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Acute temperature effects on function of the chick embryonic heart

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Abstract

Aim: We analysed the effects of acute temperature change on the beating rate, conduction properties and calcium transients in the chick embryonic heart *in vitro* and *in ovo*.

Methods: The effects of temperature change (34, 37 and 40 °C) on calcium dynamics in isolated ED4 chick hearts *in vitro* were investigated by high-speed calcium optical imaging. For comparison and validation of *in vitro* measurements, experiments were also performed *in ovo* using videomicroscopy. Artificial stimulation experiments were performed *in vitro* and *in ovo* to uncover conduction limits of heart segments.

Results: Decrease in temperature from 37 to 34 °C *in vitro* led to a 22% drop in heart rate and unchanged amplitude of Ca^{2+} transients, compared to a 25% heart rate decrease *in ovo*. Increase in temperature from 37 to 40 °C *in vitro* and *in ovo* led to 20 and 23% increases in heart rate, respectively, and a significant decrease in amplitude of Ca^{2+} transients (atrium −35%, ventricle −38%). We observed a wide spectrum of arrhythmias *in vitro*, of which the most common was atrioventricular (AV) block (57%). There was variability of AV block locations. Pacing experiments *in vitro* and *in ovo* suggested that the AV blocks were likely caused by relative tissue hypoxia and not by the tachycardia itself.

Conclusion: The pacemaker and AV canal are the most temperature-sensitive segments of the embryonic heart. We suggest that the critical point for conduction is the connection of the ventricular trabecular network to the AV canal.

Keywords arrhythmias, calcium imaging, chick embryo, conduction block, heart development, optical mapping.

The function of the embryonic heart is strongly affected by temperature. The temperature changes affect the kinetics of ion channels, pumps and the Na^+/Ca^{2+} exchanger. Changes in the kinetics of these channels are crucial for the generation and propagation of electrical activation through the embryonic heart (Sperelakis & Lehmkuhl 1967, Chen & DeHaan 1993).

Despite homoeothermy, the avian embryo retains some flexibility from its poikilothermic ancestors and tolerates (within limits) variations in incubation temperature. The effects of temperature on developing chick embryonic heart have been extensively studied. One of the most striking findings is the effect of long-lasting hypothermia (32–36 °C), which causes cardiac hypertrophy (Warbanow 1970). These changes are accompanied by increased contractility of embryonic hearts (Warbanow 1971). Another study focused on hypothermia tests haemodynamic effects of environmental hypothermia in the stage 21

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(Hamburger & Hamilton 1951) embryo. Cooling from 34.7 to 31.1 °C causes a significant decrease in heart rate by about 25%, increased vascular resistance and decrease in blood pressure and blood flow. This bradycardic response is independent of functional autonomic innervation (Nakazawa *et al.* 1985). A recent study at stage 17 shows that hypothermia is associated with bradycardia and a decrease in the peak velocity of blood during systole (Lee *et al.* 2011). The effects of environmental hyperthermia (37–40 °C) cause an increase in the heart rate by about 22% at stage 21, without changes in stroke volume (flow/rate). This study also shows a significant increase in the basal heart rate during development from stage 18 to stage 24 (Nakazawa *et al.* 1986). The long-term consequences of periods of hypothermia (31–36 °C) and hyperthermia (38–42 °C) show teratogenic and lethal effects on the chick embryo (Peterka *et al.* 1996).

An *in vitro* hypothermia-rewarming study by Sarre and colleagues shows dramatic changes in heart rate during cooling from 37 to 0 °C and subsequent rewarming to 37 °C in isolated chick embryonic hearts at stage 24. The hearts stopped beating in deep hypothermia at the critical temperature of 18 °C, and they resumed beating at the same temperature during rewarming. Changes in heart rate remained linear in the range between 34 and 37 °C (Sarre *et al.* 2006). The most recent study shows acute temperature effects on *ex vivo* zebrafish hearts studied by optical mapping, in the range from 18 to 28 °C. Cooling to 18 °C decreases heart rate by about 40% and increases atrial and ventricular APD_{50} by factors 3 and 2 respectively. It shows that the atrial APD is the most severely affected AP parameter by an acute temperature change (Lin *et al.* 2014) and that this property is conserved among vertebrates. The action potentials of atrium, atrioventricular (AV) canal, ventricle and outflow tract differ in duration – APD_{90}. The longest APD_{90} occurs in the AV canal. Arguello *et al.* (1986) suggested that prolonged APD_{90} in AV canal region was caused by longer predominance of Ca^{2+} and slow Na^+ current dependence during development, as compared to atrial or ventricular cells.

Early cardiac rhythmicity is critically dependent on intracellular dynamics of calcium ions. Calcium handling is regulated by calcium ion channels, receptors, ATPases and the Na^+/Ca^{2+} exchanger (NCX; Bers 1991), and its kinetics is strongly influenced by temperature. Embryonic cardiac rhythmicity is maintained by pacemaker cells from the developing sinoatrial node located at the inflow portion of the heart (Kamino *et al.* 1981). The pacemaker potential is generated by a specific set of ion channels. Spontaneous depolarization is initiated mainly by the funny current (I_f) or 'pacemaker' current, via the HCN channels (Na^+, K^+ currents) (DiFrancesco 1993, Moroni *et al.* 2001). A significant role during early depolarization is played also by influx of positive charges, known as the I_{NCX} during forward mode ($3Na^+$ in, $1Ca^{2+}$ out) of sodium–calcium exchanger – NCX (Haddock *et al.* 1997).

We analysed the acute effects of temperature change on electrical activity of the chick embryonic heart using *in vitro* high-speed calcium imaging. We hypothesized that temperature-dependent arrhythmias, in particular AV blocks, are due to a particular sensitivity of the AV canal to relative hypoxia. The main goal of this study was to observe changes in calcium transient dynamics as a basis for contractility and also to analyse defects in conduction through activation maps with high spatio-temporal resolution. We also performed videomicroscopy *in ovo* to compare *in vitro* temperature effects on the pacemaker *per se* with the effects on the whole embryo, in which the working heart is coupled to the vascular system.

Materials and methods

Experimental model

White Leghorn chicken eggs (Institute of Molecular Genetics, Kolec, Czech Republic) were stored at 16 °C prior to incubation. The eggs were incubated at 37.5 ± 0.5 °C in a humidified incubator until ED4 (HH stage 21–23, Hamburger & Hamilton 1951). Chick embryos were removed from the eggs and placed into Tyrode's solution (composition: NaCl 145 mmol L^{-1}, KCl 5.9 mmol L^{-1}, $CaCl_2$ 1.1 mmol L^{-1}, $MgCl_2$ 1.2 mmol L^{-1}, glucose 11 mmol L^{-1}, HEPES 5 mmol L^{-1}; pH = 7.4). Hearts were isolated from the embryos and stained in 2.5 mL Rhod-2 (1.78 mM; Invitrogen, Carlsbad, CA, USA) in Tyrode's solution for 1 h in the dark at room temperature. The hearts were then incubated in 2.5 mL Tyrode's solution for 1 h in the dark at 38 °C to de-esterify the dye loaded to the myocytes, according to manufacturer's instructions.

Optical mapping

The two-dimensional optical mapping system for embryonic hearts has been described in detail previously (Tamaddon *et al.* 2000, Rentschler *et al.* 2001). Optical calcium imaging of chick embryonic hearts was based on an established set-up (Valderrabano *et al.* 2006). We used the calcium-sensitive dye Rhod-2 with a modified set-up (Vostarek *et al.* 2014). Stained hearts were placed into a tissue bath containing 2 mL of Tyrode's solution with 0.15 μM

blebbistatin to reduce movement (Fedorov *et al.* 2007). Temperature in the glass-bottomed Petri dish (Wilco Wells, Amsterdam, the Netherlands) was maintained by a temperature-controlled stage (Linkam DC-60, Tadworth, UK). An inverted epifluorescence microscope (Nikon Eclipse TE 2000-S, Tokyo, Japan) fitted with a high-speed EM-CCD camera (Andor iXon3; Andor, Belfast, UK) was used to monitor changes in intracellular calcium concentration – visualized as changes of fluorescence over time under hypothermia (34 °C), normothermia (controls, 37 °C) and hyperthermia (40 °C). Two measurements were performed on each heart after 5 min of stabilization at different temperatures (37 and 34 °C – cooling, 34 and 37 °C – warming, 37 and 40 °C – warming). This temperature range represents the physiological range, compatible with long-term embryonic survival.

Data analysis

The obtained data were analysed by NIS Elements software (Nikon, Tokyo, Japan) and BV_Ana Analysis software (SciMedia Ltd, Costa Mesa, CA, USA). Heart rate was established from counts of calcium transients in the atrium during the 6-s interval (NIS Elements). Amplitudes of calcium transients in atrium and ventricle were analysed using NIS Elements tools. The start of the calcium transient was established as a point of beginning of the upstroke from baseline. Amplitudes were measured as the height from baseline to the peak. APD_{90} values were obtained from calcium transient recordings from atrium, AV canal and ventricle of normothermic hearts ($n = 10$), with recordings free from motion artefacts or arrhythmias. Spatiotemporal activation maps were constructed from raw data from NIS Elements using BV_Ana Analysis software. Data were filtered by high pass/low pass and median filters. The first derivative was calculated as described previously (Nanka *et al.* 2008, Sankova *et al.* 2010). Its peak was used to mark the time of activation of each pixel.

Videomicroscopy

ED4 chick embryos incubated *in ovo* were studied by videomicroscopy to measure changes in heart rate under different temperature conditions. The first measurement was performed at 34 °C, the second at 37 °C and the third at 40 °C. The embryos *in ovo* were maintained at set temperature using a custom-made styrofoam-insulated metal container filled with pre-heated Bath Armor pellets placed on a Torrey Pines Scientific chilling/heating plate. Ten-second movies were recorded for each temperature with a Nikon D7000 camera (640 × 480 px, 30 fps) mounted on a Leica 125 dissecting microscope; Leica Microsystems, Wetzlar, Germany with a 150 W halogen light source fitted with a green interference filter to enhance contrast of blood. Data analysis was performed using ImageJ software; National Institutes of Health, Bethesda, MD, USA. Two regions of interest were selected on atrium and ventricle, respectively, and heart rate values were obtained by the measurement of changes in grey-scale levels in time during blood flow (Sedmera *et al.* 1999, Kockova *et al.* 2013).

Electrical stimulation in ovo

Atrial and ventricular pacing was performed in ED4 chick embryonic hearts after spontaneous rhythm recordings were obtained. A platinum bipolar electrode was positioned over the atrium using a Narishige micromanipulator; Narishige International USA, Inc., Amityville, NY, USA. The heart was stimulated in overdrive mode at a gradually increasing/decreasing rate (from 120 to 600 beats min^{-1}), with 2-ms pulses twice the diastolic threshold, which was between 1.5 and 2.5 mA (Sedmera *et al.* 2003). The pacing frequency at which conduction disturbances appeared was considered the limit of sustainable conduction rate. Video recordings were obtained and analysed as described above.

Electrical stimulation in vitro *(voltage recordings)*

Chick ED4 embryonic hearts were isolated with a piece of adjacent body wall to allow for fixation to the dish bottom. Hearts were stained in 500 μL of voltage-sensitive dye di-4-ANEPPS (2.5 mM; Invitrogen) for 5 min. They were then briefly rinsed and pinned face-up in a custom-made oxygenated tissue bath containing 20 mL of Tyrode's solution with added 0.1 μM blebbistatin to reduce movement. Atrial and ventricular pacing was performed by a platinum bipolar electrode, which was positioned over the atrium or ventricle using a Narishige micromanipulator. The hearts were stimulated in overdrive mode at varying rates (atrium from 200 up to 400 beats min^{-1} and ventricle from 300 up to 600 beats min^{-1}) to uncover capture and conduction limits. Data acquisition and analysis were performed using the Ultima L high-speed camera; SciMedia Ltd, Costa Mesa, CA, USA and bundled software as described recently (Sankova *et al.* 2010).

Statistical analysis

The data from optical mapping were divided into two groups according to the temperature: hypothermia group (37 and 34 °C) and hyperthermia group (37 and 40 °C). Each group included at least 45 hearts,

and significance of difference was tested by two-tailed Student's paired *t*-test. The occurrence of third-degree AV block in the three groups, hypothermia, normothermia and hyperthermia hearts, was tested by Pearson's chi-square test. The number of hearts analysed *in ovo* was 19, and each heart was tested at all three temperatures. In all cases, values are presented as mean ± SD, and $P < 0.05$ was considered statistically significant.

Results

Experiments in vitro

Changes in calcium transients dynamics. Electrical activity of the chick embryonic hearts (monitored by calcium optical imaging) was strongly affected by acute temperature changes. The most striking was modulation of sinus rhythm frequency. We tested the acute effects in three temperatures. We set 37 °C as a default (baseline *in vitro*) temperature – normothermia, hypothermia at 34 °C and hyperthermia at 40 °C. We observed a nearly linear dependence of the sinus rhythm on temperature under these conditions. The rhythm changed by approx. 20% in hypothermia ($P < 0.001$) and hyperthermia ($P < 0.001$), in comparison with normothermia (Fig. 1).

We then focused on changes in amplitude of the calcium transients. The acceleration of heart rate during hyperthermia caused a significant decrease in calcium transient amplitude in both the atrium and ventricle (Fig. 1d), while no significant changes were observed during hypothermia (Fig. 1b).

We also observed a wide spectrum of rhythm disturbances (for examples, see Supplemental Video S1). These arrhythmias were divided into two main groups.

Defects in rhythm generation – direct influence on the pacemaker. Defects in generation of electrical impulse represented 28% of all arrhythmias. Most common were atrial extrasystoles and sinus pauses (missed beats), which together represented 22% of all observed arrhythmias (Table 1). Atrial extrasystoles and sinus pauses were two times more frequent during hyperthermia than in the normothermia group, while almost none were found in hypothermia. Complete cardiac arrest developed only in three of the 99 hearts analysed. We also uncovered junction rhythm activity in the AV canal in two cases during hyperthermia, at considerably high firing rates (160 and 180 beats min^{-1}).

Defects in impulse propagation. This phenomenon was observed most frequently during hyperthermia, less commonly during normothermia, and was observed only as a few conduction blocks during hypothermia. Defects in impulse propagation

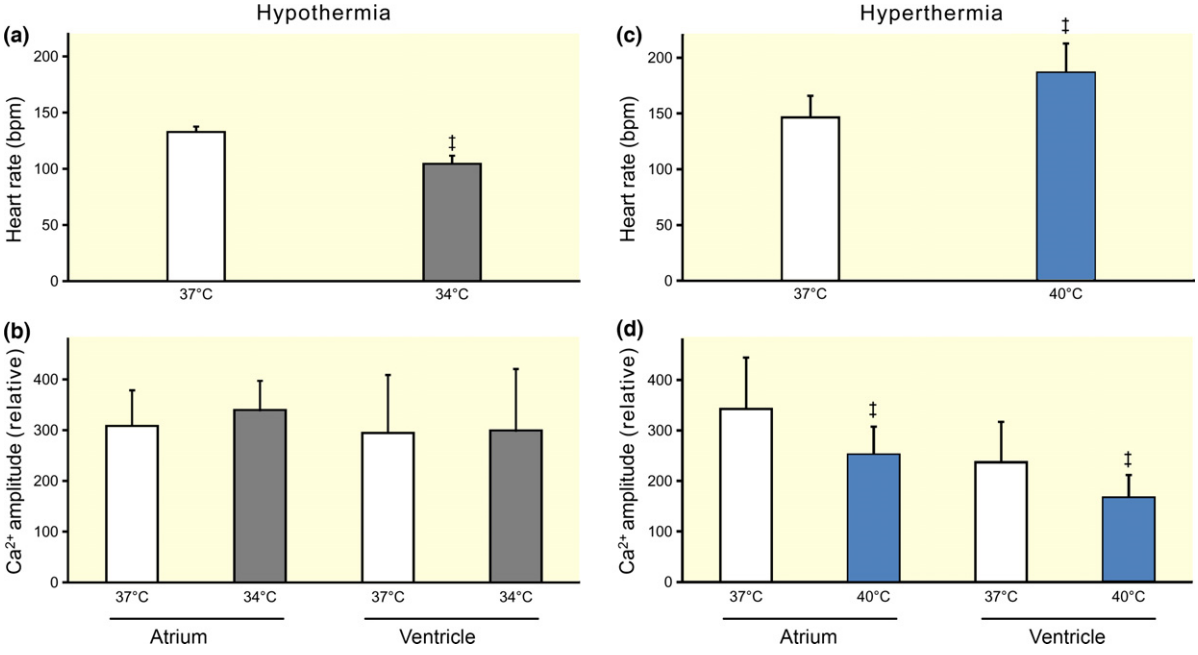

Figure 1 Effects of acute temperature changes on heart rate and amplitudes of Ca^{2+} transients in ED4 chick embryonic heart *in vitro*. (a) Decrease in temperature from 37 to 34 °C led *in vitro* to a 22% drop of intrinsic heart rate ($n = 45$). (b) Hypothermia caused no significant changes in amplitude of Ca^{2+} transients ($n = 45$). (c) Increase in temperature from 37 to 40 °C lead *in vitro* to a 20% acceleration of intrinsic heart rate ($n = 54$). (d) Hyperthermia caused a significant decrease in the amplitude of Ca^{2+} transients ($n = 54$, atrium −35%, ventricle −38%, mean ± SD, ‡$P < 0.001$).

Table 1 Arrhythmias observed in ED4 chick embryonic hearts *in vitro*. Complete spectrum of observed arrhythmias in hypothermia, normothermia and hyperthermia (total number of observed arrhythmias = 106)

Type of arrhythmia	Occurrence (%)
Complete cardiac arrest	3
Atrial extrasystoles	10
Sinus pauses	12
Junction rhythm	2
Atrial block	3
Second-degree AV block	22
Third-degree AV block	35
Mid-ventricular block	2
Ventriculo-conotruncal block	8
AV re-entry	3
Total	100

AV, atrioventricular.

represented 72% of all observed arrhythmias ($n = 106$, Table 1). In three cases, conduction blocks were observed in conduction through the atrium (atrial block), in two cases in the middle of the ventricle (mid-ventricular block) and in 8% of cases ($n = 106$ observed arrhythmias) at the boundary between the ventricle and outflow tract (ventriculo-conotruncal block). Our attention was especially focused on conduction in the AV canal. Development of conduction blocks in the AV canal (second- or third-degree AV block) presented 57% of all observed arrhythmias. The most common defect was a complete AV block, which developed in one case during hypothermia ($n = 45$ hearts, Pearson's chi-square test $P < 0.03$), in 15% of cases in normothermia (stabilization, $n = 99$ hearts) and 39% in hyperthermia ($n = 54$ hearts, Pearson's chi-square test $P < 0.001$). Third-degree AV block represented 35% of all observed arrhythmias ($n = 106$). The second most common defect was second-degree AV block (22% of all arrhythmias), which developed in 11% of cases during hypothermia ($n = 45$), in 10% of cases in normothermia (stabilization, $n = 99$) and 14% in hyperthermia ($n = 54$). The most common was an intermittent second-degree AV block in 65% of second-degree AV blocks, Wenckebach phenomenon represented 30% of second-degree AV blocks, and Mobitz II was observed only in one case. Occasionally, we noted rare arrhythmias such as AV re-entry, but only in three cases of the 106 arrhythmias observed (Table 1).

Locations of AV blocks – optical activation maps. Optical mapping allowed us to focus specifically on the localization of third-degree AV blocks. Activation maps were created from calcium data in BV_Ana Analysis software. Comparison of standard conduction in normothermia and various types of the third-degree AV block is shown in Figure 2. The locations of block were variable within the AV canal. In 59% of cases, the conduction stopped uniformly across the AV canal. In the remaining 41% of cases, we observed a different AV block pattern, wherein conduction stopped either at the inner curvature or at the AV canal–ventricular boundary, along the outer curvature (Fig. 2f). The most critical part of the AV canal was the distal region at the boundary of the ventricle. The distal region of AV canal developed 53% of AV blocks. By contrast, the proximal AV region developed 37% of blocks and conduction stopped in the middle part of the AV canal in the remaining 10% of blocks.

In ovo *experiments*

We decided to compare the *in vitro* findings with an *in ovo* experiment that included vascular coupling. We studied acute temperature effects *in ovo* by videomicroscopy ($n = 19$ hearts). The experimental temperatures were chosen in the same range as the *in vitro* studies. We obtained heart rates 120 ± 11 beats min^{-1} in hypothermia, 160 ± 21 beats min^{-1} in normothermia and 197 ± 27 beats min^{-1} in hyperthermia. Increase in temperature from 34 to 37 °C leads to a 25% change of baseline heart rate and increase from 37 to 40 °C lead to 23% acceleration *in ovo* ($P < 0.001$). This dependence of heart rate on temperature was almost linear ($R^2 = 0.999$) and corresponded well with the *in vitro* findings.

We also observed rhythm disturbances, which developed in some embryos during hyperthermia. The most frequent were sinus pauses. On occasion, second-degree AV block developed (see Fig. 3). Third-degree AV block was not observed.

Electrical stimulation of the atrium and ventricle. We hypothesized that third-degree AV blocks found *in vitro* were caused by relative tissue hypoxia, which most profoundly affects conduction through the AV canal (Tran *et al.* 1996, Sedmera *et al.* 2002). To test this hypothesis, we measured the ability of the AV canal to propagate high frequencies induced by electrical stimulation from the atrium to the ventricle. The measurements of calcium transients *in vitro* (37 °C) showed that the APD_{90} under spontaneous rhythm was longest in the AV canal of whole ED4 hearts. The longer APD_{90} in the AV canal (330 ms) predisposes this cardiac segment to be a limiting factor in conduction of higher beat frequencies between the atrium and the ventricle. The APD_{90} in the ventricle

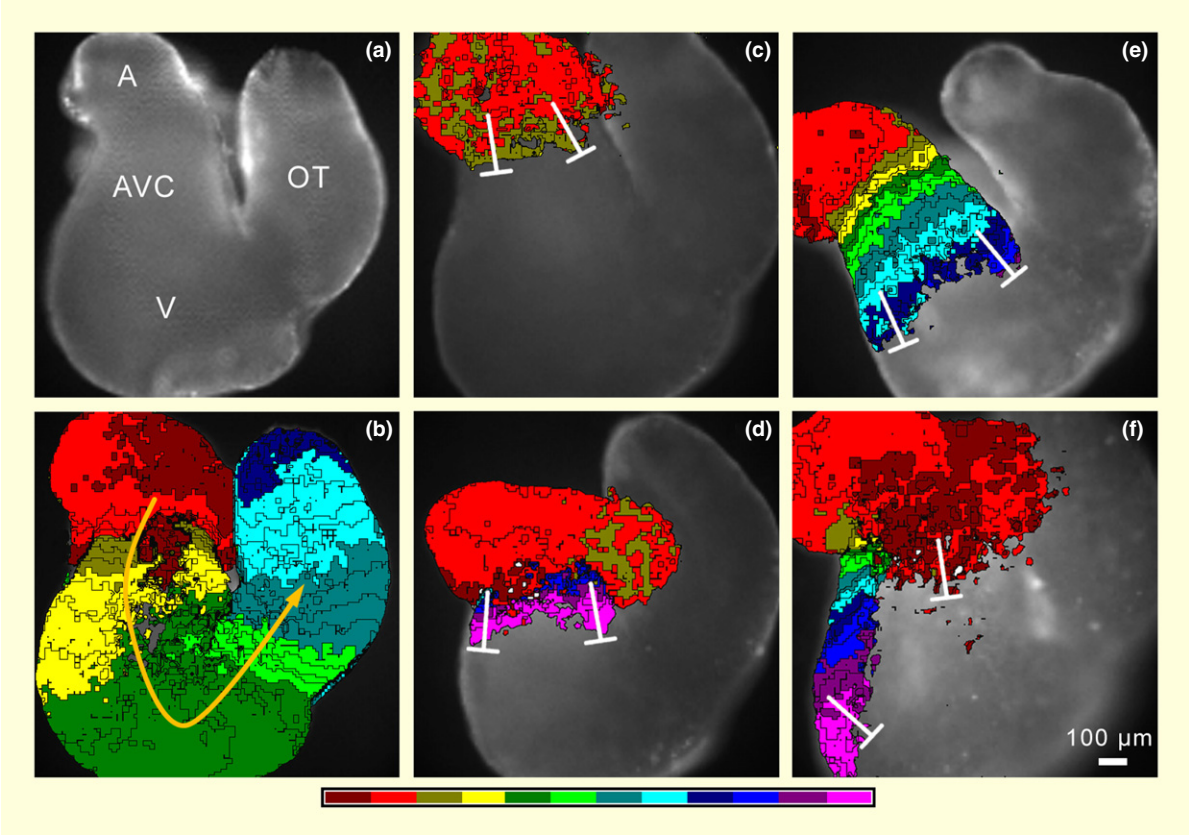

Figure 2 Comparison of third-degree atrioventricular (AV) block locations – different localization of conduction disturbances in the AV canal. (a) ED4 chick heart. (b) Standard conduction. (c) Proximal AV block. (d) Mid-AV block. (e) Distal AV block. (f) Distal AV block with preferential conduction along the outer curvature of the AV canal. Colour bands are in 32 ms (b) and 16 ms intervals (c–f), isochrones are in 8 ms intervals. A, atrium; AVC, AV canal; V, ventricle; OT, outflow tract.

(282 ms) was longer than in the atrium (223 ms, $P < 0.001$).

Electrical stimulation experiments *in ovo* showed that the AV canal of all experimental hearts ($n = 7$) was capable of propagating frequencies of up to 300 beats min^{-1}. AV canal conduction limit reached 360 beats min^{-1} during atrial stimulation in one case (Fig. 4a,b). During stimulation of atrium, an atrial conduction limit of 360 beats min^{-1} was found. This being said, second-degree AV block regularly occurred when pacing rates exceeded 300 beats min^{-1}. Hearts were capable of beating at high frequencies for only a few seconds without developing conduction block. Stimulation of the ventricle uncovered a maximal capture threshold at 600 beats min^{-1} (Fig. 4c). These results support our hypothesis that hypoxia could be one of several parameters that would explain our findings, as we never observed such high rates in sinus rhythm, even during the highest tachycardias in hyperthermia *in vitro* (270 beats min^{-1}) without AV block.

We performed electrical stimulation experiments on isolated ED4 hearts *in vitro* to uncover the conduction limits of isolated hearts without blood flow and vascular coupling ($n = 23$). The AV conduction limit (1 : 1 AV capture) was breached at 261 beats min^{-1} (see Fig. 4d). In the same heart, a maximum frequency of 353 beats min^{-1} was reached in the atrium, but second-degree AV block developed (see Fig. 4e). The next two highest heart rates successfully propagated through the AV canal in other hearts were 232 beats min^{-1} and 200 beats min^{-1}. The remaining hearts did not even reach 200 beats min^{-1} with normal AV conduction. A capture limit for the ventricle was reached at 476 beats min^{-1} (see Fig. 4f).

Discussion

Arrhythmias in the embryonic heart

High-speed imaging of calcium in the embryonic mouse heart (Valderrabano *et al.* 2006) results in increased sensitivity compared to imaging of voltage-sensitive dye. This modality also allows for signal detection in the AV canal, enabling the detection of various arrhythmias. Our experimental set-up (Vostarek *et al.* 2014) for imaging normal and stressed

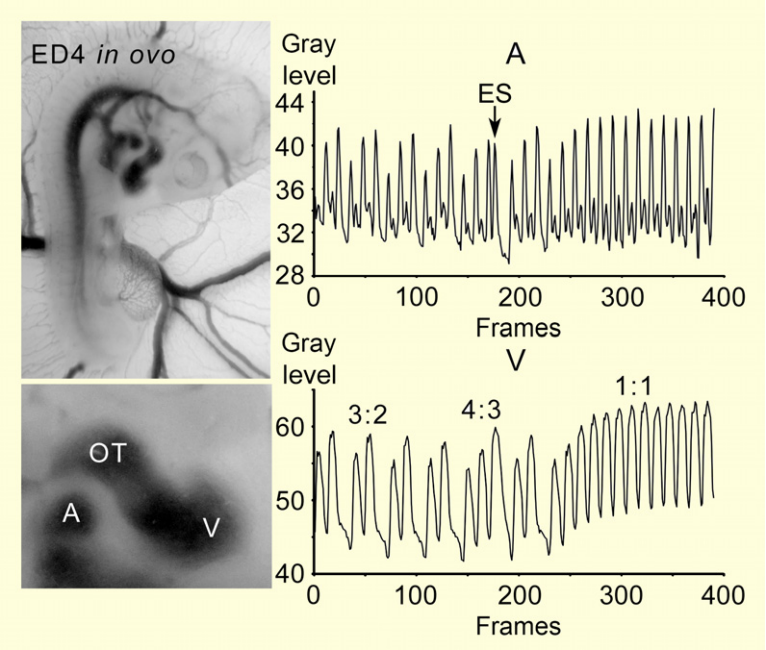

Figure 3 Second-degree atrioventricular (AV) block during hyperthermia *in ovo*. *In ovo* videomicroscopy recording of a transient second-degree AV block (Wenckebach phenomenon) during hyperthermia. Traces represent changes in grey level in time due to edge motion. Each peak represents one contraction. The *x*-axis is presented in frames (acquisition speed 30 fps). A, atrium; V, ventricle; OT, outflow tract; ES, atrial extrasystole.

embryonic hearts represents a significant technological improvement. Among other advances, it enables precise localization of sites of conduction block and the uncovering of ectopic pacemakers (Hoogaars *et al.* 2007, Leaf *et al.* 2008, Ammirabile *et al.* 2012, Benes *et al.* 2014) in the isolated embryonic heart model.

We measured correlation of PQ intervals in hypothermia, normothermia and hyperthermia, but no significant dependences were observed. PR interval was not significantly influenced by temperature or heart rate, similar to the results of Sarre *et al.* (2006). However, a modest trend towards the prolongation of PR interval was noted in their follow-up study, which included analysis of the bradycardic effects of ivabradine (Sarre *et al.* 2010). This probably reflects the limitation of ion pumps responsible for the restoration of membrane potential at higher heart rates during the pre-innervation stages of cardiogenesis. On the other hand, the mechanical PQ interval was described as negatively correlated with heart rate in human foetuses. It is notable that these more mature foetal hearts were measured under *in vivo* conditions and with full autonomic innervation (Tomek *et al.* 2011).

Temperature effects on heart rate and calcium transients

Results from calcium transient dynamics measurements *in vitro* showed that heart rate is linearly dependent on the temperature in the range from 34 to 40 °C. This corresponds well with a previous study using a ramp protocol (Sarre *et al.* 2006). Changes in heart rate were caused by direct influence on the pacemaker changing its kinetics. In the hypothermia group, we tried to change the temperature in both directions – cooling from 37 to 34 °C and warming from 34 to 37 °C. We did not observe any significant difference in heart rate change or arrhythmogenesis related to the direction of temperature change.

We expected changes in temperature to influence calcium transient amplitudes. During hyperthermia, we observed a significant decrease in amplitudes of calcium transients. We hypothesize that this is caused by higher heart rate with less time for calcium channels and pumps to establish calcium transients. We suggest that lowering of calcium transients could result in weaker contractility, which may lead over time to negative effects on pumping efficiency. These negative effects on cardiac output are equalized by higher heart rate, but limitations of energetic metabolism are crucial. On the other hand, we observed no significant changes during hypothermia. This is probably due to adaptation of chick embryos to hypothermia in natural conditions. Decreased activity of calcium transporters is balanced by a longer period available for establishing the equilibrium concentration. Cardiac output is maintained through Frank-Starling compensation by increased stroke volume (Benson *et al.* 1989).

Temperature and cardiac output

Experiments *in ovo*, including vascular coupling and blood flow, showed the same linear dependence of heart rate on temperature as *in vitro*. Chick ED4 hearts are not innervated, but β-adrenergic receptors are expressed at

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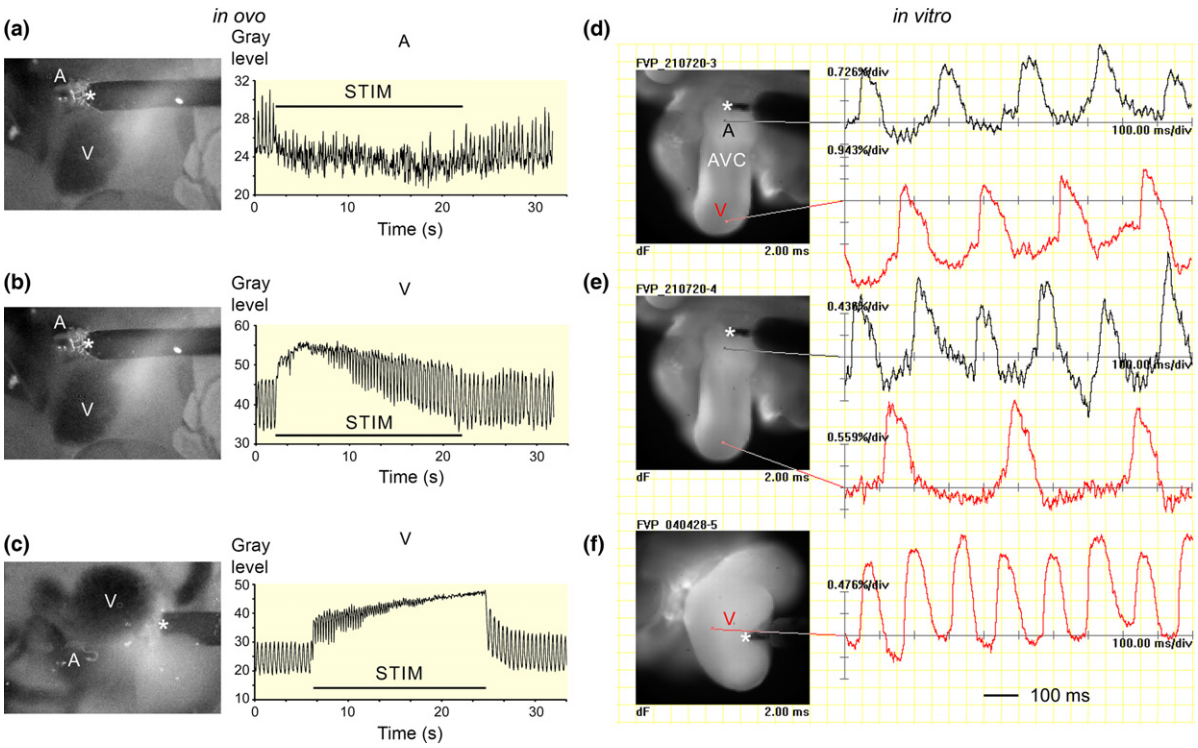

Figure 4 Artificial stimulation uncovers conduction limits. Left side shows recordings during electrical stimulation *in ovo* (n = 7). Atrioventricular (AV) canal conduction limit of chick ED4 heart *in ovo* reached 360 beats min^{-1} during electrical stimulation of the atrium. Atrium was stimulated gradually from 400 to 120 beats min^{-1}. (a) Motion signal from the atrium. (b) Edge movement signal from the ventricle. (c) Maximal measured conduction limit of ventricle *in ovo* reached 600 beats min^{-1}. Ventricle stimulated from 200 to 600 beats min^{-1}. Right side shows voltage imaging of electrical stimulation of ED4 chick hearts *in vitro* (n = 23). (d) Maximal measured AV canal conduction frequency of 261 beats min^{-1}. Atrium stimulated up to 300 beats min^{-1}. (e) Maximal measured conduction limit of atrium was 353 beats min^{-1}. Second-degree AV block (2 : 1) developed. Atrium stimulated up to 400 beats min^{-1}. (f) Maximal measured conduction limit of ventricle *in vitro* reached 476 beats min^{-1}. Ventricle stimulated up to 600 beats min^{-1}. A, atrium; AVC, AV canal; V, ventricle.

this stage. It was shown that ED4 chick heart responded to adrenaline stimulation by a significant increase in heart rate (up to 60%). Treatment by different β-blockers leads to a significant decrease of heart rate in ED4 chick hearts (Kockova *et al.* 2013). Heart function in ectothermic animals, such as fish or reptiles, is strongly affected and limited by hyperthermia, even when autonomic innervation is fully developed. The main regulating mechanisms are the same as in the embryonic heart of the chick, which physiologically develops at constant temperature maintained by the hen incubating the egg. The embryo is unable to generate its body heat by itself and therefore is well adapted to brief periods of hypothermia. A crucial limiting factor is oxidative phosphorylation in mitochondria and especially sufficient temperature-dependent ATP synthesis (Power *et al.* 2014).

Mechanisms of temperature-induced conduction defects

Increased temperature increases metabolic demands, and the availability of oxygen can become a limiting factor. This is especially the case *in vitro* where the thick AV region is probably not optimally oxygenated, as it physiologically receives nutrients and oxygen from the heart lumen. We thus focused on defects in impulse propagation in this cardiac segment, as almost 60% of all observed arrhythmias (n = 106) were caused by conduction block in the AV canal (Table 1). We analysed in detail various types of AV block (Fig. 2). Third-degree AV block developed in one case during hypothermia (n = 45 hearts), in 15% of cases in normothermia (stabilization, n = 99 hearts) and 39% of cases in hyperthermia (n = 54 hearts). This suggests that development of complete AV block is influenced by increased temperature or by another condition connected to hyperthermia. It probably corresponds with the increase of metabolic needs of hearts and concomitant decreases in oxygen concentration in the tissue bath with increasing temperature.

The slowing of conduction velocity in the AV canal is highly influenced by the presence of cardiac jelly and the endocardial cushions (Bressan *et al.* 2014). The

morphology of the AV canal is determined by large extracellular spaces, scarce membrane contacts and anionic extracellular matrix resulting in a low margin of conduction safety (Arguello *et al.* 1986). This principle is supported by new findings regarding ephaptic conduction of action potential between myocytes without recourse to gap junctions. This might happen via electric field mechanism or ion transients within the extracellular space between two tightly, <15 nm apposed myocytes, occurring, for example, in the perinexus – the cleft formed at the edge of gap junctions (Rhett & Gourdie 2012, Veeraraghavan *et al.* 2014).

The primitive cardiac tube is characterized by uniformly slow conduction velocity and expresses only one gap junction protein, Connexin45. The conduction in the AV canal is slow but robust, as noted already by Paff *et al.* (1964) and later by Sedmera *et al.* (2002), who noted that AV block could not be induced pharmacologically or by anoxia/reoxygenation prior to ED3. Chamber myocardium is characterized by Connexin40 expression, among other specific gene products, a gap junction protein essential for fast conduction. Transition between the slowly conducting AV canal and the ventricle might include heterotypic Cx45/Cx40 gap junctional coupling, which could represent a tissue sector with increased probability of functional block, similar to the substrate for sinoatrial blockade. In isolated cardiomyocytes, it was observed that cooling decreases the frequency of gap junction channel opening at all conductance levels (Chen & DeHaan 1993).

Action potentials with a low rate of rise and longer duration are typical for the AV canal (Sanders *et al.* 1984, de Jong *et al.* 1987, 1992). We measured APDs in the atrium, AV canal and ventricle in normothermia, to test the hypothesis that longer APDs predispose the AV canal to be the limiting segment of the heart. We obtained higher values than standard APDs measured by microelectrodes (Arguello *et al.* 1986), likely because of the prolongation of APD by blebbistatin – similar to the effects of cytochalasin D (Sedmera *et al.* 2006).

Our experiments showed that the pacemaker and AV canal were the most temperature-sensitive segments of the embryonic heart. The most common location of AV block was at the transition between the slowly conducting tissue of the AV canal and the fast conducting tissues of the ventricle. We suggest that the most critical region for the propagation of impulse is the connection site of trabecular network to the AV canal. It corresponds with similar finding of Coppen *et al.* in the embryonic and mature rodent heart. This observation uncovered the analogous sharp transitional interface between the Cx45-expressing component of the mouse AV node and Cx40-expressing His bundle (Coppen *et al.* 1999).

Hypoxia in the developing heart

During cardiac development, hypoxic regions are found in several locations of the myocardium (Nanka *et al.* 2006, 2008, Wikenheiser *et al.* 2006). These hypoxic regions correlate with areas of conduction system formation (Wikenheiser *et al.* 2006). Coincidentally, hypoxia is also detected in the thickest regions of the myocardium (AV canal, interventricular septum, outflow tract myocardium), and it is believed that hypoxia is a powerful stimulus for coronary vasculogenesis (Nanka *et al.* 2008). As the AV canal is one of the thickest areas of the embryonic myocardium, it lacks trabeculae and is separated from the oxygen-carrying blood in the lumen by the cardiac cushions, and it thus comes as no surprise that it is very sensitive to hypoxia. Because the normal routes of oxygenation in the chick embryonic heart prior to ED9 (establishment of coronary perfusion) is through the lumen, it is not surprising that hearts were more prone to develop AV block *in vitro*, where the direction of oxygen diffusion, as well as its concentration gradient, is perturbed.

We tested the ability of the AV canal to propagate high beat frequencies by electrical stimulation. The main point was to prove that AV blocks induced during comparatively mild tachycardia in hyperthermia were not due to the intrinsic absolute conduction limit of the AV conduction. Electrical stimulation experiments showed that the conduction limit of the AV canal was much higher *in ovo* (360 beats min^{-1}) than *in vitro* (261 beats min^{-1}). Also the conduction limits of the atrium and ventricle, respectively, were higher *in ovo* (atrium 360 beats min^{-1}, ventricle 600 beats min^{-1}) than *in vitro* (atrium 353 beats min^{-1}, ventricle 476 beats min^{-1}). This is probably caused by better oxygenation of the hearts from blood flow through the lumen. These results suggest that the observed AV blocks could be caused by relative tissue hypoxia and not by a low ability of the AV canal to propagate high frequencies.

In conclusion, this study provides a quantitative evaluation of temperature effects on conduction in the chick embryonic heart. Hypothermia is tolerated better than hyperthermia, the former of which embryos seem to be well adapted to. The most common arrhythmia observed under hyperthermia was AV block, which was observed typically at the transition between the AV canal and ventricle. Thus, morphological and molecular distinctions between different compartments of the developing heart have physiological consequences manifesting under increased metabolic demands.

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Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Video S1. Examples of arrhythmias observed in the embryonic chick heart using calcium imaging.

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