. only about 5% of the *bre* larvae survived, compared with 90% of wild-type animals. Afterwards survival remained almost constant, but at this temperature that of wild-type larvae was also remarkably low compared to lower temperatures. From 6 d.p.f. on it decreased continuously and very few larvae survived until the end of our observation period. In 6 of 10 batches none of the *bre* larvae survived until 9 d.p.f. Of the 1082 wild-type eggs at the onset of the experiment 809 larvae hatched, and on average only 30% of the wild-type larvae were still alive at 9 d.p.f.

A detailed analysis of changes in chamber-specific heart rate with development in homozygous *bre* mutant animals is summarized in Fig. 4, including a comparison with data on wild-type animals. Data on cardiac activity of wild-type zebrafish have been published before (Pelster and Burggren, 1996; Barrionuevo and Burggren, 1999; Jacob et al., 2002). To avoid repetition only certain stages were repeated for wild-type animals. Table 1 presents the *N*-values for the various groups; note that different mortality rates (Fig. 3) reduced the number of experimental animals in certain groups. A striking observation was that in homozygous *bre* larvae, at 28°C and 31°C, ventricular contraction rate in the 2:1 rhythm was about 80 beats min^{-1}, and there was almost no change in heart rate

with proceeding development. In animals raised at 25°C ventricular contraction rate in the 2:1 rhythm was also about 80 beats min^{-1} or slightly lower, although it was a little more variable than at the higher temperatures. Comparison with the heart rate of wild-type animals revealed that atrial contraction frequency of *bre* hearts beating in the 2:1 rhythm was almost always significantly lower than that of wild-type animals (see Table 2 for significance).

At 25°C most of the homozygous *bre* mutants showed a 1:1 rhythm. Heart rate started at about 110 beats min^{-1} at (3 d.p.f.), but then increased to 190 beats min^{-1} (8 d.p.f.), and dropped again to about 140 beats min^{-1} (13 d.p.f.; Fig. 4). These changes in cardiac activity with proceeding development paralleled the developmental changes in cardiac activity observed in wild-type animals, but the heart rate of *bre* mutants beating with the 1:1 rhythm was consistently lower than those of wild-type animals. Thus, throughout development, even with a 1:1 rhythm, *bre* mutants had a significantly lower heart rate (about 30–40 beats min^{-1}) than wild-type animals (see Table 2 for significance). The bradycardia of *bre* mutants contracting in the 1:1 rhythm was even more pronounced at 28°C and 31°C. At both temperatures contraction rate in the 1:1 rhythm was 100–120 beats min^{-1}, and there was no significant change with development (Fig. 4). In wild-type animals raised at these temperatures heart rate initially increased with development and than decreased, similar to the pattern observed in *bre* animals at 25°C.

Like heart rate, the stroke volume of larvae contracting with the 2:1 rhythm showed very little change with development at all three temperatures (Fig. 5). At 25°C the stroke volume of wild-type animals was significantly higher, but at 28°C lower, than in *bre* mutants (at 5 and 7 d.p.f. differences were statistically significant), except for the last few days of the observation period. After 11 d.p.f., stroke volume decreased in mutant animals, while that of wild-type animals increased, finally exceeding values of homozygous *bre* mutants. At 31°C, stroke volume of wild-type individuals was always lower than that of *bre*-mutants, and this difference was significant until 9 d.p.f.

Cardiac output showed some variation at all three temperatures (Fig. 6). Compared to wild-type animals raised at 25°C, 28°C or at 31°C, cardiac output of mutants was severely reduced. This difference was especially pronounced towards the end of the observation period at 25°C and at 28°C, when cardiac output of the growing wild-type larvae increased significantly, while in *bre* mutants it decreased.

In an additional series of experiments blood pressure was measured in the ventricle and in the bulbus arteriosus of wild-type larvae and *bre* mutants at 2.5–3 d.p.f. (body mass 0.2–0.3 mg). At this early developmental stage, pressure could be measured by penetrating the body wall with the glass pipette, whereas at later stages the body wall became too thick, necessitating a preparation, which might have interfered with the pressure signal. While peak systolic pressure appeared to be similar in both experimental groups (Fig. 7), mean arterial blood pressure was slightly lower in *breakdance* larvae (0.31±0.02 mmHg; N=12), in contrast to 0.37±0.04 mmHg for wild-type larvae (N=17, Table 3; P<0.05). A comparison of

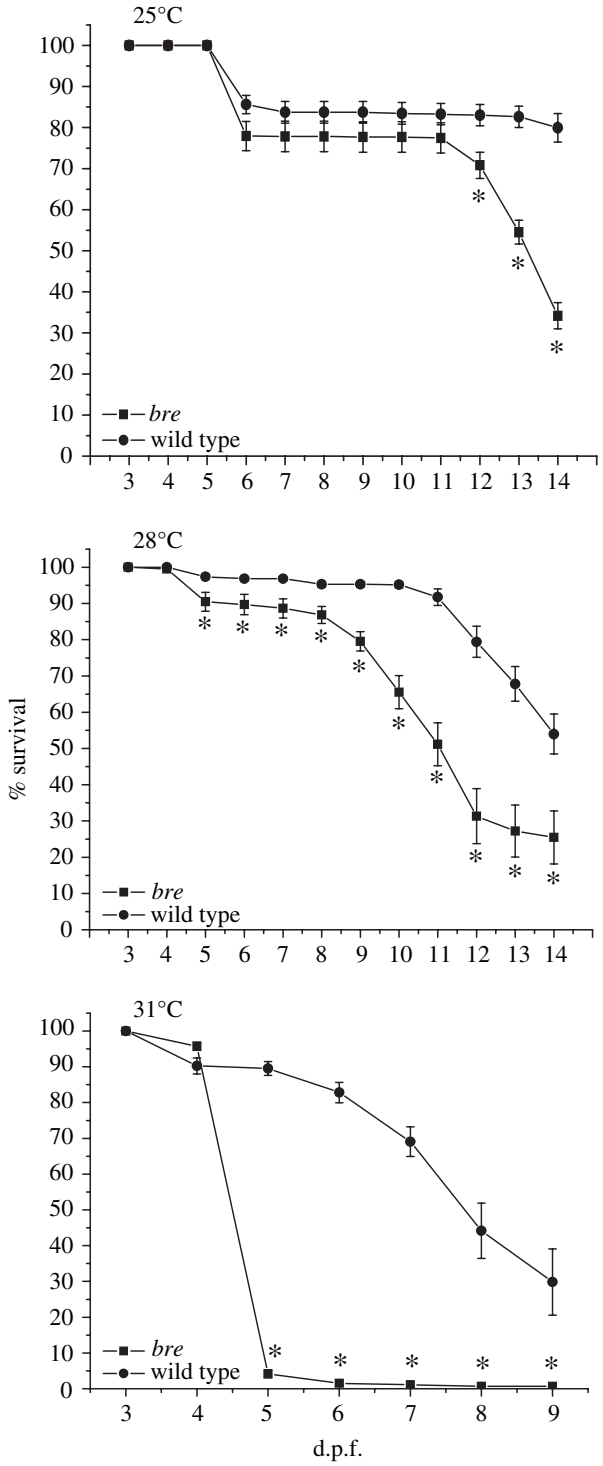

Fig. 3. Survival rate of wild-type and *bre* zebrafish larvae raised at 25°C, 28°C or 31°C during the first days of development. For each group 10 separate batches of animals were observed, with about 100 animals in each batch. Total number of hatched larvae (N) at the beginning of the experiment were 826 (25°C), 802 (28°C) and 467 (31°C) for *bre* larvae, and 435 (25°C), 716 (28°C) and 809 (31°C) for wild-type larvae, respectively.

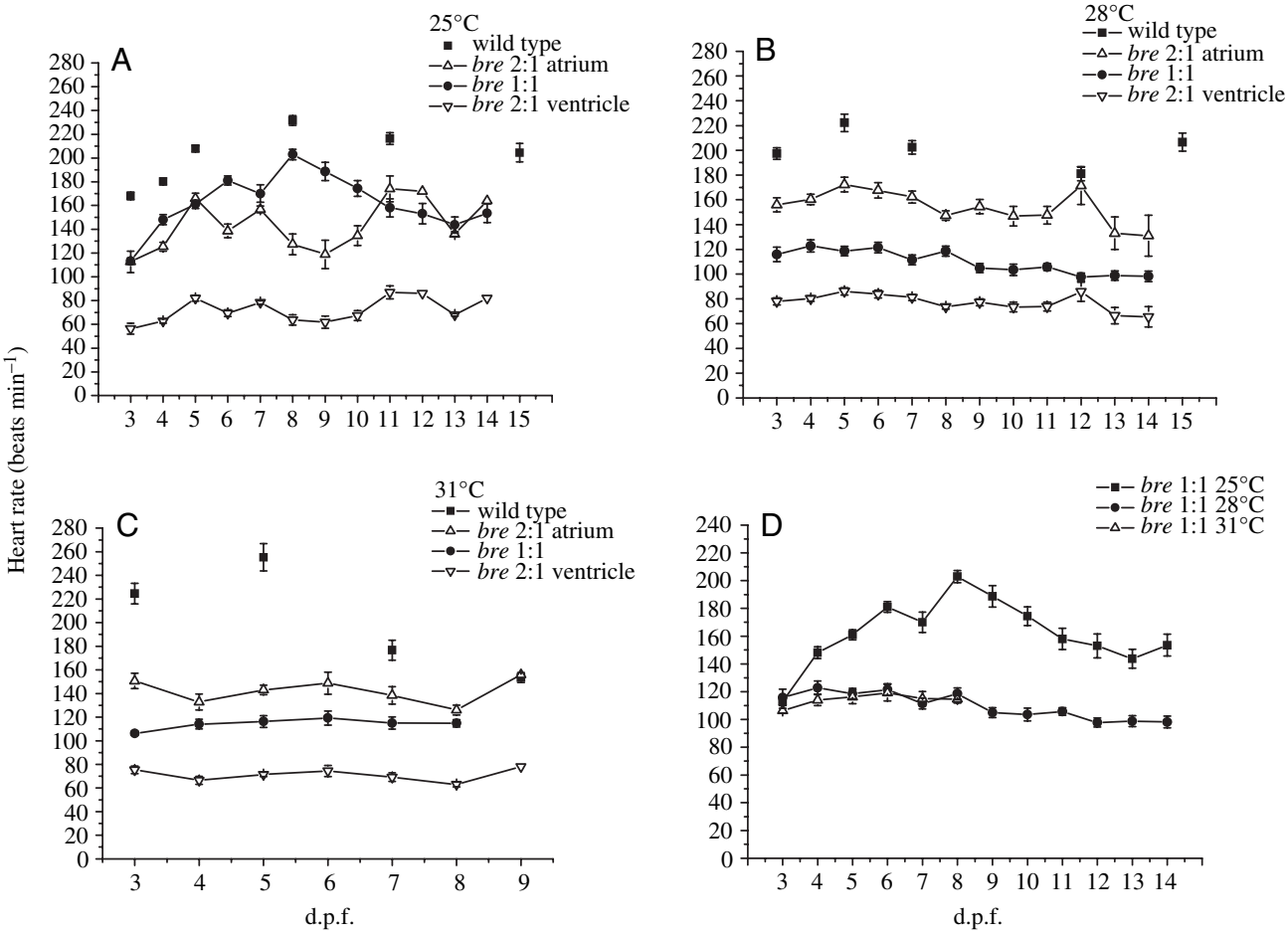

Fig. 4. Changes in heart rate with development in homozygous *bre* mutant animals raised at 25°C, 28°C or 31°C. (A–C) Chamber-specific heart rate (i.e. atrial rate and ventricular rates) in the 2:1 rhythm, and heart rate at the 1:1 rhythm for the three different temperatures. For comparison, heart rate of wild-type animals is included. (D) Comparison of heart rate of mutants in the 1:1 rhythm at the three different temperatures in order to demonstrate the inverse temperature sensitivity of heart rate during the 1:1 rhythm. *N*-values for the separate stages and groups as well as a statistical comparison of the various stages and groups are listed in Tables 1 and 2.

pressure traces obtained from wild-type and *bre* animals showed that in mutants every second ventricular contraction was completely missing, and during this time ventricular and bulbus arteriosus pressure reached a plateau at about diastolic ventricular pressure. Compared to wild-type animals the final part of ventricular relaxation was extended. In wild-type animals the time between the increase of ventricular pressure at the onset of contraction and the return of the pressure trace to the diastolic level was 332.6±1.8 ms, while in *bre* mutants it was 464.7±3.8 ms (N=5; P<0.01). In the pressure traces this prolongation of ventricular contraction was sometimes clearly visible as a shoulder during the relaxation period (Fig. 7A). The delayed relaxation was not seen in either the pressure traces of the ventral artery (Fig. 7A), or in the bulbus arteriosus (Fig. 7C). Mostly the atrium contracted during this phase without emptying into the ventricle, inducing a visible back flow of erythrocytes away from the heart (Fig. 7D). The extended time period between subsequent ventricular contractions appeared to result in a slightly increased blood accumulation in the sinus venosus. Infrequently the ventricle took over additional blood from the second atrial contraction.

Discussion

The *breakdance* mutant of zebrafish has been described as cardiac arrhythmia in which only every second atrial contraction is followed by a ventricular one (Chen et al., 1996). Analyzing the video recordings we recognized, however, that the heart of homozygous *bre* zebrafish larvae did not show the 2:1 rhythm all the time. In almost all of the homozygous *bre* larvae, besides the 2:1 an additional 1:1 rhythm was observed, i.e. every atrial contraction was followed by a ventricular contraction. Switching between the two patterns of cardiac activity occurred without any regularity and was not noted in the initial characterization of the *bre* mutation or in the morpholino knockdown embryos of *Zerg* (zebrafish ortholog of HERG; Chen et al., 1996; Langheinrich et al., 2003), and the severe bradycardia of *bre* mutants was also not mentioned in these original studies. It can be speculated that through

Table 1. N-*values for the individual groups analyzed for cardiac activity (Fig. 4)*

	*bre*1:1			*bre*2:1			Wild type		
d.p.f.	25°C	28°C	31°C	25°C	28°C	31°C	25°C	28°C	31°C
3	12	12	12	12	12	12	12	12	12
4	12	12	12	11	12	11	12		
5	12	12	12	9	12	10	12	12	12
6	12	12	8	6	12	8			
7	12	12	9	12	12	9		12	12
8	12	12	8	6	12	9	27		
9	12	12		10	12	1			12
10	12	12		13	5				
11	12	12		4	5		18		
12	12	12		1	4			9	
13	12	12		2	4				
14	12	12		1	4				
15							18	11	

Table 2. *Data summarizing significant differences in heart rate, stroke volume and cardiac output in wild-type and* bre *animals as shown in Fig. 4, at three different temperatures*

Temperature (°C)	25	28	31	25/28	28/31	25/31
Heart rate	*bre* 1:1/wdt (3–14) Atrium *bre* 2:1/wdt (3–14) Ventricle *bre* 2:1/wdt (3–14)	*bre* 1:1/wdt (3–14) Atrium *bre* 2:1/wdt (3–12) Ventricle *bre*2:1/wdt (3–14)	*bre* 1:1/wdt (3–9) Atrium *bre*2:1/wdt (3–7) Ventricle *bre*2:1/wdt (3–9)	1:1/1:1 (4–14) 2:1/2:1 (none)	1:1/1:1 (none) 2:1/2:1 (none)	1:1/1:1 (3–9) 2:1/2:1 (none)
Stroke volume	*bre*/wdt (3–11)	*bre*/wdt (5 and 7)	*bre*/wdt (3,7,11)	*bre*/wdt (8,11,12 and 13)	*bre*/wdt (4 and 8)	*bre*/wdt (3)
Cardiac output	*bre*/wdt (3–14)	*bre*/wdt (3–14)	*bre*/wdt (3 and 5)	*bre*/wdt (3–10)	*bre*/wdt (4,7,8)	*bre*/wdt (3)

To read the table: at 25°C, heart rate of *bre* mutants beating with the 1:1 rhythm was significantly different from heart rate of wild-type (wdt) animals in all stages between 3 and 14 d.p.f. (P<0.05).

generations of inbreeding of homozygous *bre* mutants, a line of fish with a lower expressivity or with a *bre* modifier was selected. The experiments described in this study were performed soon after receiving the homozygous parent animals from the Max-Planck-Institute of Developmental Biology in Tübingen. In addition, for micropressure measurements, for example, homogygous *breakdance* animals were obtained from mating heterozygous *breakdance* parents (TL × bre, ID fish zebrafish stockcenter Tübingen). Mutant animals were sorted out by identification of the typical phenotype of a 2:1 ratio between atrium and ventricular contraction. Thus, offspring from heterozygous and homozygous parents showed the typical switching between 2:1 and 1:1 rhythm. We are therefore convinced that the temperature-dependent switching behaviour is not the result of continuous inbreeding.

It is generally accepted that arrhythmias represent the end product of abnormal ion-channel function, which can result from genetic mutations, altered levels or spatial patterns of expression or from a modulation of activity caused by ischaemia, for example (Vandenberg et al., 2002). Metabolic effects such as oxygen shortage can be excluded due to the small size of the embryos, which do not need the circulatory system for oxygen supply and are apparently well oxygenated just by bulk diffusion through the skin (Territo and Altimiras, 2001; Pelster, 1999, 2002). Thus, the slowed repolarization of the ventricle can probably be attributed to a modification of an ion channel. An unspecific cation channel, a Ca^{2+} channel and a K^+ channel (delayed rectifier channel) have been discussed in context with cardiac arrhythmia (Baker et al., 1997). The phenotype of the *bre*-mutation appears to phenocopy the human Long QT Syndrome (Vandenberg et al., 2002; Shanbag et al., 2002; Curran et al., 1995), which is due to a mutation in a gene encoding a K^+ channel (Sanguinetti et al., 1995). It has been identified as a miss-sense mutation in the HERG-K^+ channel (HERG=human ether-a-go-go related gene) (Huang et al., 2001; Piippo et al., 2000), which results in an abnormality in atrio-ventricular conductance and a prolonged QT interval, and thus in an extended repolarization. Accordingly it appears possible that a defective K^+ channel is also underlying the *bre* mutation in zebrafish, and Langheinrich et al. (2003) did indeed identify a mutation in the *Zerg* gene that could be responsible for the *bre* mutation. As a consequence of an extended QT repolarization, the time course of the relaxation period following ventricular contraction may be extended, and

this was indeed observed in our pressure measurements. Compared to wild-type animals the relaxation time of the ventricle was significantly prolonged in *bre* mutants. This observation supports the conclusion of Langheinrich et al. (2003) that the *Zerg* gene is responsible for this mutation, and that the 2:1 rhythm of *bre* animals is the consequence of an AV-block.

Not only was the relaxation time prolonged in *bre* mutants, but we also observed a significant bradycardia in these animals in the 2:1 rhythm (atrial frequency), and even more pronounced in the 1:1 rhythm, especially at 28°C and 31°C. This suggested that not only the QT interval of the conductance system was affected, but that the pacemaker cells in the sinoatrial node must

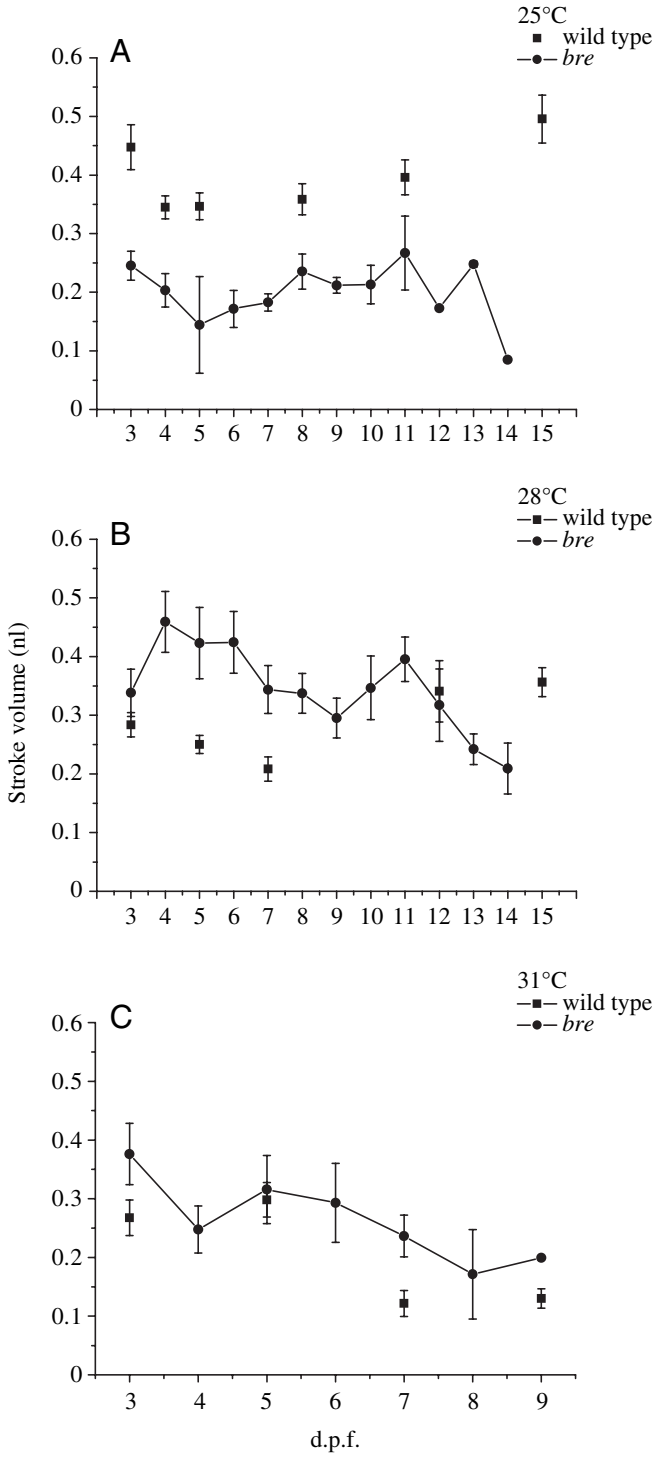

Fig. 5. Changes in stroke volume (2:1 rhythm) with development in homozygous *bre* mutant animals raised at 25°C, 28°C or 31°C. For comparison, data for wild-type zebrafish are included. For statistical significance and *N*-values, see Tables 2 and 3.

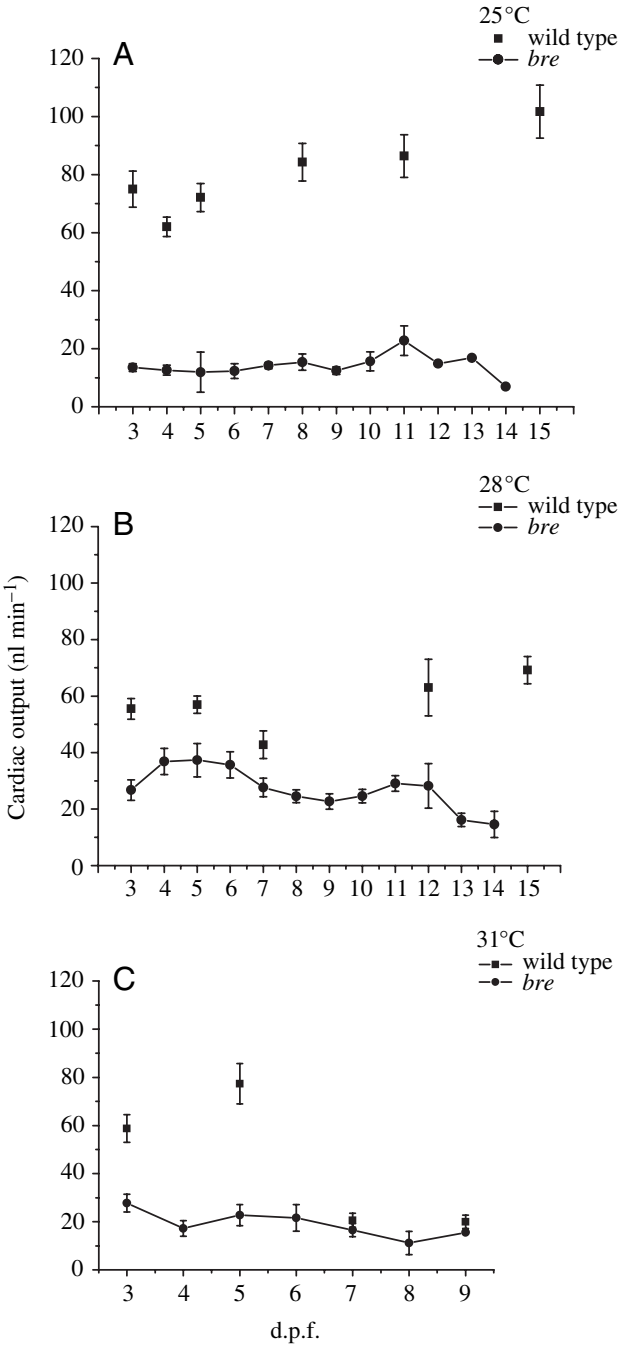

Fig. 6. Changes in cardiac output (2:1 rhythm) with development in homozygous *bre* mutant animals raised at 25°C, 28°C or 31°C. For comparison, data for wild-type zebrafish are included. For statistical significance and *N*-values, see Tables 2 and 3.

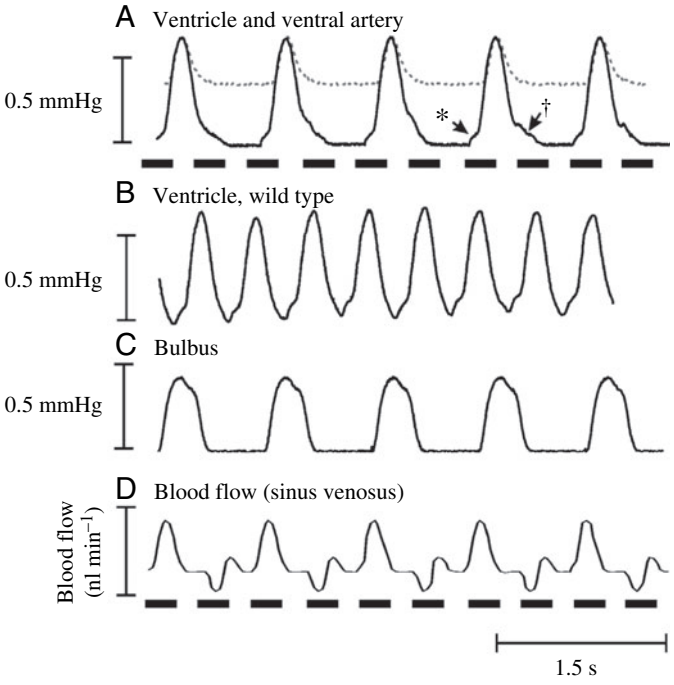

Fig. 7. Blood pressure and blood flow in homozygous *bre* zebrafish mutants (A,C,D) and blood pressure in wild-type zebrafish (B) (2.5–3 d.p.f.). (A) Blood pressure in the ventricle and in the ventral artery. Horizontal bars show duration of atrial contractions. Dotted line, trace of blood pressure in the ventral artery; *atrial contraction; †second atrial contraction. (B,C) Blood pressure in the ventricle and in the bulbus arteriosus. (D) Blood flow to the heart, measured by video imaging in the sinus venosus. Horizontal black bars, duration of atrial contractions.

be affected as well. Electrophysiological studies revealed that the ERG channels (I_{Kr}) are expressed in mouse pacemaker cells and pharmacological block of these channels results in a bradycardia (Clark et al., 2004). The bradycardia observed in *bre* animals thus provides additional support for the conclusion that this mutation is due to a defect in the delayed rectifier potassium channel. *Slow mo* has been described as another zebrafish mutant with a chronically reduced heart rate, and a defective pacemaker current (I_h) has been identified as responsible for this mutation (Baker et al., 1997).

The influence of development

Our data clearly demonstrate that expression of the arrhythmia was dependent on the developmental state of the larvae. With proceeding development the expression of the arrhythmia was significantly reduced in animals raised at 28°C and at 31°C. After the first beat, heart rate typically increases with development, reaching a maximum after the first few days of development and than decreasing slowly to an adult level (Burggren and Warburton, 1994; Pelster, 1997). In zebrafish, the highest heart rates are typically recorded until about 5 d.p.f. and 8 d.p.f., depending on temperature. During this time, the expression of the arrhythmia had already dropped from about 55% at the onset of cardiac activity to about 40% in 28°C animals, and at 31°C this decrease was even more pronounced. Thus, in this phase of development the reduction in the expression of the arrhythmia cannot be related to a concomitant change in heart rate. In animals raised at 25°C the appearance of the 2:1 rhythm dropped until 5 d.p.f. and then increased again until 7 d.p.f. Several studies have discussed possible reasons for development-related changes in membrane conductance in pacemaker cells, but there appears to be no obvious explanation for this change in the expression of the 2:1 rhythm with proceeding development. One possibility might be a change in the expression pattern of ion channels involved in the conductance of the electrical signal in cardiomyocytes. During development different isoforms or even different ion channels may be integrated into the cell membrane (Berthier et al., 2002; Schmidt et al., 1999) and so there may be an exchange of the defective channels by functioning ones. Another possibility appears to be that the voltage dependency of a channel changes with development, which has been shown for the pacemaker current in rat ventricle, for example (Robinson et al., 1997). This can be the consequence of a drifting of genes, a change in the structure of the channel depending on the age of the larvae, or the presence or absence of associated proteins. Growth of cells might also modify the electrical properties and thus contribute to this phenomenon (Verheijck et al., 2002).

Apart from the prolonged relaxation period, comparison of the individual contraction of the ventricle of a *bre* mutant and of wild-type animals did not provide any indication for an impairment of ventricular performance in mutant animals at the beginning of cardiac activity (3 d.p.f.). Peak systolic blood pressure was similar in mutants and in wild-type animals, and normally during the second atrial contraction ventricular filling did not increase, obviously because ventricular pressure was still elevated and atrial pressure could not open the

Table 3. *Mean values of heart rate, stroke volume, cardiac output and blood pressure in slightly anesthetized wild-type animals and in homozygous* bre *mutants measured at 2.5–3 d.p.f.*

	Heart rate (beats min^{-1})	Cardiac output (nl min^{-1})	Stroke volume (nl)	Blood pressure in ventral aorta (mmHg)	*N*
Breakdance	85±2*	38±4*	0.44±0.2	0.31±0.02*	12
Wild type	175±5	71±2	0.41±0.3	0.37±0.04	17

Temperature = 28°C

*Significant differences between *breakdance* and wild-type animals (P<0.05).

atrioventricluar valve. Very rarely we observed that the ventricle took over additional blood from the second atrial contraction. Usually a retrograde movement of blood cells was observed in the sinus venosus during the second contraction, and occasionally the shape change of the chambers during the contraction without emptying caused a backwards movement of red cells in the aorta dorsalis, but these cells did not return into the ventricle. These observations clearly suggest a proper functioning of all valves within the heart. Thus, the individual contraction of the ventricle appeared to be normal except for the prolonged relaxation, and during arrhythmia every second ventricular contraction was missing. This is in line with previous descriptions of the mutant (Chen et al., 1996; Langheinrich et al., 2003), which stated that the arrhythmia was the only phenotype.

While the bradycardia was consistently observed in all mutants, irrespective of the developmental stage and the temperature, the situation was different for stroke volume. At 25°C, stroke volume was lower in *bre* mutants than in wild-type animals, but at 28°C and at 31°C stroke volume was elevated compared to wild-type animals. In animals raised at 25°C the bradycardia together with the reduced stroke volume resulted in a significantly lower cardiac output in mutants, but even in animals raised at higher temperatures the increase in stroke volume could only partly compensate for the reduced heart rate, so that in all mutants cardiac output was significantly lower than in wild-type animals. Until 8 d.p.f. at 28°C and 11 d.p.f. at 25°C the viability of mutants was not impaired compared to wild-type animals, so that the reduced cardiac output did not result in an increased mortality. This can probably be attributed to the fact that in early developmental stages oxygen supply is achieved by bulk diffusion, and convective oxygen transport is not required to ensure aerobic metabolism of the tissues (Territo and Altimiras, 2001; Pelster, 1999, 2002). Between 12 and 14 d.p.f., convective oxygen transport becomes essential to ensure adequate oxygen supply to tissues (Schwerte et al., 2003; Jacob et al., 2002; Rombough, 2002). The increased mortality of mutants observed at about 10 and 12 d.p.f. may therefore, at least in part, be related to the fact that at about this stage convective oxygen transport becomes essential to meet the oxygen requirements of the larvae, and the reduced cardiac output is not sufficient to take on this task. At 31°C the situation was aggravated even more. In our experiments at 31°C the viability of wild-type larvae was actually significantly reduced compared to lower temperatures, despite 31°C being listed as a normal temperature for zebrafish development. The mortality of mutants was extremely high from 5 d.p.f., and can probably be attributed to the very high metabolic rate at this temperature, which requires an earlier onset of convective oxygen transport in order to ensure aerobic metabolism of all tissues.

The elevation of stroke volume observed at 28°C and at 31°C obviously represented a partial compensation for the bradycardia caused by the mutation in order to stabilize cardiac output. The pressure measurements together with the data for cardiac output indicated that in mutants compensatory changes also occurred in total peripheral resistance, which can be calculated from the pressure difference ΔP divided by blood flow. While flow (cardiac output) was reduced by about 50% or more compared to wild-type animals, systolic pressure was similar in mutants and in wild-type animals, and mean arterial pressure was reduced by only 20% in *bre* mutants, so that total peripheral resistance must be significantly elevated in mutants. This suggests that a vasoconstriction may be present in order to stabilize blood pressure in mutant animals.

Temperature dependence

A striking observation was an increasing expression of the arrhythmia at higher temperatures. Screening of the larvae revealed that at 25°C only a low percentage of the larvae expressed the 2:1 rhythm during the first days of cardiac activity, while at 31°C the 2:1 rhythm was observed in more than 70% of the animals. It is well established that heart rate increases with increasing temperature, and this is also true for vertebrate larvae during early development (Pelster, 2002), which suggests that the expression of the 2:1 rhythm might be especially pronounced at higher frequencies; but this conclusion was not supported by the changes in the expression of the 2:1 rhythm observed with development (see above). At 25°C, for example, expression of the 2:1 rhythm was higher at 3 d.p.f. than at 5 d.p.f., while heart rate usually increases with development at this stage (see Fig. 4, and also Jacob et al., 2002). At 28°C and at 31°C expression of the 2:1 rhythm was high in the earliest stages, while the highest heart rate is observed about 2 days later (see Fig. 4). Nevertheless, a prolongation of the QT interval will most likely induce a 2:1 rhythm at high frequencies. At low frequencies, low enough to permit completion of the repolarization despite the fact that this repolarization is prolonged compared to wild-type animals, the 1:1 rhythm should occur. If heart rate increases, however, repolarization cannot be completed, and as a consequence the second atrial depolarization reaches the ventricle in its refractory phase, and atrio-ventricular conductance is interrupted (AV-block). The heart starts to beat with a 2:1 rhythm. This may explain why expression of the 2:1 rhythm was much lower at 25°C, but obviously cannot explain all of the temperature-related changes in the expression of the 2:1 rhythm.

Studies on mutated ion channels of *Drosophila* revealed that temperature may modulate the overall activity of ion channels. *napts*, for example, is a recessive mutation affecting a sodium channel so that at higher temperatures action potentials are lost (Kernan et al., 1991). Temperature has also been reported to regulate a potassium channel in *Drosophila* selectively (Chopra and Singh, 1994). Therefore it may be that the effect of temperature on the expression of the 2:1 rhythm is due to temperature related changes in the activity of an ion channel.

Another striking observation was the temperature insensitivity of the mutant heart, compared to wild-type animals. Ventricular frequency during the 2:1 rhythm remained at about 80–85 beats min^{-1}, irrespective of incubation temperature and development. In wild-type zebrafish larvae the

Q_{10} (i.e. the increase in activity encountered during a 10°C increase in temperature) for heart rate varied between 1.2 and 2.5 (Jacob et al., 2002; Barrionuevo and Burggren, 1999). Accordingly, heart rate can be expected to increase with rising temperature, but this was not observed after 5 d.p.f. at 28°C and 31°C and in mutants with the 2:1 rhythm. In the 2:1 rhythm mutants, ventricular rate appeared to be independent of temperature, i.e. the Q_{10} was about 1. An increase in incubation temperature, however, caused a significant increase in the number of mutant animals expressing the arrhythmia (see above).

A similar situation was observed when comparing heart rate in the 1:1 rhythm of animals raised at 28°C and 31°C. At both temperatures heart rate remained at about 110–120 beats min^{-1}, demonstrating insensitivity to temperature. At 25°C the situation was different. In these animals heart rate showed some changes with development and reached values of about 160 beats min^{-1}, which exceeded even that observed at higher temperatures, resulting in a reversed temperature effect between 25°C and 28°C. The results obtained with the 25°C group differed from the results of the higher temperature animals in several respects. In contrast to the 28°C and 31°C group, in this class developmental changes in heart rate were observed, and atrial contraction rate during the 2:1 rhythm and heart rate during the 1:1 rhythm were similar except for the time between 8 and 10 d.p.f. In the other two groups heart rate in the 1:1 rhythm was significantly lower than atrial rate during the 2:1 rhythm. Based on the general effect of temperature we would expect that heart rate in the 25°C group is lower than in the 28°C and 31°C class. Accordingly, this observation may again indicate that higher temperatures, and thus higher heart rates, facilitate the expression of the 2:1 rhythm, and that the negative aspects of this mutation can best be compensated at lower temperatures.

In summary, we can say that the *bre* mutation does not imply the continuous expression of a 2:1 rhythm. An elevated ventricular pressure during the relaxation phase and the bradycardia support the conclusion that an ion channel involved in the repolarization of the ventricle is defective in mutant animals. A possible candidate for this ion channel would be a potassium channel, as proposed by Langheinrich et al. (2003), but the mutation not only affects the signal transduction to the ventricle, but also the pacemaker itself. This was clearly demonstrated by the temperature sensitivity of mutant hearts, which differed largely from the temperature sensitivity of wild-type hearts. The bradycardia inevitably resulted in a significantly reduced cardiac output, although at 28°C and at 31°C a compensating elevation of stroke volume was observed. Measurements of blood pressure and of cardiac output also indicated that in mutants peripheral resistance is elevated in order to stabilize blood pressure. Nevertheless, at least in later stages, the viability of mutants is significantly reduced compared to wild-type animals, although *bre* mutants may survive until adulthood, especially at lower temperatures.

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References

Alexander, J. and Stainier, D. Y. R. (1999). Mutations affecting cardiac development in zebrafish. In *Heart Development* (ed. R. P. Harvey and N. Rosenthal), pp. 91-110. San Diego, London, Boston, New York, Sydney, Tokyo, Toronto: Academic Press.

Alexander, J., Stainier, D. Y. R. and Yelon, D. (1998). Screening mosaic F1 females for mutations affecting zebrafish heart induction and patterning. *Dev. Genet.* **22**, 288-299.

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

Barrionuevo, W. R. and Burggren, W. W. (1999). O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2. *Am. J. Physiol.* **276**, R505-R513.

Berthier, C., Monteil, A., Lory, P. and Strube, C. (2002). a1H mRNA in single skeletal muscle fibres accounts for T-type calcium current transient expression during fetal development in mice. *J. Physiol.* **539**, 681-691.

Burggren, W. W. and Warburton, S. (1994). Patterns of form and function in developing hearts: Contributions from non-mammalian vertebrates. *Cardioscience* **5**, 183-191.

Clark, R. B., Mangoni, M. E., Lueger, A., Couette, B., Nargeot, J. and Giles, W. R. (2004). A rapidly activating delayed rectifier K^+ current regulates pacemaker activity in adult mouse sinoatrial node cells. *Am. J. Physiol*. **286**, H1757-H1766.

Chen, J.-N. and Fishman, M. C. (1997). Genetic dissection of heart development. In *Development of cardiovascular systems: Molecules to organisms* (ed. W. W. Burggren, and B. B. Keller), pp. 7-17. Cambridge: Cambridge University Press.

Chen, J.-N., Haffter, P., Odenthal, J., Vogelsang, E., Brand, M., van Eeden, F. J., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Heisenberg, C.-P. et al. (1996). Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development* **123**, 293-302.

Chopra, M. and Singh, S. (1994). Developmental temperature selectively regulates a voltage-activated potassium current in *Drosophila*. *J. Neurobiol*. **25**, 119-126.

Curran, M. E., Splawski, I., Timothy, K. W., Vincent, G. M., Green, E. D. and Keating, M. T. (1995). A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. *Cell* **80**, 795-803.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z. et al. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37-46.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., Eden, F. J. M. V., Jiang, Y.-J., Heisenberg, C.-P. et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1-17.

Hou, P.-C. L. 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.

Huang, L., Bitner-Glindzicz, M., Tranebjaerg, L. and Tinker, A. (2001). A spectrum of functional effects for disease causing mutations in the Jervell and Lange-Nielson syndrome. *Cardiovasc. Res.* **51**, 670-680.

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

Kernan, M. J., Kuroda, M. I., Kreber, R., Baker, B. S. and Ganetzky, B. (1991). napts, a mutation affecting sodium channel activity in *Drosophila*, is an allele of mle, a regulator of X chromosome transcription. *Cell* **66**, 949-959.

Langheinrich, U., Vacun, G. and Wagner, T. (2003). Zebrafish embryos express an orthologue of HERG and are sensitive towards a range of QT-prolonging drugs inducing severe arrhythmia. *Toxicol. Appl. Pharmacol*. **193**, 370-382.

Milan, D. J., Peterson, T. A., Ruskin, J. N., Peterson, R. T. and MacRae, C. A. (2003). Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation* **107**, 1355-1358.

Pelster, B. (1997). Oxygen, temperature, and pH influences on the development of non-mammalian embryos and larvae. In *Development of*

cardiovascular systems (ed. W. W. Burggren and B. B. Keller), pp. 227-239. Cambridge: Cambridge University Press.

Pelster, B. (1999). Environmental influences on the development of the cardiac system in fish and amphibians. *Comp. Biochem. Physiol.* **124A**, 407-412.

Pelster, B. (2002). Developmental plasticity in the cardiovascular system of fish, with special reference to the zebra fish. *Comp. Biochem. Physiol.* **133A**, 547-553.

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

Piippo, K., Laitinen, P., Swan, H., Toivonen, L., Viitasalo, M., Pasternack, M., Paavonen, K., Chapman, H., Wann, K. T., Hirvela, E. et al. (2000). Homozygosity for a HERG potassium channel mutation causes a severe form of long QT syndrome: identification of an apparent founder mutation in the finns. *J. Am. Coll. Cardiol.* **35**, 1919-1925.

Robinson, R. B., Yu, H., Chang, F. and Cohen, I. S. (1997). Developmental change in the voltage-dependence of the pacemaker current, if, in rat ventricle cells. *Eur. J. Physiol.* **433**, 533-535.

Rombough, P. J. (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.

Rottbauer, W., Baker, K., Wo, Z. G., Mohideen, M. A., Cantiello, H. F. and Fishman, M. C. (2001). Growth and function of the embryonic heart depend upon the cardiac-specific L-type calcium channel alpha 1 subunit. *Dev. Cell* **1**, 265-275.

Sanguinetti, M. C., Jiang, C., Curran, M. E. and Keating, M. T. (1995). A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{Kr} potassium channel. *Cell* **81**, 299-307.

Schmidt, K., Eulitz, D., Veh, R. W., Kettenmann, H. and Kirchhoff, F. (1999). Heterogeneous expression of voltage-gated potassium channels of the *shaker* family (Kv1) in oligodendrocyte progenitors. *Brain Res.* **843**, 145-160.

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

Sehnert, A. J., Huq, A., Weinstein, B. M., Walker, C., Fishman, M. C. and Stainier, D. Y. R. (2002). Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. *Nat. Genet*. **31**, 106-110.

Shanbag, P., Govindakumar, P. T., Vaidya, M., Joshi, V. and Shahid, S. K. (2002). The congenital long QT syndrome. *Indian J. Pediatr.* **69**, 141-144.

Territo, P. R. and Altimiras, J. (2001). Morphometry and estimated bulk oxygen diffusion in larvae of *Xenopus laevis* under chronic carbon monoxide exposure. *J. Comp. Physiol.* **171B**, 145-153.

Vandenberg, J. I., Walker, B. D. and Campbell, T. J. (2002). HERG K^+ channels: friend and foe. *Trends Pharmacol. Sci.* **22**, 241-246.

Verheijck, E. E., Wilders, R. and Bouman, L. N. (2002). Atrio-sinus interaction demonstrated by blockade of the rapid delayed rectifier current. *Circulation* **105**, 880-885.