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RESEARCH ARTICLE

Effects of prolonged anoxia on electrical activity of the heart in crucian carp (*Carassius carassius*)

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ABSTRACT

The effects of sustained anoxia on cardiac electrical excitability were examined in the anoxia-tolerant crucian carp (*Carassius carassius*). The electrocardiogram (ECG) and expression of excitation–contraction coupling genes were studied in fish acclimatised to normoxia in summer (+18°C) or winter (+2°C), and in winter fish after 1, 3 and 6 weeks of anoxia. Anoxia induced a sustained bradycardia from a heart rate of 10.3 ± 0.77 beats min^{-1} to 4.1 ± 0.29 beats min^{-1} ($P < 0.05$) after 5 weeks, and heart rate slowly recovered to control levels when oxygen was restored. Heart rate variability greatly increased under anoxia, and completely recovered under re-oxygenation. The RT interval increased from 2.8 ± 0.34 s in normoxia to 5.8 ± 0.44 s under anoxia ($P < 0.05$), which reflects a doubling of the ventricular action potential (AP) duration. Acclimatisation to winter induced extensive changes in gene expression relative to summer-acclimatised fish, including depression in those genes coding for the sarcoplasmic reticulum calcium pump (Serca2a_q2) and ATP-sensitive K$^+$ channels (Kir6.2) ($P < 0.05$). Genes of delayed rectifier K$^+$ (*kcnh6*) and Ca^{2+} channels (*cacna1c*) were up-regulated in winter fish ($P < 0.05$). In contrast, the additional challenge of anoxia caused only minor changes in gene expression, e.g. depressed expression of Kir2.2b K$^+$ channel gene (*kcnj12b*), whereas expression of Ca^{2+} (*cacna1a*, *cacna1c* and *cacna1g*) and Na$^+$ channel genes (*scn4a* and *scn5a*) was not affected. These data suggest that low temperature pre-conditions the crucian carp heart for winter anoxia, whereas sustained anoxic bradycardia and prolongation of AP duration are directly induced by oxygen shortage without major changes in gene expression.

KEY WORDS: Anoxia tolerance, Bradycardia, Fish heart, Electrical excitability, Excitation–contraction coupling, Seasonal acclimatisation

INTRODUCTION

Contraction of the vertebrate heart is initiated and regulated by the orderly spread of electrical excitation through atrial and ventricular myocardia (Coraboeuf, 1978). In fish, the rate and rhythm of the heartbeat are determined by spontaneous activity of the ring-shaped pacemaker tissue at the border zone between the sinus venosus and the atrium (Yamauchi and Burnstock, 1968; Saito, 1969; Haverinen and Vornanen, 2007; Newton et al., 2014); a fast propagating atrial

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action potential (AP) provokes atrial systole. Impulse transmission is delayed at the atrioventricular canal to allow sufficient time for ventricular filling, before a fast propagating AP occurs and contraction is elicited in the ventricular wall (Saito and Tenma, 1976; Sedmera et al., 2003). Electrical excitation of the fish heart accommodates cardiac function to systemic circulatory demands under different environmental stresses, including variations in oxygen availability (Stecyk et al., 2008). This occurs by modifying heart rate (f_H) and force generation so that cardiac output ($\dot{Q}$) matches the metabolic demand of tissues, and guarantees uninterrupted blood flow under all conditions including that of oxygen deficit. Furthermore, AP duration needs to be matched to f_H to maintain the balance between durations of systole and diastole when the length of the cardiac cycle changes.

A common response of the fish heart to reduced oxygen availability (hypoxia) (Satchell, 1961; Spitzer et al., 1969; Wood and Shelton, 1980; Randall, 1982; Fritsche, 1990) or complete absence of oxygen (anoxia) (Butler and Taylor, 1975; Nilsson et al., 1993) is bradycardia, i.e. reduction of f_H (Farrell, 2007; Gamperl and Driedzic, 2009). The $\dot{Q}$ of hypoxaemic fish is not necessarily compromised by a low beat frequency, as bradycardia is usually associated with a compensatory increase in stroke volume (Holeton and Randall, 1967; Butler and Taylor, 1975; Wood and Shelton, 1980). Indeed, hypoxic bradycardia is assumed to be beneficial to fish, because it may improve oxygen uptake in gills (Satchell, 1960; Randall et al., 1967; Perry and Desforges, 2006), increase oxygen delivery to the heart by reducing diffusion distance in the distended myocardial walls (Farrell, 2007), or allow better perfusion of the coronary vessels (Gamperl et al., 1995; Farrell, 2007). These measures could protect the heart against energy deficiency, an unavoidable consequence when oxidative ATP production is compromised. However, none of these mechanisms are available for protecting cardiac function in fish during prolonged anoxia, as no oxygen is available.

Hypoxic bradycardia is also typical for diving mammals (Andersen, 1966) and mammalian fetuses (Singer, 1999). In contrast to fishes, adult mammals usually respond to hypoxia with tachycardia (Kontos et al., 1967). In this case, cardiac protection is provided by the shortening of ventricular AP, due to the opening of ATP-sensitive K$^+$ channels (Noma, 1983). Under normoxic conditions, these channels are kept closed by a high cytosolic energy charge, but reductions in the rate of ATP production reduce the ATP/ADP ratio, which triggers their opening (Flagg and Nichols, 2011). The outward K$^+$ current ($I_{K(ATP)}$) reduces plateau duration of the cardiac AP, and hence contractility of cardiac myocytes and thus energy usage by the cell. Analogous to mammalian hearts, shortening of the ventricular AP via opening of the ATP-sensitive K$^+$ channels has been suggested to protect hypoxic goldfish (*Carassius auratus*) hearts (Chen et al., 2005; Cameron et al., 2013). However, these observations are based on short-term *in vitro* studies, while direct demonstration of hypoxic shortening of the QT interval *in vivo* under prolonged oxygen shortage is lacking.

List of symbols and abbreviations

AP	action potential
CV	coefficient of variation
DSD	diastole/systole duration
EAD	early after-depolarisation
E–C coupling	excitation–contraction coupling
ECG	electrocardiogram
f_H	heart rate
HRV	heart rate variability
RT-qPCR	real-time quantitative reverse transcription PCR
$\dot{Q}$	cardiac output
SR	sarcoplasmic reticulum

Whether bradycardia and shortening of cardiac AP are viable responses of the fish heart under prolonged oxygen shortage, characteristic conditions for the natural habitat of some anoxia-tolerant species like the crucian carp (*Carassius carassius*), have not been critically examined. Crucian carp tolerate the complete absence of oxygen for several months in winter, if the animals have properly acclimatised to the ambient conditions of cold and oxygen-deficient waters (Piironen and Holopainen, 1986). Lowering temperature is the seasonal cue that usually pre-conditions these fish for winter, e.g. by stimulating glycogen storage and modifying the cardiac excitation–contraction (E–C) coupling machinery (Tiitu and Vornanen, 2001; Rissanen et al., 2006; Stensløkken et al., 2010; Varis et al., 2016). The heart of summer-acclimatised crucian carp responds to short-term oxygen shortage (1–16 h) at +20°C with a strong bradycardic reflex, which is mediated by increased cholinergic tone (Vornanen, 1994b; Vornanen and Tuomennoro, 1999). In contrast, a previous study reported that when exposed to anoxia, crucian carp showed only a transient (2 day) bradycardia, after which f_H and $\dot{Q}$ reverted to normoxic control levels, suggesting a surprising ability to maintain normoxic f_H in the complete absence of oxygen (Stecyk et al., 2004). However, those experiments were conducted at high temperature (+8°C) and were of relatively short duration (5 days) in comparison to the cold temperature (0 to +4°C) and prolonged duration (up to 4 months) of anoxia in their natural habitat, suggesting that the fish were not properly acclimatised to winter. Therefore, the response to prolonged and cold anoxic conditions in winter remains unclear (Stecyk et al., 2008), and the proposed hypoxic/anoxic shortening of the cardiac AP has not been tested under these conditions.

Under prolonged exposure to energy-limited conditions, survival of animals is generally based on depression of metabolic rate and organ functions (Hochachka, 1988). We therefore hypothesised that winter-acclimatised crucian carp will elicit a sustained bradycardia in anoxic and cold waters to reduce energy consumption, and that prolongation of the ventricular AP is necessary to maintain a constant diastole/systole duration (DSD) for uninterrupted circulation of blood. If, however, there were no reduction in f_H in anoxia (Stecyk et al., 2004), then protection to the heart might be provided by the shortening of the AP duration via increased activity of ATP-sensitive K^+ channels (Chen et al., 2005). To test these hypotheses, seasonally acclimatised winter fish were exposed to controlled anoxia in the laboratory for several weeks at their natural habitat temperature in winter. Electrical activity of the heart was monitored (by electrocardiogram, ECG) and the underlying molecular mechanisms were examined by transcript expression (by quantitative PCR) of relevant E–C coupling genes in summer- and winter-acclimatised fish, and winter-acclimatised carp exposed to prolonged anoxia.

MATERIALS AND METHODS
Animals
Winter-acclimatised crucian carp, *Carassius carassius* (Linnaeus 1758), of two size classes were captured from local ice-covered lakes (62°36′N, 29°45′E) in central Finland in late October–November. Fish with a body mass of 590–725 g (654±39 g mean± s.e.m., N=3) were captured from lake Mustalampi, while fish caught from lake Kalattomatlammit ranged between 46 and 153 g (80± 14 g, N=7). Fish captured late in autumn have already built up full glycogen reservoirs of the body, are physiologically acclimatised to low temperature, have started their natural winter fast and therefore possess a full anoxia-tolerance capacity (Vornanen and Paajanen, 2006). In the laboratory, fish were maintained in 500 l metal tanks at +2°C under a constant oxygen concentration of ca. 11 mg O_2 l^{-1}. Winter-acclimatised crucian carp do not forage in the wild and under laboratory conditions do not eat if given free access to food; therefore, fish were not fed during the study. Summer-acclimatised fish (16.4–20.7 g, 19.0±0.8 g mean±s.e.m., N=5) were caught in August and maintained in the lab at +18°C under constant oxygenation and daily feeding with goldfish fodder (Tetra). All experiments were conducted with permission of the National Ethical Committee for Animal Experimentation (permission number STH252A).

ECG recordings
In vivo ECG recordings were made as previously described (Campbell et al., 2004; Vornanen et al., 2014; Badr et al., 2016). Briefly, crucian carp of varying size were narcotised in neutralised tricaine methanesulphonate (MS-222, 0.3 mg l^{-1}; Sigma, St Louis, MO, USA) and placed ventral side up on an operating table, and the gills were irrigated with cold tap water. Two recording electrodes (7-strand Teflon-coated wire, length 40 cm, diameter 0.23 mm; A-M Systems, Carlsborg, WA, USA) were hooked into the end of a 24 gauge hypodermic needle and obliquely inserted from the ventral surface at the level of the pectoral fins forward, close to the pericardium. The electrode wires were secured by glue and sutures to the belly of the fish and in front of the dorsal fin. Small fish (N=7) were placed in individual 2.2 l Erlenmeyer bottles and larger fish (N=3) were placed individually into 9 l Plexiglas cylinders, which were immersed in 500 l stainless steel aquaria regulated to +2±0.5°C (Computec Technologies, Joensuu, Finland). f_H recording was started immediately. The fish were allowed to recover from the operation for several days before being exposed to sustained anoxia for 39–57 days. Water was made anoxic with nitrogen gassing ([O_2] <0.1 mg l^{-1}; WTW CellOxi 325, Germany) and the bottles and cylinders were tightly sealed. The aquaria were covered by black plastic and the electrodes extended about 30 cm outside the tank so that they could be connected to the amplifier without disturbing the fish. ECG tracings were collected via a differential bioamplifier (ML 136, ADInstruments, Colorado Springs, CO, USA) to a digital recording system (PowerLab, ADInstruments). Temperature was recorded in the same file using a thermocouple. ECGs were considered to be of sufficiently good quality when P, QRS and T waves could be clearly recognised.

f_H, heart rate variability (HRV), RT interval and duration of the QRS complex were determined from ECG recordings at a sampling frequency of 400 Hz using LabChart 7.1 software (ADInstruments). f_H was calculated from mean interbeat (RR) intervals. HRV is the variation in cardiac activity, determined from continuous ECG recordings of at least 2 h in duration, and visualised as tachograms for overall HRV and Poincaré plots for variability between successive RR intervals. In the latter representation, long-term

HRV runs along the diagonal axis from the origin, and beat-to-beat variations occur along the normal to the axis maxima. For statistical analyses, HRV was determined as a coefficient of variation (CV), which is the standard deviation of RR intervals (σ) divided by the mean RR interval (μ), i.e. $CV=\sigma(s)/\mu(s)$. RT interval represents the period between ventricular depolarisation (the peak R wave) and repolarisation (the end of the T wave), and is a measure for the average duration of the ventricular AP. The duration of the QRS complex was determined as the width of this waveform at the zero voltage level, indicating the time needed for AP depolarisation to propagate through the ventricular myocardium, and is therefore a measure of the rate of impulse conduction.

Anoxic gene expression

The experiment consisted of five test groups: normoxic (11 mg O_2 l^{-1}) summer-acclimatised fish kept at +18°C for a minimum of 3 weeks, normoxic winter-acclimatised fish (at +2°C), and winter fish exposed to anoxia for 1, 3 or 6 weeks (anoxic groups); fish were allowed to recover from the 6 week anoxia for 1 week in normoxia. For anoxic exposure, 1–3 fish (4.2–22.2 g, 15.6±0.8 g mean±s.e.m., N=32) were placed in a 2.2 l Erlenmeyer bottle and oxygen was driven off from the water with vigorous nitrogen bubbling (Holopainen et al., 1986; Crawshaw et al., 1989; Vornanen and Haverinen, 2016). When the oxygen concentration was <0.1 mg O_2 l^{-1}, the bottle was sealed with a rubber stopper and placed on the bottom of a fish tank regulated to +2±0.5°C. Details of this experimental setting are provided by Vornanen and Haverinen (2016).

mRNA levels of 21 E–C coupling genes were measured from the ventricular tissue (Table 1). Transcript expression was measured for 16 genes that we cloned from hearts of crucian carp in our earlier studies. In addition, five novel genes coding for ATP-sensitive K^+ channels Kir6.1 (*kcnj8*), Kir6.2a (*kcnj11a*) and Kir6.2b (*kcnj11b*) and sulfonylurea receptors Sur1 (*abcc8*) and Sur2 (*abcc9*) were included in the analysis. Partial cDNA sequences for these genes were cloned by PCR from cardiac cDNA prepared from DNase-treated total RNA using RevertAid Premium Reverse Transcriptase

(ThermoFisher Scientific) and random hexamers (Promega, Madison, WI, USA). PCR was performed using Phusion High Fidelity DNA polymerase (ThermoFisher Scientific), cardiac cDNA and primers (Table 2) under the following conditions: initial denaturation at +98°C for 1 min followed by 35 cycles at +98°C for 10 s, +60°C for 30 s and +72°C for 40 s and final extension at +72°C for 2 min. The PCR products were run on an agarose gel; desired products were extracted from the gel by GeneJet Gel extraction kit (ThermoFisher Scientific) and cloned to pGEM-T Easy vector (Promega). Two clones from each gene were sequenced (GATC Biotech) bidirectionally. The cloned *kcnj8* (GenBank no. KU885440), *kcnj11a* (KU885441) and *kcnj11b* (KU885442) sequences shared 85.5%, 88.9% and 89.8% identity with corresponding zebrafish (*Danio rerio*) genes (NM_001039827, NM_001039827 and NM_001012387, respectively), indicating that they are orthologous genes. Similarly, crucian carp *abcc8* (KU885443) and *abcc9* (KU885444) sequences shared 88.3% and 89.9% identity with zebrafish *abcc8* (NM_001172647) and *abcc9* (NM_001030154), respectively.

For real-time quantitative reverse transcription PCR (RT-qPCR), atrial and ventricular samples were quickly excised, frozen in liquid nitrogen and stored at −80°C. RNA was extracted with TriReagent (ThermoFisher Scientific) according to the manufacturer's instructions, quantified by NanoDrop spectrophotometer and qualified by agarose gel electrophoresis. A 1 µg sample of RNA was treated with RQ1 RNase-free DNase (Promega) and first strand cDNA was synthesised by Maxima cDNA synthesis kit (ThermoFisher Scientific) using both oligo(dT) and random hexamers. A control cDNA synthesis performed without RT enzyme was performed on every sample to control for possible DNA contamination. The RT-qPCR was performed on every sample in triplicate using Maxima SYBR Green qPCR Kit (ThermoFisher Scientific) and primers (Table 3), using AriaMX Real-Time PCR System (Agilent Technologies) with cycling conditions of +95°C for 10 s, +58°C for 20 s and +72°C for 30 s, followed by melting curve analysis from +65°C to +95°C. Gene expression was normalised to that of the reference gene *dnaja2* (Hassinen et al., 2008a).

Statistics

Statistically significant differences between variables, obtained by each research method (effect of anoxia on ECG, f_H, RT interval, HRV and cardiac gene expression) were assessed at the 5% level ($P<0.05$) using one-way ANOVA after checking normality of distribution and equality of variances, and making necessary transformation of variables. Paired comparisons between two means were done by Tukey's or Dunnett's T3 honestly significant difference *post hoc* tests.

RESULTS

ECG of anoxic fish

Under controlled laboratory conditions, f_H of normoxic fish at +2°C was 10.3±0.77 beats min^{-1}, and when exposed to sustained anoxia, deep bradycardia gradually developed to 4.1±0.29 beats min^{-1} after 5 weeks ($P<0.05$). In most fish, the majority of f_H depression occurred within 2 days of anoxia; the lowest recorded anoxic f_H value was just 2 beats min^{-1}. Restoration of normoxic conditions resulted in slow recovery of f_H toward control levels, and after 20 days f_H was statistically indistinguishable from control values (8.3±0.53 beats min^{-1}, $P>0.05$; Fig. 1).

Anoxia was associated with significant changes in the ECG waveform. RT interval, representing the average duration of

Table 1. Targets of gene expression studies

Gene	Protein	Function
kcnj2	Kir2.1	Inward rectifier K^+ current (I_{K1})
kcnj12a	Kir2.2a	
kcnj12b	Kir2.2b	
kcnj14	Kir2.4	
kcnj8	Kir6.1	ATP-sensitive K^+ current ($I_{K(ATP)}$)
kcnj11a	Kir6.2a	
kcnj11b	Kir6.2b	
abcc8	Sur1	
abbc9	Sur2	
kcnh2	Kv11.1	Delayed rectifier K^+ current (I_{Kr})
kcnh6	Kv11.2	
kcnq1	Kv7.1	Delayed rectifier K^+ current (I_{Ks})
kcne1	MinK	Delayed rectifier K^+ current (I_{Ksr})
scn4a	Nav1.4a	Na^+ current (I_{Na})
scn5a	Nav1.5a	
cacna1a	Cav2.1	P/Q-type Ca^{2+} current ($I_{CaP/Q}$)
cacna1c	Cav1.2	L-type Ca^{2+} current (I_{CaL})
cacna1g	Cav3.1	T-type Ca^{2+} current (I_{CaT})
atp2a2a_q1	Serca2a_q1	SR Ca^{2+} pump
atp2a2a_q2	Serca2a_q2	
fkbp1a	Fkbp12.1	Modulator of ryanodine receptors

Excitation–contraction coupling genes, corresponding proteins and their main function in the fish heart are presented.

Table 2. Primers used for cloning

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product (bp)
kcnj8	AGCGCGCTTCATCGCCAAGA	CCGAACTTGGAGTAGTCCAC	908
kcnj11a	ATGTTGTCCAGAAAAGGACTC	TGGTGATGCCGGTGGTCT	889
kcnj11b	ATGGTGGACCTGAAATGGCA	CTGGTTTGGTCCAGCTCCTT	880
abcc8	AGACCTCCAACTTCCCCAAA	AACTCGCTGAGCTTCTGAAC	1450
abcc9	CTTCTCTTCATCACCTTCCC	TCCAGCTGTCGTCTCCAAT	1711

ventricular AP, was prolonged from the control value of 2.8±0.34 s to 5.8±0.44 s under anoxia ($P<0.05$; Fig. 2A,B). The longest RT intervals were about 7 s in duration. Restoration of normoxic conditions caused recovery of RT interval to the control level (3.2± 0.4 s; $P>0.05$). The duration of the QRS complex (ventricular depolarisation) was increased from the normoxic value of 356± 45 ms to 910±68 ms in anoxia ($P<0.05$; Fig. 2C). When oxygen was restored, the QRS value recovered to 538±45 ms ($P>0.05$).

Tachograms and Poincaré plots indicate marked changes in HRV between normoxic and anoxic conditions (Fig. 3A,B). Although HRV was present in normoxic fish, anoxia exposure caused a striking increase in HRV. Restoration of normoxia was associated with recovery of HRV, indistinguishable from the normoxic controls.

Expression of genes involved in E–C coupling
The most prominent changes in crucian carp ventricular mRNA expression of 21 genes involved in cardiac E–C coupling appeared between winter fish acclimated to +2°C and summer fish acclimated to +18°C (Fig. 4). Transcript levels of proteins involved in Ca^{2+} uptake and release by the sarcoplasmic reticulum (SR)-Serca2a_q2 and Fkbp1a were strongly depressed (82% and 53%, respectively) in winter fish ($P<0.05$). Genes responsible for the inward rectifier K^+ current (I_{K1}) were also modified by seasonal acclimatisation. There were no seasonal changes in the main Kir2 isoform, Kir2.4 ($P>0.05$). In contrast, of the two Kir2.2 paralogues, Kir2.2a was depressed (81%) in winter ($P<0.05$) while Kir2.2b remained unaltered ($P>0.05$). This resulted in a prominent change to the Kir2.2a/Kir2.2b ratio from 5.6 in summer to 1.0 in winter. Kir6 channels and sulfonylurea receptors generate the ATP-sensitive K^+

current ($I_{K(ATP)}$). Of the three Kir6 channels, Kir6.2a was clearly the main isoform in the crucian carp ventricle, and Sur2 (*abcc9*) was the main sulfonylurea receptor type. Both of these were strongly depressed (55% and 54%, respectively) in winter-acclimatised fish ($P<0.05$). There are two delayed-rectifier K^+ currents in the crucian carp heart, I_{Kr} and I_{Ks}. The gene encoding the I_{Kr} channel, *kcnh6* was strongly (3.3-fold) up-regulated in winter ($P<0.05$). Changes in expression of *kcnq1* and *kcne1* (I_{Ks} channel) were not statistically significant ($P>0.05$).

In contrast to prominent expression changes between summer- and winter-acclimatised fishes, anoxia exposure of winter fish at +2°C did not cause any large-scale changes in gene expression relative to winter-acclimatised fish held under normoxic conditions (Fig. 4). Indeed, there were only a few changes in ion channel expression, e.g. a significant reduction of 77%, 87% and 99% in the expression of Kir2.2b channels after 1, 3 and 6 weeks of anoxia, respectively ($P<0.05$). Expression of *scn5a* was reduced by 51.3% after 6 weeks of anoxia when compared with expression levels after 1 week of anoxia ($P<0.05$), demonstrating a progressive response. All changes in gene expression levels, except for Kir2.2b, were reversible 1 week after return to normoxia ($P>0.05$). It is notable that Kir6 channel and sulfonylurea receptor genes did not respond to anoxia.

DISCUSSION
Low temperature pre-conditions cardiac E–C coupling for winter by altering gene activity
Although the significance of low temperature in adjusting crucian carp physiology for winter anoxia is documented by several previous studies (Vornanen, 1994b; Tiitu and Vornanen, 2001;

Table 3. Primers used in RT-qPCR

| Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product (bp)
kcnj2	ATGAGCTGGCAATCCTGAAC	TCATGTCGAGGGGTTCTCTC	103
kcnj12a	AGCAATCTCAGCGCTACCTC	AGCTCAGGACAAATGCAAGG	99
kcnj12b	TCCGTCACCCAACTGTAATG	TCGTCATCCTCTGCATCATC	102
kcnj14	CCTAAACCTGGCGTAGAGCA	TACGCCCACCAAATCCTATC	103
kcnj8	GACCAGAAAGGGGACGACTT	CGCCAAAACCTATGGTCACT	103
kcnj11a	GACCAGAAAGGGGACGACTT	CGCCAAAACCTATGGTCACT	103
kcnj11b	CCTGTGCCATGTGTCACTTC	CCTCAGTCACCATTCGTCCT	103
abcc8	CTCGCTAACGCTTACGTCCT	ATTCCAGTCTCGATGGCAAC	101
abcc9	GATGGGGTCCTATCACTTGC	GGGAAATTGGCGGTTTCTAT	105
kcnh2	CCTGTATCTGGTTCGCCATC	GATGCTGCTGTTGTGTGGTT	101
kcnh6	CCGTATTGGAGGCATGAAGA	GATAGAGGGGCCGGAGAAG	97
kcnq1	GCTGGATGCCGGAGTAAATA	ACAATAGAGGCCACCACCAC	101
kcne1	GATATCCACGCTCTGCTGGT	ATGCACAGGTGGATGATGTG	102
scn4a	GCAACAGTGATAACCTGACCA	GGTTCGCTGGGTTTTCAATA	102
scn5a	CCTTCAGACAACAGCAGCAC	CTTGGCTCCTTCCACTTTGA	105
cacna1a	CCATTATGAAGGCCATGGTT	TGCCCATGTAGAACTCAACG	99
cacna1c	CGCCAGAACTCTGAGAAACC	CTTGGTGGCAGTCTCTCCAT	96
cacna1g	CCTATCGGGAACATTGTCGT	TCCCCATGACACACAAAGAA	101
atp2a2a_q1	GTGTAACGCCCTCAACAGTCT	GCAGCGGCTCCACGTAGA	134
atp2a2a_q2	GTGTAACGCCCTCAACAGTCT	GCAGTGGGTCTACATAGA	134
fkbp1a	CGGAGATGGGAGGACTTTC	AAATGGCTTCTCACGGTCAC	100
dnaja2	AGGACTTGTACGACCGTTATGG	CGCCAAAGATATGGGAAAAGAT	93
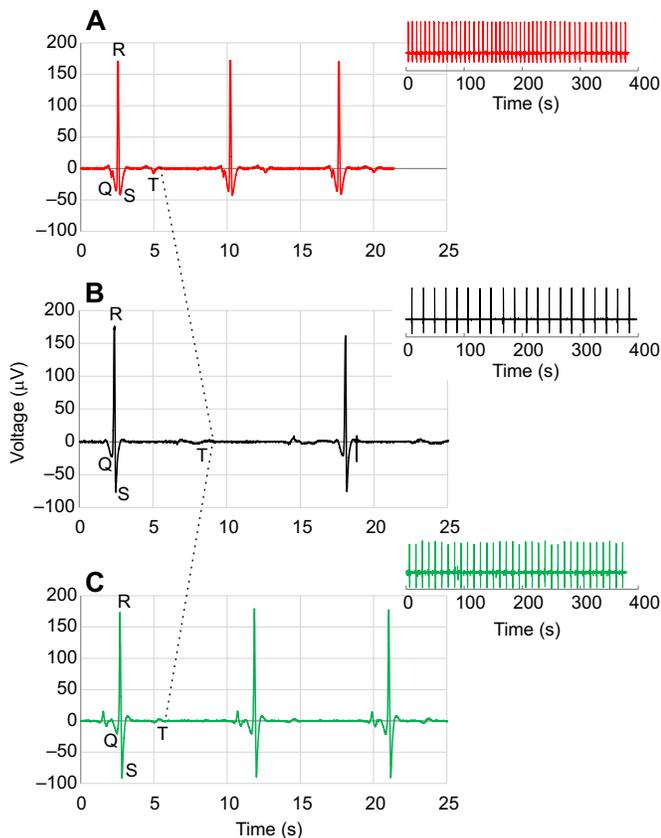
Fig. 1. An example electrocardiogram (ECG) of the winter-acclimatised crucian carp. (A) ECG in normoxia (control), (B) ECG after 5 weeks of anoxia and (C) ECG after 20 days of recovery from anoxia. R and T denote the R wave (ventricular depolarisation) and T wave (ventricular repolarisation) of the ECG. Note that the distance between R and T waves (average duration of ventricular action potential, dotted line) is increased under anoxia.

Rissanen et al., 2006; Stensløkken et al., 2010; Varis et al., 2016), this is the first study to examine the effects of temperature and anoxia on transcript expression of E–C coupling genes. Comparison of expression data between summer- and winter-acclimatised crucian carp indicates large differences in the activity of genes contributing to the molecular machinery of cardiac E–C coupling, similar to previous reports for crucian carp acclimated to different temperatures in the laboratory (Hassinen et al., 2008b, 2011; Korajoki and Vornanen, 2014). These seasonal temperature changes apparently pre-condition the heart for the anoxic and cold

conditions of winter in small ice-covered lakes, a typical habitat of these fish in northern latitudes. In contrast, summer crucian carp (caught in June) acclimated in the laboratory to +8°C and +13°C showed few changes in transcript expression among the 19,584 gene clones examined using the common carp (*Cyprinus carpio*) cDNA microarray (Stensløkken et al., 2014). This suggests that the change from 13°C to 8°C unlike the change from 18°C to 2°C is not sufficient to induce anoxic pre-conditioning of the crucian carp heart.

Prominent changes occurred in gene transcripts of Ca^{2+}-transport molecules between seasons. The SR Ca^{2+} pump (Serca2a_q2) was strongly suppressed during transition from summer to winter. Similarly, transcripts of the FK binding protein (Fkbp12.6), a regulator of SR Ca^{2+}-release channels, was down-regulated in winter fish. It is somewhat surprising to see such large seasonal changes in gene expression of SR molecules, considering the small contribution of SR Ca^{2+} release to ventricular contraction in crucian carp (Vornanen, 1989). However, gene expression data are consistent with the higher rate of SR Ca^{2+} uptake and faster relaxation of contraction in warm-acclimated than cold-acclimated crucian carp, and functional studies suggesting a lower contribution of SR Ca^{2+} management to E–C coupling of the cold-acclimated crucian carp heart (Matikainen and Vornanen, 1992; Vornanen, 1994b; Aho and Vornanen, 1998). Fkbp12.6 is assumed to enhance Ca^{2+}-induced Ca^{2+} release from the SR by sensitising the cardiac Ca^{2+} release channel (ryanodine receptor). The depression of *fkbp12.6* transcripts in winter fish is consistent with decreased Fkbp12.6 protein expression after cold acclimation (Korajoki and Vornanen, 2014). Collectively, the current evidence suggests that the relatively sluggish SR Ca^{2+} handling of crucian carp ventricular myocytes is further depressed in winter. It should be noted, however, that the Ca^{2+}-storing capacity of cardiac SR is strikingly large in both warm- and cold-acclimated crucian carp (Haverinen and Vornanen, 2009a), and therefore may play a significant role in intracellular Ca^{2+} buffering throughout the year despite depressed Ca^{2+} kinetics in winter fish. As leakage of SR Ca^{2+} stores and malfunction of SR Ca^{2+} cycling can be origins for several forms of cardiac arrhythmia (Volders et al., 2000; Zhao et al., 2012), the reduced intracellular Ca^{2+} cycling in winter-acclimatised fish may be protective against dysfunction in the cold and oxygen-deficient conditions (see 'Avoidance of cardiac arrhythmia', below).

Potassium repolarising currents maintain the negative resting membrane potential (I_{K1}) and promote shortening of AP duration (I_{Kr}, I_{Ks}, I_{K1}). Several seasonal differences were evident in transcript expression of K^+ channel subunits. A major change occurred in genes (*kcnh2*, *kcnh6*) coding for the rapid component of the delayed
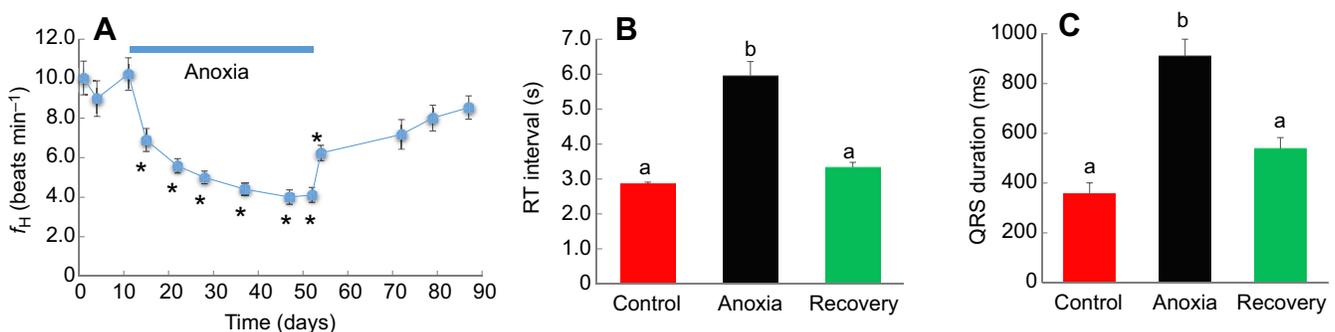

Fig. 2. Effects of prolonged anoxia on heart rate (f_H), RT interval and QRS duration. (A) Effect of anoxia on f_H in crucian carp. Results are means±s.e.m. from 10 fish. (B) RT interval in normoxia, after 5 weeks of anoxia, and after recovery from anoxia (means+s.e.m.; N=4). (C) Duration of QRS complex in normoxia, after 5 weeks of anoxia and after recovery from anoxia (means+s.e.m.; N=8). Different letters indicate statistically significant differences ($P<0.05$) between treatments.
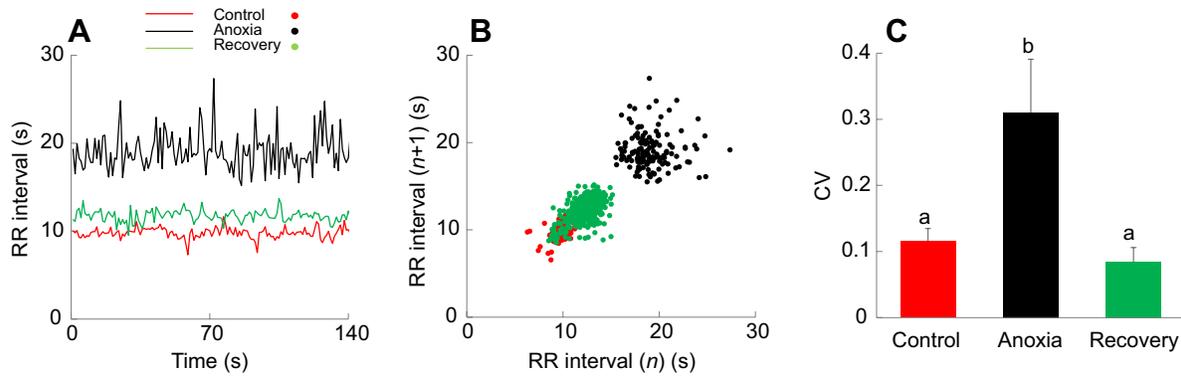
Fig. 3. Effect of anoxia on heart rate variability (HRV) in crucian carp. (A) A representative example of HRV from an individual fish under normoxia, after 5 weeks of anoxia and after recovery from anoxia, shown as progressive tachograms. Note the periodic fluctuations in RR interval that are accentuated under anoxia. (B) The same data presented in the form of Poincaré plots. Note that anoxia increases HRV principally as a result of beat-to-beat variations (normal to the axis maxima). (C) Mean results of HRV as coefficient of variation (CV) under normoxia, after 5 weeks of anoxia and after recovery from anoxia. Results are means+s.e.m. (*N*=9). Different letters indicate statistically significant differences (*P*<0.05) between treatments.

rectifier K^+ current, I_{Kr}, the major repolarising current of the fish hearts (Vornanen, 2016). *kcnh6* was strongly up-regulated in winter, consistent with earlier findings showing that I_{Kr} is up-regulated under cold acclimation in practically all fish species that have been studied (Vornanen et al., 2002; Hassinen et al., 2008a; Galli et al., 2009; Haverinen and Vornanen, 2009b; Abramochkin and Vornanen, 2015). In contrast, expression of the slow component of the delayed rectifier (I_{Ks}) was not changed by temperature acclimatisation, in agreement with a previous study (Hassinen et al., 2011). It is also notable that some of the inward rectifier K^+ channel

(Kir) genes were down-regulated in winter fish. The seasonal depression of Kir2.2a, the warm-adapted paralogue of the Kir2.2 pair, was an expected finding in the light of previous acclimation studies using crucian carp and rainbow trout (Hassinen et al., 2007, 2008b). Perhaps more surprising was the strong depression of genes coding for the components of ATP-sensitive K^+ channels, including both the pore-forming Kir6.2a subunit and the sulfonylurea receptor Sur2. This indicates that pre-conditioning of the crucian carp heart for anoxic winter survival does not involve increases in the molecular components of ATP-sensitive K^+ current, the hypoxia-
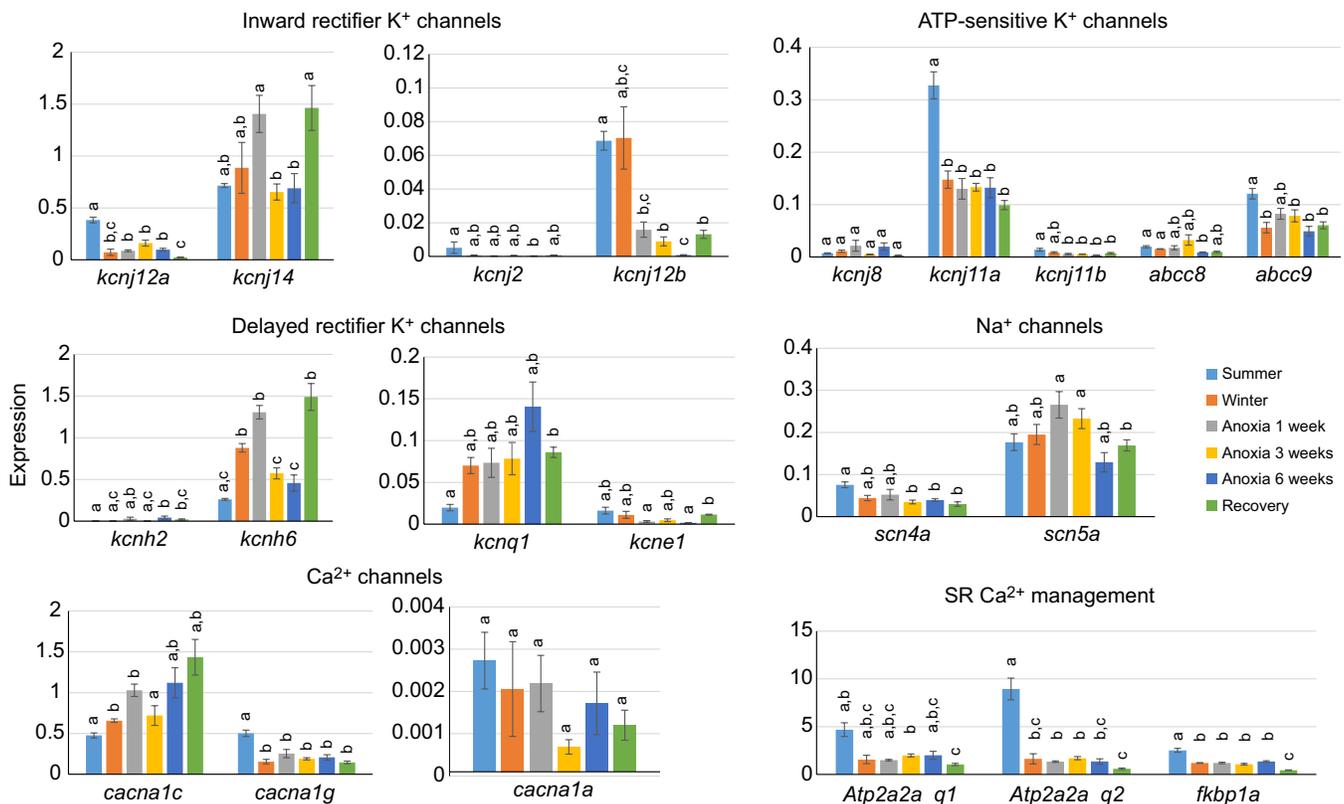

Fig. 4. Effect of seasonal acclimatisation and anoxia on cardiac gene expression. The bar graphs show gene expression changes among functionally similar genes. Results are means±s.e.m. of 5–6 fish, and expression levels are given relative to that of *dnaja2*. SR, sarcoplasmic reticulum. Different letters indicate statistically significant differences (*P*<0.05) between groups.

responsive K^+ current of the vertebrate heart (Nichols et al., 1991). It is clear that the responses of the crucian carp cardiac K^+ channels to seasonal temperature acclimatisation are mixed, probably reflecting their functional diversity. Studies on the anoxia-tolerant turtle (*Trachemys scripta*) have shown a cold-induced pre-conditioning of cardiac excitability to anoxia associated with up-regulation of the ventricular I_{K1}, similar to that of the crucian carp (Stecyk et al., 2007; Hassinen et al., 2008b). In both species, prolonged anoxia exposure was without effect on I_{K1} (Paajanen and Vornanen, 2003; Stecyk et al., 2007). It should be noted, however, that the patch-clamp studies were conducted under aerobic conditions and thus were lacking the homeostatic control of ion channel activity.

Interestingly, expression of Ca^{2+} channel genes between seasons was also variable. The L-type Ca^{2+} channel gene *cacna1c* (Cav1.2) was modestly up-regulated, while the T-type Ca^{2+} channel gene *cacna1g* (Ca$_v$3.1) was strongly down-regulated in winter fish. Therefore, seasonal acclimatisation is associated with a shift from almost equal expression of L-type and T-type Ca^{2+} channels (ratio 0.95) in summer to strong overexpression of L-type Ca^{2+} channels in winter (ratio 4.23). The functional significance of T-type Ca^{2+} channels in contractile regulation of ventricular myocytes is not clear, but they are known to be involved in growth and proliferation of cardiac myocytes (Lory et al., 2006). Therefore, the higher expression of *cacna1g* in summer fish may be indicative of proliferation and regeneration of the heart in the active growth season (Holopainen et al., 1997). Transcript levels of the L-type Ca^{2+} channel are consistent with seasonal changes in the protein expression (assessed by dihydropyridine binding), indicating that the channel number is stable around the year with the exception of a short up-regulation in May–June (Vornanen and Paajanen, 2004). However, in both turtle and crucian carp, the ventricular L-type Ca^{2+} current (I_{CaL}) is down-regulated by cold acclimation (Vornanen and Paajanen, 2004; Stecyk et al., 2007), possibly reflecting covalent regulation of channel activity.

Bradycardia and prolongation of ventricular AP are induced by anoxia

Gene expression data suggest that seasonal temperature changes pre-condition the crucian carp heart for winter anoxia by down- or up-regulation of several key components of E–C coupling, while only a few changes were induced by exposure of winter fish to anoxia. The prominent functional changes under anoxia – bradycardia and AP prolongation – are therefore mainly outcomes of homeostatic regulation (the regulation of body functions by humoral and neural factors using mainly negative feedback, and involving covalent modification of proteins) of cardiac physiology.

Bradycardia

When the winter-acclimatised fish were exposed to anoxia, there was a prominent bradycardia that occurred almost immediately, was sustained and was slowly reversible on restoration of normoxia. The recovery from bradycardia was much slower than the development of bradycardia, possibly because in the wild, recovery from anoxia occurs in warming waters. A strong hypoxic bradycardia, mediated by cholinergic influence (increased vagal tone), has previously been shown for summer-acclimatised crucian carp under short-term hypoxia (1–16 h) at +20°C, with f_H depression from 75 to ca. 29 beats min^{-1} (Vornanen, 1994a). A transient bradycardia was also seen when carp were acclimated to +8°C, where f_H dropped from 15 to 7 beats min^{-1} for 2 days, but then recovered to the initial normoxic level (Stecyk et al., 2004). Such a recovery in the face of

continued metabolic challenge is counter-intuitive, and without explanation in terms of the mechanism of energy savings under anoxia. The present findings show that winter-acclimatised fish at the typical winter temperature of their habitat respond to anoxia with an energetically appropriate, sustained bradycardia. Hypoxic bradycardia is assumed to allow more time for oxygen transfer from water to blood in the gills, and from blood to cardiac myocytes (Gamperl et al., 1995; Farrell, 2007). In anoxia, where molecular oxygen is not available, such physiological benefits of bradycardia are less apparent and maintenance of f_H under complete anoxia can be considered non-adaptive from an energetic point of view, because energy savings from AP arrest are not obtained. Because cardiac arrest is clearly undesirable, as vascular stasis would impair tissue substrate delivery/metabolite removal, a more likely explanation for anoxic bradycardia is reduction of energy consumption. This saves vital carbohydrate stores and thereby improves survival of the heart (and hence the whole animal) under prolonged seasonal anoxia, retaining viability for spring arousal from torpor. Given the reduced efficiency of ATP production by non-oxidative metabolism, this offers an obvious selective advantage. A major proportion of energy consumption in the heart occurs in ATPase activities of myofilaments for contraction, and pumps of the sarcolemma and SR for ionoregulation (Schramm et al., 1994). Bradycardia demands less myofilament ATPase activity, and fewer APs mean a reduced requirement for restoration of Na^+, K^+ and Ca^{2+} ion gradients of the sarcolemma and Ca^{2+} gradient of the SR. Indeed, AP arrest (bradycardia) can save energy in ion pumping via a reduction in the number of functional ion channels. The anoxic f_H at +2°C (4.1 beats min^{-1}) is about 40% of the normoxic f_H at the same temperature (10.3 beats min^{-1}), while the anoxic whole-body metabolic rate at +2°C is only about 10% of its normoxic value (Vornanen, 1994a; Eskelinen, 2011). Even if stroke volume does not increase in anoxia, the reduction in $\dot{Q}$ must be less than the depression of metabolic rate between normoxic and anoxic fish. Thus, the working anoxic crucian carp heart should be able to satisfy systemic metabolic demands by circulating metabolites and hormones around the body, hence maintaining whole-body homeostasis.

Bradycardia was associated with a marked increase in HRV. This is consistent with the assumption that bradycardia is mediated by increased vagal tone, as activation of the parasympathetic nervous system drives increased HRV in both mammals and fishes (De Vera and Priede, 1991; Campbell et al., 2005; Acharya et al., 2006). Interestingly, HRV almost completely disappeared when animals were returned to normoxia, suggesting that there was a reversal of parasympathetic drive and a likely increase in sympathetic tone. However, the importance of beat-to-beat variation in RR intervals suggests that global cellular excitability is augmented by other, probably ionic, mechanisms.

AP duration

The length of the AP plateau determines the duration of cardiac systole, which varies with f_H to allow both sufficient time for ventricular filling and a force of contraction that maintains a steady $\dot{Q}$ at all f_H. *In vivo* ECG recordings of roach heart (*Rutilus rutilus*) show that over a large range of f_H, the normal ventricular DSD is between 1 and 1.5, i.e. diastole is slightly longer in duration than systole (Badr et al., 2016), and crucian carp conforms to this rule (normoxic carp 1.10, anoxic carp 1.48). Despite a strong anoxic bradycardia (60% lower f_H), the relative duration of diastole increases by only 35%, presumably to maintain adequate force according to the Frank–Starling mechanism (Shiels et al., 2002).

Relative maintenance of DSD, which is achieved by strong prolongation of the ventricular AP duration (RT interval), is important for constancy of tissue perfusion at low f_H in anoxia. Shortening of ventricular AP duration in ischaemia and under oxygen shortage, as often happens in hypoxia-sensitive vertebrates (Trautwein et al., 1954; McDonald and MacLeod, 1973), would probably be detrimental for the anoxic crucian carp as short systole could lead to cessation of blood flow towards the end of long diastolic periods, in part due to greater blood viscosity in the cold and attendant potential for haemostasis.

In contrast to mammalian hearts, where hypoxia and metabolic blockade result in shortening of ventricular AP duration via opening of the ATP-sensitive K^+ channels (Nichols et al., 1991; Venkatesh et al., 1991), AP in the heart of crucian carp and flounder (*Platichthys flesus*) (Lennard and Huddart, 1992) is greatly prolonged under anoxia. Although an ATP-sensitive K^+ current exists in crucian carp ventricular myocytes, it is not activated under prolonged anoxia (Paajanen and Vornanen, 2002, 2003). Conversely, it has been suggested that in the heart of warm-acclimated (+21°C) goldfish (*Carassius auratus*), an anoxia-tolerant related species, hypoxia causes a slight shortening (15%) of ventricular AP via opening of the ATP-sensitive K^+ channels (Chen et al., 2005). Whether the difference between crucian carp and goldfish is species specific, related to highly different acclimation and experimental temperatures, or indicates a real difference in their responses to hypoxia versus anoxia, remains to be shown.

The AP plateau is maintained by a balance between inward L-type Ca^{2+} current (I_{CaL}) and outward K^+ currents (I_K). At this point the membrane resistance is high (i.e. ion conductance is very low) and only small changes in ion currents are needed to change the AP duration. A prerequisite for the generation of ultra-long ventricular APs – of several seconds in duration – is that the 'window' I_{CaL} (due to a small proportion of Ca^{2+} channels that do not inactivate or are reactivated at the AP plateau) is opposed by slowly activating K^+ currents, so that repolarisation is delayed (Qu and Chung, 2012). In principle, anoxic prolongation of the AP plateau could be achieved by changes in either the number or activity of ion channels. Because there were only minor changes in sarcolemmal ion channel expression in anoxia, the former seems unlikely. A progressive reduction of the Kir2.2b channel transcripts with duration of anoxia could produce a reduced outward I_{K1}, but Kir2.2b transcripts form only a small proportion of the whole Kir2 channel population (7.6±2.1% in winter-acclimatised carp), and I_{K1} mainly exerts its effect at the final phase-3 repolarisation of AP, not at plateau voltages. Transcripts of T- and L-type Ca^{2+} channels remained unchanged, suggesting that the inward currents are not increased in anoxic hearts. Clearly, the duration of the ventricular AP under anoxia is physiologically regulated, e.g. via direct effects of oxygen shortage on ion channel activity or by indirect frequency-dependent changes on ion currents (Boyett and Jewell, 1980; Vleugels et al., 1980). Depression of f_H is known to increase the amplitude of I_{CaL} and duration of the ventricular AP in rainbow trout (Harwood et al., 2000). The observed bradycardia suggests this autoregulatory mechanism of AP duration may also occur in the anoxic crucian carp heart, possibly augmented by depressed SR Ca^{2+} cycling due to weaker Ca^{2+}-dependent inactivation of I_{CaL}. Indeed, ventricular AP and contraction of the cold-acclimated crucian carp show great propensity for prolonged duration, in particular if extracellular Ca^{2+} concentration is reduced (as seen in plasma; Vornanen, 1996). Frequency dependence of K^+ currents could be also involved. Crucian carp cardiac myocytes express two delayed rectifier K^+ currents, I_{Kr} and I_{Ks}, which should be active at AP plateau (Hassinen et al., 2008a, 2011). Because of incomplete deactivation at low temperatures, I_{Kr} may accumulate at normoxic f_H but less so during anoxic bradycardia, although the role of K^+ currents in AP prolongation requires confirmation by electrophysiological experiments.

Avoidance of cardiac arrhythmia

In mammals, excessive prolongation of ventricular AP predisposes the heart to early after-depolarisations (EADs) and ventricular tachyarrhythmia (Qu and Chung, 2012). In contrast, no arrhythmia was evident in the ECG of anoxic crucian carp, despite ultra-long APs, suggesting some protective mechanisms must be operative in the fish heart. EADs are considered to be due to reactivated or non-inactivated I_{CaL}, which generates inward current transients by the 'window' I_{CaL} during the plateau of ventricular AP (January and Riddle, 1989). In addition, EADs are promoted by spontaneous Ca^{2+} release from the SR that triggers the inward Na^+/Ca^{2+}-exchange current (Choi et al., 2002). Consistent with these mechanisms, in mammalian ventricular myocytes, EADs can be abolished by reducing the size of the window I_{CaL} (Madhvani et al., 2015) or by blocking the SR Ca^{2+} release with ryanodine (Volders et al., 1997). Interestingly, in crucian carp ventricular myocytes, cold acclimation (+4°C) reduces the size of the maximum window I_{CaL} from ca. 6% to 3% of the warm-acclimated fish peak I_{CaL} (Vornanen, 1998), while a reduced contribution of SR Ca^{2+} release to E–C coupling is also found (Matikainen and Vornanen, 1992; Vornanen, 1994b; Aho and Vornanen, 1998). Collectively, these data suggest that cold acclimation pre-conditions the crucian carp heart against cardiac arrhythmia, with modulation of sarcolemma I_{CaL} and SR Ca^{2+} cycling protecting against EADs.

Impulse conduction

It is notable that anoxic prolongation of ventricular AP was associated with the lengthening of QRS complex duration. Because QRS originates from depolarisation of the ventricle, this indicates slower conduction of APs through the ventricular wall. This may be functionally appropriate in matching the rate of conduction to lower f_H and prolonged AP duration of the anoxic heart. The mechanistic basis of this response remains elusive. The rate of impulse propagation in the heart is directly related to the rate of AP upstroke, i.e. the density of the Na^+ current, I_{Na}, and inversely related to the axial resistance of the myocardium, which is mainly determined by gap junction conduction (Kléber and Rudy, 2004). Accordingly, the slowed conduction could result from reductions in I_{Na} density and/or gap junction conduction. Although there was a progressive anoxic reduction in transcripts of the Nav1.4a channel, the minor Na^+ channel isoform, anoxia-induced changes in the total Na^+ channel population were insignificant. Therefore, putative anoxic depression of I_{Na} must assume homeostatic regulation of Na^+ channel function. However, anoxia-induced changes in connexin expression and gap junction conduction cannot be excluded.

Conclusions

The present findings show that a seasonal drop in temperature activates a gene expression programme that pre-conditions the crucian carp heart for maintenance of regulated activity in the winter. When anoxia develops, the homeostatic response to oxygen shortage involves a strong bradycardic reflex and remarkable lengthening of ventricular AP duration. Anoxic bradycardia probably delivers energy savings via AP arrest, while the ultra-long ventricular AP keeps blood flowing even with heart beat intervals of 15–20 s. These responses contrast with those of hypoxia-sensitive endotherms, where $\dot{Q}$ is maintained by tachycardia and energy savings in hypoxic

myocytes are achieved by shortening of AP duration (Kontos et al., 1967; Vogel and Harris, 1967; Noma, 1983). These remarkable adaptations of the crucian carp heart are probably necessary for the exceptional anoxia tolerance of the species, driven by selection pressure in shallow lakes, which may freeze down to the bottom during the long northern winters (Nikolsky, 1963; DeVries, 1971; Holopainen and Oikari, 1992).

Data availability
The following genes were deposited in GenBank: *kcnj8* (GenBank no. KU885440), *kcnj11a* (KU885441), *kcnj11b* (KU885442), *abcc8* (KU885443) and *abcc9* (KU885444).

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