TITLE:

A rapid intrinsic heart rate resetting response with thermal acclimation in rainbow trout, *Oncorhynchus mykiss*

RUNNING TITLE

Pacemaker rate resetting in rainbow trout

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KEYWORDS

Temperature acclimation; heart; intrinsic heart rate; pacemaker; rainbow trout; HCN proteins

SUMMARY STATEMENT

In response to warm temperature, rainbow trout rapidly reset intrinsic heart rate resetting within 1 h without associated changes in mRNA expression of related cardiac proteins.

ABSTRACT

We examined cardiac pacemaker rate resetting in rainbow trout following a reciprocal temperature transfer. In a first experiment performed in winter, 4°C-acclimated fish transferred to 12°C reset intrinsic heart rate after 1 h (56.8 ± 1.2 to 50.8 ± 1.5 bpm) and 12°C acclimated fish transferred to 4°C reset intrinsic heart rate after 8 h (33.4 ± 0.7 to 37.7 ± 1.2 bpm). However, in a replicate experiment, performed on a different brood year in the summer, intrinsic heart rate was not reset, even after 10 weeks. Using this serendipitous opportunity, we examined mRNA expression of a suite of proteins in sinoatrial node (SAN), atrial and ventricle tissue after 1 h and longer than 3 weeks to assess changes associated with pacemaker rate resetting in both experimental groups. In addition to the changes in mRNA expression associated with thermal acclimation in contractile cardiac chambers, we observed downregulation of *NKA α1c* in the atrium and ventricle, and upregulation of *HCN1* in the ventricle after > 3 weeks of warm acclimation associated with pacemaker resetting. However, we found no SAN mRNA expression changes at any time during warm acclimation, including the first hour, unique to the fish with pacemaker rate resetting. Thus, despite identifying changes in mRNA expression of contractile cardiac tissues, there was absence of changes in mRNA expression directly involved with the initial, rapid pacemaker rate resetting with warm acclimation. Importantly, pacemaker rate resetting with thermal acclimation does not always occur in rainbow trout.

INTRODUCTION

In ectothermic organisms such as fishes, acute increases in temperature result in an increase in metabolic oxygen demand due to a thermodynamic response. Nevertheless, over time, many fish species can acclimate to a new temperature and improve function. Thermal acclimation, as a whole, involves generalized (e.g., homeoviscous adaptation) and tissue specific changes, likely over different timescales (Fangue et al., 2014; Fu et al., 2018). This study focuses on the resetting of the intrinsic cardiac pacemaker rate known to occur in rainbow trout (*Oncorhynchus mykiss*) during thermal acclimation.

Intrinsic heart rate is the rate of the heartbeat without any neural or humoral influences. These neural and humoral effects are largely cholinergically and adrenergically mediated in fishes and respond to temperature (Altimiras and Axelsson, 2004; Ekström et al., 2016; Gilbert et al., 2019; Graham and Farrell, 1989; Jose and Taylor, 1969; Wood et al., 1979). Thus, intrinsic heart rate can be

measured *in vivo* by removing these influences with pharmacological agents (Ekström et al., 2016; Jose and Taylor, 1969). Also, intrinsic heart rate can be measured *in vitro* by placing the heart in physiological saline and physically removing it from these influences, as in the present study, because the fish heart is myogenic and beats rhythmically by itself (Graham and Farrell, 1989; Hiroko et al., 1985). The myogenic heartbeat is a direct result of specialised pacemaker cells found in the sinoatrial node (SAN) that generate spontaneous action potentials (Farrell and Smith, 2017; Haverinen and Vornanen, 2007; Newton et al., 2014; Stoyek et al., 2015; Stoyek et al., 2016). Thus, the spontaneous depolarisation in SAN pacemaker cells sets intrinsic heart rate, which is then modulated by neural and humoral influences to set heart rate *in vivo*.

Intrinsic heart rate is not necessarily fixed throughout the life of an animal. For instance, in ectotherms, acute warming typically increases the rate of SAN pacemaker cell depolarisation driving an increase in intrinsic heart rate (Aho and Vornanen, 2001) and thermal acclimation can reset the pacemaker rate. The effects of thermal acclimation on intrinsic heart rate have been studied in a wide range of ectothermic species, including in rainbow trout, goldfish, perch, guppy, medaka, American bullfrogs and common and wood frogs (Bowler and Tirri, 1990; Harri and Talo, 1975; Hiroko et al., 1985; Lotshaw, 1977). Specifically, warm acclimation resets the cardiac pacemaker to a slower rate in rainbow trout: 4°C-acclimated rainbow acclimated to 17°C decreased intrinsic heart rate from 31 bpm to 25 bpm when measured at 4°C and from 74 bpm to 61 bpm when measured at 14°C (Aho and Vornanen, 2001). Thus, thermal acclimation in rainbow trout resets the pacemaker rate in the opposite direction to that of the acute thermal response, which may then allow for further increases in heart rate, for example with acute warming or exercise, before reaching the maximum heart rate at the new acclimation temperature. What is less clear concerning pacemaker resetting for rainbow trout is the timescale for this thermal acclimation response. General assumptions are that pacemaker resetting takes several weeks at the new temperature, as reported by Aho and Vornanen (2001). Indeed, Ekström et al. (2016) concluded that pacemaker rate resetting in rainbow trout must occur over longer than 6 weeks because they found no change in intrinsic heart rate between 1 day and 6 weeks after rainbow trout were moved from 9°C to 16°C. However, alternative explanations for this result could be that either pacemaker resetting may occur rapidly, or under certain conditions it may not occur at all. In fact, the ability to reset pacemaker rate with thermal acclimation is currently assumed to be a taxon-specific response (Matikainen and Vornanen, 1992), and may even only occur over a restricted temperature range and under certain conditions. For example, crucian carp acclimated to 5°C and 15°C did not reset pacemaker rate when measurements were made between 4°C and 35°C (Matikainen and Vornanen, 1992). Similarly, pacemaker rate was not reset in either Atlantic cod or bald rock cod (Egginton and Campbell, 2016; Lurman et al., 2012).

Therefore, the aims of our study were two-fold: a) to resolve the time course of pacemaker rate resetting in rainbow trout; and b) to provide new mechanistic insights into intrinsic heart rate resetting by measuring gene expression of a suite of proteins potentially involved in the ionic currents associated with cardiac activity in the sino-atrial node [SAN], atrium and ventricle because such studies are quite limited for salmonids (Aho and Vornanen, 2001; Hassinen et al., 2007; Hassinen et al., 2008; Hassinen et al., 2017; Haverinen and Vornanen, 2007; Korajoki and Vornanen, 2009; Korajoki and Vornanen, 2012). We specifically targeted proteins central to two competing models advanced for pacemaking in mammals (Lakatta and DiFrancesco, 2009): a) the calcium clock hypothesis, which suggests that spontaneous depolarisation is caused by spontaneous release of calcium sparklets from the sarcoplasmic reticulum via ryanodine receptors; and b) the membrane clock hypothesis, which suggests that spontaneous depolarisation is caused by hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels resulting in a "funny" current. In doing so, and unexpectedly, we found that pacemaker resetting did not occur in both our acclimation experiments with rainbow trout. As a result, we used a Fluidigm microfluidics qPCR platform to evaluate mRNA expression of 28 functional proteins in 3 different cardiac tissues from rainbow trout acclimated to either 4°C or 12°C for more than 3 weeks (4-10 weeks). Differences in mRNA expression of functional genes in the SAN, atrium and ventricle were established, and differences associated with thermal acclimation were identified for each tissue type. These characterizations then allowed identification of functional genes with mRNA expression patterns uniquely associated with the demonstrated slowing of pacemaker rate with warm acclimation in the first acclimation experiment. Thus, by comparing gene expression patterns across these experiments, we were able to distinguish the changes in mRNA expression potentially associated with pacemaker rate resetting responses during warm acclimation from the more general cardiac responses to warm acclimation.

MATERIALS AND METHODS

Study design

The time-course of pacemaker rate resetting was followed during both cold-acclimation (4°C) and warm-acclimation (12°C). We measured intrinsic heart rate after as little as 1 h, and as long as 10 weeks after transferring rainbow trout either from 4°C to 12°C, or from 12°C to 4°C (Table 1). We predicted that pacemaker rate resetting would take longer to occur with cold acclimation than with warm acclimation due to basic thermodynamics. However, mRNA expression in SAN, atrial and ventricle tissues was only examined during warm acclimation. The experiments were performed in accordance with the Animal Care Guidelines at the University of British Columbia (UBC) (Permit Number - A15-0035).

Fish supply, temperature treatments and sampling times

Rainbow trout were obtained from the Sun Valley Trout Hatchery, where they had been reared in outdoor, flow-through raceways. Two replicate experiments were performed, one over winter from November through December of 2016, and a second on a subsequent brood year of trout from the same source over early summer from March through June of 2017.

Overall experimental design was the same for the two experiments. First, fish were pre-acclimated to either 4°C (±1°C) or 12°C (±1°C) in recirculating tanks inside an environmental chamber for 3-12 weeks. Daily replacement with temperature-adjusted freshwater ensured good water quality, ammonia and oxygen levels (monitored daily). Fish were fed a daily maintenance diet of BioTrout 4.0 mm (Skretting, Vancouver) and held at a 12:12 h photoperiod. A subset of fish was sampled directly from the pre-experimental acclimation tanks (0 h; > 3weeks; 10-12 weeks). Sub-groups of fish were then placed into individual, 19 L mesh-covered buckets and moved into a new experimental tank at the opposite temperature, and were sampled 1 h, 8 h, and 24 h at the new acclimation temperature *(Fig. S1-A)*. This facilitated taking rapid samples during the initial stages following transfer. As control for fish handling, a group of 4°C-acclimated fish were placed in buckets and returned to the 4°C experimental tank and sampled after 1 h.

Most of the fish used in experiment 1 were obtained in May 2016 (43 fish) and were held in outdoor flow-through tanks at UBC until October 2016, after which they were moved into temperature-controlled tanks for the pre-acclimation period. In November of 2016, additional fish of the same brood year (27 fish) were obtained from the same source added to the pre-acclimation tanks to allow increased sample sizes. Molecular samples were taken over 2 days in December from fish acclimated for 1 h to 12°C, for 1 h at 4°C (a fish handling control group) and for > 3 weeks at both 4°C and 12°C (7 weeks and 4 weeks, respectively). Heart rate measurements were taken between late November and late December 2016. The fish used in the experiment had body mass = 337 ± 9 g (mean ± s.e.m.); n= 70.

Fish for experiment 2 were placed directly into temperature-controlled pre-acclimation tanks at UBC in March 2017. The experiment was conducted as described above. Molecular samples were taken during one day in May from fish acclimated for 10 weeks to either 4°C or 12°C (referred to as > 3 weeks acclimation). Heart rate measurements were taken between early April and early June 2017. The fish used in this experiment had body mass = 258 ± 8 g; n=55. Consequently, the fish used in these two experiments were of were the same strain, a similar size and received the same

experimental treatments, but they were from different brood years and were acclimated at different times of the year.

Intrinsic heart rate measurements

Fish were individually euthanized with a blunt blow to the head, followed by destruction of the brain and spinal cord. The heart, including much of the sinus venous to ensure an intact SAN, was quickly excised (<2 min) and was placed (with the ventricle laterally cut to aid saline perfusion) in an oxygenated (100% oxygen) saline bath held at the appropriate acclimation temperature (either 4 ± 1°C or 12 ± 1°C) maintained by a circulating water bath and recirculating chillers. The temperature of the physiological saline was monitored directly and contained: 140 mM NaCl, 2.8 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 3.9 mM TES free acid, 6.1 mM TES Na salt and 10 mM glucose with a pH of 7.8 at 10°C. The electrocardiogram (ECG) signal was recorded using two custom-made stainless steel electrodes, one placed next to the SAN the other away from the heart *(Fig. S1-B)*. The ECG signal was amplified (100-1000x) and filtered (high pass: 0.3 Hz, low pass: 0.1 kHz) with a Grass P55 AC amplifier (Astro-Med Inc). Data acquisition used a PowerLab ML870 and ECG signals were digitally filtered (60 Hz line filter, 0-5 Hz high pass, 45 Hz low pass) and analysed using automated beat detection in LabChart 7 Pro software (AD instruments). After a stabilization period of 30 min in the saline bath, the stable intrinsic heart rate (bpm) was recorded as an average from consecutive ECG waveforms (> 1 min) using automated beat detection. Intrinsic heart rates are presented as mean ± s.e.m. for n individuals. Fish were discarded if either the saline temperature occasionally drifted outside the experimental range after 30 min, or heart rate occasionally became arrhythmic or unstable. One-way ANOVAs with a post hoc Tukey test and student t-tests were conducted using Sigmaplot (ver. 13.0; Systat Software) and differences were considered significant when $P<0.05$.

Quantitative reverse transcriptase (qRT-) PCR

CARDIAC TISSUE SAMPLING

Hearts from fish undergoing temperature acclimation were similarly removed into oxygenated saline to allow dissection of the ventricle (335 ± 20 mg), atrium (58 ± 4 mg) and SAN-regions (34 ± 2 mg) *(Fig. S1-C).* Each of these regions was placed into individual Eppendorf tubes for flash freezing with liquid nitrogen. The SAN region can include the sino-atrial canal (Haverinen and Vornanen, 2007; Newton et al., 2014; Stoyek et al., 2015; Stoyek et al., 2016), and so the considerable sample of the sinus venosus tissue included a minimal amount of atrial tissue to ensure inclusion of pacemaker cells. Tissues were stored at -80°C until analysed.

Cardiac genes for qRT-PCR targeted those potentially implicated in pacemaker function and temperature acclimation (Table A1). All genes had been previously sequenced in rainbow trout (D'Souza et al., 2014; Hassinen et al., 2008; Korajoki and Vornanen, 2009; Korajoki and Vornanen, 2012) with the exception of the HCN genes, which have not been previously sequenced. Primer sequences *(Table A1)* were designed using Primer Express 3 (Life Technologies, Carlsbad, California, USA) with melting temperatures of 58-60°C (default settings). Specificity of each primer set was tested by cloning and sequencing. Each primer set was run individually on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, California, United States) with SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, California, United States) and a mixed cardiac tissue sample. Cycling conditions were: 95°C for 10 min followed by 40 repeats of 95°C for 15 s and 55°C for 1 min. Products were cloned using TOPO™ TA Cloning™ Kit for Subcloning (Invitrogen, Carlsbad, California, United States), as per manufacturer's protocol. One Shot™ TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, California, United States) were used for most assays; however, HCN isoforms were tested using Shot™ TOP10F' E. *coli* cells (Invitrogen, Carlsbad, California, United States) as these produced a higher yield. Plasmid extraction was performed using GeneJET Plasmid Miniprep Kit (Thermo Scientific, Waltham, Massachusetts, United States) and the resulting product was sequenced by either the NAPS core facility at UBC (Vancouver, BC, Canada) or Macrogen USA (Rockville, MD). Primers tested produced at least 9-10 clones of the expected sequence, and if not, the primers were discarded. In most cases and apart from isoforms of HCN, we did not try to distinguish between paralogues, unless previously identified in other work. When primer sequences were obtained from previous studies *(Table A1),* the products of these primers were not cloned and sequenced.

RNA EXTRACTION

RNA was extracted according to the manufacturer's protocol from all tissue samples using MagMAX™-96 for Microarrays Total RNA Isolation Kits (Ambion, Austin, Texas, United States). Briefly, a tissue sample was weighed, and if necessary divided into a tissue sections weighing <90 mg. A tissue section was then placed into 200 µL of TRI reagent® (Sigma-Aldrich, St Louis, Missouri, United States) and briefly homogenised using a pellet pestle. More TRI reagent® was added to give a final TRI reagent® volume of 15 µL per 1 mg of tissue. 0.2 µL of external standard mRNA (500 pg μL^{-1}) per mg of tissue was then added to the tube with □10 @ 1.0 mm ceria-stabilized zirconium oxide beads (Next Advance, Averill Park, New York, United States) in each tube. The tissue samples were fully homogenised with Bullet Blender24 (Next Advance, Averill Park, New York, United States) before adding 0.1 mL of 1-bromo-3-chloropropane per 1 mL of Tri reagent® to each tube and centrifuging at 12,000 g for 10 min. The aqueous layer was removed and frozen at -80°C for storage. The aqueous layer for tissues weighing >90 mg, which had been processed in sections up till this point, were

recombined with volumes relative to section weight, to make up 150 µL [(section weight/ sample weight) x 150 µL]. When all samples had been processed to this point, the aqueous layers were defrosted and placed in 96 well plates. RNA extraction then proceeded as in the 'spin' protocol using a Biomek FXP (Beckman Coulter, Brea, California, United States) automated liquid handling instrument. Purity was assessed using 260/280 ratios (1.97-2.45) and yield was calculated using 260 nm absorbance. The RNA concentration was then normalised using the BioMek FXP (Beckman Coulter, Brea, California, United States) automated liquid handling instrument to 62.5 ng μL^{-1}.

FLUIDIGM QRT-PCR

qRT-PCR was run using the Fluidigm BioMark™ microfluidics platform (Fluidigm, South San Francisco, California, United States) with Evagreen® assays as fully described in Jeffries et al. (2014) for 33 target genes and 8 reference genes *(Table 2.1)*. Briefly, total RNA was used to synthesize cDNA with SuperScript™ VILO™ MasterMix (Invitrogen, Carlsbad, California, United States) as per the manufacturer's protocol. cDNA was pre-amplified in a specific target amplification (STA) step using all the primer pairs (*Table A1)* and TaqMan™ Preamp Master Mix (Life Technologies, Carlsbad, California, USA). The BioMark protocol for pre-amplification was then followed (1.25 µL of cDNA, 1.25 µL of 200 nM pooled primer mix and 2.5 µL of Master Mix). Any primers that were unincorporated were then removed using ExoSAP-IT™ High-Throughput PCR Product Clean Up (MJS BioLynx Inc, Brockville, Ontario, Canada) (2 µL of ExoSap-IT per 5 µL of post-PCR reaction volume), followed by 1:5 dilution in DNA Suspension Buffer (TEKnova, Hollister, California, United States).

qRT-PCR was run with 96 by 96 dynamic arrays using the Biomark HD™. A sample premix (2.5 µL 2x SsoFast™ EvaGreen® Supermix with low ROX (Biotium, Fremont, California, United States), 0.25 µL 20x DNA Binding Dye Sample Loading Reagent (Fluidigm, South San Francisco, California, United States) and 2.25 µL of the diluted pre-amplified product) and an assay premix (2.5 µL of 2x Assay Loading Reagent (Fluidigm, South San Francisco, California, United States), 2.25 µL 1x DNA Suspension Buffer (TEKnova, Hollister, California, United States) and 50 µM each of mixed forward and reverse primers) were made. These were loaded onto the 96.96 dynamic array and mixed using an IFC controller HX (Fluidigm, South San Francisco, California, United States). qRT-PCR was then run using the recommended protocol for Evagreen, GE Fast 96 x 96 PCR+Melt v2 protocol with Biomark HD™ (A thermal mixing protocol of 70°C for 40 min and 60°C for 30 s, then a hot start protocol of 95°C for 60 s, followed by 30 qRT-PCR cycles of 96°C for 5 s and 60°C for 20 s. A melting protocol of 60°C for 3 s followed using a 1°C increase every 3 s up to 95°C).

QUALITY CONTROL

The same number of samples (n=7) were used for each experimental group to ensure a balanced statistical design with ANOVA analyses. mRNA extraction and qPCR was performed on all original 7-10 tissue samples per group and sample sizes were reduced by first excluding any individual where the SAN, atrial or ventricle sample was unavailable, and then by excluding the last samples that were homogenised.

While using the Fluidigm approach offers the advantage of running many assays simultaneously, it can be difficult to optimize the conditions so that all assays perform well with differing mRNA expression levels. As the aim of this study was only to gain a broad-scale picture, we did not optimise and rerun assays that failed quality criteria, and instead we chose to exclude data for any genes that did not meet our stringent quality control filters. Regardless, we report all efficiencies, r^2 and experimental Ct ranges in *Table S1.*

Of 33 possible target genes, 28 met our quality control standards and were ultimately used for qRT-PCR analysis. For the target genes that were excluded, the standard curves for α_{1A}-*adrenoceptor*, β_2-*adrenoceptor*, *HCN4b* and *NKA α1b* were insufficient, while for *ANP* Ct values for some samples were too low for the Fluidigm system (*i.e.*, 2.5Ct). For the remaining 28 target genes, a measurement was excluded from further analysis if the difference between technical replicates was greater than 1 cycle (*i.e.*, $Na_v1.4$ in a single SAN sample from the fish acclimated to 4°C for 1 h with a pacemaker rate resetting response, and $Na_v1.6$ in a single ventricle sample from fish acclimated to 4°C for >3 weeks without a pacemaker rate resetting response*; Table S1)*.

We utilized data for two reference genes (*CCDC84* and *SEP15*) that have been used previously with salmonids (*CCDC84*; Jeffries et al., 2014) and zebrafish (*SEP15*; Xu et al., 2016). Both were confirmed as a suitable combination for an endogenous control using Normfinder (combined stability value of 0.019). Six of the reference genes tested (*18s*, *β-actin*, *EF1α*, the external standard, *DnaJA2* and *MprPL40*) were excluded using one of the following strict quality criteria: a) Ct values were outside the recommended detection limit (*i.e.*, 6-25 Cts) for the Fluidigm system (*i.e.*, *18s*, *β-actin*, *EF1α*, *DnaJA2* and *MprPL40*) *(Table S1)*; b) the efficiency was ≤ 80% or ≥ 120% (*18s* and the external standard, respectively); or c) r^2 was ≤ 0.980 (both *18s* and the external standard). Stricter quality criteria were applied for to the reference genes than to the target genes because inaccurate reference gene measurements will affect every assay, whereas inaccurate target gene measurements will only affect that single assay.

Standard curves, run in duplicate on both plates to give quadruplicates, were calculated from serial dilutions (0.2x, 0.04x, 0.008x and 0.0016x) from a cDNA mixture of all samples that was made up

before the STA step (Bustin et al., 2009). The undiluted sample (1x) did not have a linear relationship with the other dilutions for any of the assays, and so was not used in the standard curve. When replicates had a range greater than 1 Ct, we excluded this dilution. If fewer than 3 dilutions remained, normally in association with high Ct values, we excluded the whole assay (*i.e.*, α_{1A}-*adrenoceptor*, β_2-*adrenoceptor*, *HCN4b* and *NKA α1b*)

CALCULATING GENE EXPRESSION

$E^{-\Delta Ct}$ (Normalised Quantity)

$E^{-\Delta Ct}$, expression normalised to a housekeeping gene, was calculated for a semi-quantitative comparison of the expression of different target genes. This measure was obtained as in Scott et al. (2004) using the geometric mean of the reference genes as in Hellemans et al. (2007) *(Eqn. A1)*.

$E^{-\Delta\Delta Ct}$ (Calibrated Normalised Quantity)

$E^{-\Delta\Delta Ct}$, expression of the target gene normalised to the expression of the reference genes then an inter-run calibrator sample (IRC), a 0.04x dilution of pooled sample (the highest standard curve measurement used), was calculated as in Pfaffl (2001) using the geometric means of the reference genes, as in *(Eqn A2)*.

Fold Change in $E^{-\Delta\Delta Ct}$ with Warm Acclimation (Calibrated Normalised Relative Quantity)

Fold change in $E^{-\Delta\Delta Ct}$ of the target gene with warm acclimation was calculated for each pacemaker rate response group, by dividing $E^{-\Delta\Delta Ct12^{\circ}C}$ *(Eqn A2)* by the $E^{-\Delta\Delta Ctaverage4^{\circ}C}$ *(Eqn A3),* in a method that is mathematically identical to Hellemans et al. (2007).

STATISTICAL ANALYSIS OF mRNA EXPRESSION

PCA analysis was performed using the PRCOMP package in R Studio (Team, 2014) with 68% confidence ellipses used to depict one standard deviation. FDR adjustments by GraphPad Prisim 7.00 (GraphPad Software; CA) and other statistical analysis was performed Sigmaplot 14.0 (Systat; CA).

Four primary questions were examined with our qRT-PCR study. First, how does mRNA expression differ among cardiac tissues? - by comparing the mRNA expression in the SAN, atrium and ventricle in fish acclimated to 4°C for at least 3 weeks (Experiment 1). Second, how does cardiac tissue mRNA expression change with thermal acclimation? - by comparing mRNA expression in fish acclimation to either 4°C or 12°C after at least 3 weeks (Experiment 1). Third, what changes in mRNA expression are specifically associated with pacemaker rate resetting? - by comparing fish in Experiment 1 and Experiment 2 that had been acclimated for more than 3 weeks to either 4°C or 12°C. To answer these question, genes were placed into one of three groups:

1) *mRNA changes associated with warm acclimation, but not pacemaker rate resetting:* For these genes, the fold change in mRNA expression was significantly different between 4°C and 12°C in Experiments 1 and 2, but not different with warm acclimation between the two Experiments.

2) *mRNA changes only associated with pacemaker rate resetting*: For these genes, the fold change in mRNA expression was significantly different between 4°C and 12°C only in Experiment 1, which meant a significant difference with warm acclimation between the two Experiments.

3) *mRNA changes associated with no pacemaker rate resetting*: between 4°C and 12°C, only in Experiment 2, which meant a significant difference with warm acclimation between the two Experiments.

Lastly, we examined if the mRNA expression changes associated with pacemaker rate resetting during full thermal acclimation could also be driving the initial resetting of pacemaker rate by comparing mRNA expression in control fish moved from 4°C to 4°C for 1 h, and experimental fish moved from 4°C to 12°C for 1 h. The mRNA expression changes seen within this first hour were then compared to those changes in mRNA only associated with pacemaker rate resetting.

COMPARISON OF mRNA EXPRESSION IN CARDIAC TISSUE TYPES

Differences in mRNA expression patterns were compared between cardiac tissue types with principal component analysis, using $E^{-\Delta\Delta Ct}$. A 68% confidence limit elipse was plotted around each tissue type to graphically ilustrate one standard deviation for the distribution.

Significant differences in genes between cardiac tissues were identified by comparing $E^{-\Delta\Delta Ct}$ mRNA expression using a one-way ANOVA with a Tukey's *post-hoc* test. If the test for normality (Shapiro-Wilk) or equal variance (Levene's) failed, the non parametric Kruskal-Wallis ANOVA with a Dunn's *post-hoc* test was run.

For mRNA expression of the membrane clock and calcium clock proteins, relative expression is as important as overall expression. Therefore, pie charts were created to qualitatively compare the relative expression for each tissue type from average $E^{-\Delta Ct}$ values for HCN expression (*Fig. S2*) and calcium clock expression (*Fig. S3).*

A semi-quantitative measure of total mRNA expression for each functional gene group was calculated from the total $E^{-\Delta Ct}$ values of all the genes in each group for each individual. These were

compared among cardiac tissues, using a one-way ANOVA with a Tukey's *post-hoc* test. If the test for normality (Shapiro-Wilk) or equal variance (Levene's) failed, the non parametric Kruskal-Wallis ANOVA with a Dunn's *post-hoc* test was run. FDR adjustment was performed within each thermal acclimation group

COMPARISON OF mRNA EXPRESSION BETWEEN WARM- AND COLD-ACCLIMATED FISH

Differences in mRNA expression patterns were compared between warm- and cold- acclimated fish with principal component analysis, using $E^{-\Delta\Delta Ct}$. A 68% confidence limit elipse was plotted around each tissue type to graphically ilustrate one standard deviation for the distribution.

Significant differences in genes between warm- and cold-acclimated fish were established by comparing $E^{-\Delta\Delta Ct}$ mRNA expression, within tissue and treatment groups, with Student's t-test (Fenna, 2013; Hassinen et al., 2008; Korajoki and Vornanen, 2012). If the test for normality (Shapiro-Wilk) or equal variance (Levene's) failed, the non-parametric Mann-Whitney test was run.

FDR adjustment was performed within each tissue for each temperature and thermal acclimation group.

COMPARISON OF THE FOLD CHANGE IN mRNA EXPRESSION WITH WARMING

Signficant differences between the pacemaker rate resetting responses were established by comparing the fold change in $E^{-\Delta\Delta Ct}$ with warm acclimation for mRNA expression between fish with and without a pacemaker rate resetting response using Student's t-test. If the test for normality (Shapiro-Wilk) or equal variance (Levene's) failed, the non-parametric Mann-Whitney test was run.

FDR adjustment was performed within each tissue for each thermal acclimation group.

CORRECTING FOR MULTIPLE COMPARISONS

Multiple comparisons increase the likelihood of false positive results. Therefore, false discovery rate (FDR) adjustments were applied to all ANOVAs and Student's t-tests using a two-stage step-up method (Benjamini et al., 2006). However, unadjusted *P*-values are also presented for comparison.

DATA PRESENTATION

Data presentation, including Venn diagrams for comparisons of cold- and warm-acclimation and comparisons of the fold changes in mRNA expression with warm acclimation, was done using R

Studio (Team, 2014), GraphPad Prism 7.00 (GraphPad Software; CA) and Inkscape 0.92 (Inkscape Team).

RESULTS

Pacemaker rate resetting

In Experiment 1, fish rapidly reset intrinsic heart rate during both warm acclimation and cold acclimation *(Fig. 1)*. When intrinsic heart rate was measured at 4°C, it was significantly higher after 4-6 weeks in 4°C-acclimated fish (36.1 ± 0.9 bpm) compared with 12°C-acclimated fish (33.4 ± 0.7 bpm) (P=0.044 Student's t-test) *(Table 1)*. After transferring 4°C-acclimated fish to 12°C, resetting of the pacemaker rate (from 56.8 ± 1.2 bpm to 50.8 ± 1.5 bpm) was first seen after 1 h, and the new rate persisted through until the 24 h measurement (50.3 ± 1.5 bpm) *(Fig. 1)*. Fish-handling control fish transferred back to 4°C, however, maintained a heart rate of 57.9 ± 1.9 bpm after 1 h, confirming that the pacemaker rate resetting was caused by the temperature change. Similarly, significant pacemaker rate resetting (from 33.4 ± 0.7 bpm to 37.7 ± 1.2 bpm) was seen when 12°C-acclimated fish were moved to 4°C, but this was first detected after 8 h, rather than 1 h, and again persisted unchanged for the 24 h measurement (37.7 ± 1.2 bpm) *(Fig. 1)*.

In Experiment 2, fish did not reset pacemaker rate with either temperature acclimation. When 4°C-acclimated fish were moved to 12°C, intrinsic heart rate was unchanged after both 24 h (52.7 bpm ± 2.2 bpm *vs* 57.5 ± 2.7 bpm) and 10-12 weeks (53.3 ± 2.8 bpm *vs* 52.2 ± 2.3 bpm) *(Table 1)*. Similarly, when 12°C-acclimated fish were moved to 4°C, intrinsic heart rate was unchanged after both 24 h (30.6 ± 1.7 bpm *vs* 29.2 ± 0.6 bpm) and 10-12 weeks (29.9 ± 1.1 bpm *vs* 29.4 ± 1.1 bpm) *(Table 1)*.

mRNA expression

mRNA EXPRESSION DIFFERENCE BETWEEN TISSUES ACCLIMATED TO 4°C FOR MORE THAN 3 WEEKS

Principal component analysis revealed mRNA expression in the ventricle was distinct from both SAN and atrium. SAN and atrium had a minor overlap of 68% confidence intervals for PC1, and PC1 and PC2 explained 35.9% and 19.4% of variance in gene expression, respectively *(Fig. 2-A)*, perhaps in part due to the minor atrial content of the SAN sample. Nevertheless, mRNA expression in the SAN and atrium did differ specifically for the membrane clock component *HCN1*, the calcium clock component *$Ca_v1.3$* and *collagen α1a (Fig. 3)*. Furthermore, the mRNA expression of membrane clock proteins was highest for SAN *(Fig. S2)*, unlike the calcium handling protein mRNA expression *(Fig. S3)* which were not significantly different among the three cardiac tissues.

mRNA EXPRESSION CHANGES AS A RESULT OF WARM ACCLIMATION

Principal component analysis also revealed differences in mRNA expression between warm acclimation and cold acclimation; PC1 and PC2 explained 24.0% and 22.1% of variance in gene expression, respectively *(Fig. 2-B)*. Warm acclimation and pacemaker rate resetting in Group 1 was associated with differential expression of 20 genes: 6 in SAN, 5 in atrium and 9 in ventricle *(Fig. 4)*. Specifically, a membrane clock component (*HCN2a2*) was significantly downregulated with warm acclimation in the SAN, but not in the atrium *(Fig. 4)*. Notably, *HCN4a1/2* was the dominant HCN isoform and was upregulated relative to total HCN expression in all three tissue types *(Fig. S2)*. Warm acclimation also downregulated mRNA expression of calcium clock components *calsequestrin* and *SERCA2* in both SAN and atrium, while mRNA expression of *S100* was downregulated only in SAN and mRNA expression of *RYR3* was downregulated only in the atrium *(Fig. 4)*. mRNA expression of *NKA α1a* and *NKA α1c* were also downregulated in both SAN and atrium. However, mRNA expression of $K_{ir}2.1$ and $K_{ir}2.2$, *ERG*, *Cx43* and *collagen 1α1* did not change significantly in either atrium or ventricle, and neither *SERCA2* or *calsequestrin* were downregulated in the ventricle for fish that reset pacemaker rate *(Fig. 4)*.

mRNA EXPRESSION CHANGES ASSOCIATED WITH PACEMAKER RATE RESETTING

Of the 20 gene expression changes associated with warm acclimation, 9 fitted the criteria for 'mRNA changes only associated with pacemaker rate resetting' (*i.e.*, they were observed only in Experiment 1) *(Fig. 4, Fig. 5, Fig. 6 and Fig. S4)*. Specifically, downregulation of calcium clock components *S100* and *SERCA2* mRNA expression occurred only in SAN, downregulation of *calsequestrin* mRNA expression occurred only in atrium, and upregulation of *HCN1*, *HCN4a1/2* and *NKA α3* mRNA expression occurred only in ventricle. Downregulation of *NKA α1c* expression with warm acclimation was the only change common to SAN, atrial and ventricular tissue that was unique to the fish in Experiment 1 *(Fig. 4, Fig. 5 and Fig. S4)*. However, after FDR adjustment, only the upregulation of *HCN1* in ventricle and the downregulation of *NKA α1c* in atrium remained significant *(Fig. 4, Fig. 5, Fig. 6 and Fig. S4)*.

Of the 14 gene expression changes observed with warm acclimation in Experiment 2 (5 in the SAN, 6 in the atrium and 3 in the ventricle), 6 fitted the criteria for 'mRNA changes associated with no pacemaker rate resetting' (*i.e.*, they were observed only in Experiment 2) *(Fig. 6)*. Specifically, upregulation of membrane clock component *HCN3* and *TGF-β1* occurred in both SAN and atrium, downregulation of *HCN2a1* and *HCN2a2* and an upregulation of *collagen 1α1* occurred only in atrium, and a downregulation of *calsequestrin* occurred only in ventricle *(Fig. 4, Fig. 5 and Fig. S4)*.

However, after FDR adjustment only the upregulation of membrane clock component *HCN3* in SAN and atrium remained significant *(Fig. 4, Fig. 5, Fig. 6 and Fig. S4)*.

Gene expression changes that fitted the criteria for 'mRNA changes associated with warm acclimation, but not pacemaker rate resetting' *(Fig. 6)* included downregulation of the membrane clock component *HCN2a2* and the calcium clock component *calsequestrin* mRNA expression in SAN, downregulation of *RYR3* mRNA expression in atrium, and an upregulation of *HCN3* mRNA expression in ventricle *(Fig. 4, Fig. 5 and Fig. S4)*. However, after FDR adjustment none of these expression changes remained significant (*Fig. 4, Fig. 5, Fig. 6 and Fig S4)*.

mRNA expression changes observed 1 h after warm acclimation in fish with a pacemaker rate resetting response *(Fig. 7)* included upregulation the membrane clock component *HCN2b1/2* and calcium clock component *NCX* in SAN, downregulation of *TGF-β1* in SAN and atrial tissues, upregulation of *calsequestrin* in atrium, upregulation of $Na_v1.5$ in ventricle and downregulation of *TRPC1* in ventricle. However, none of these changes were significant after FDR adjustment. None of these genes showed expression patterns that fitted the criteria for 'mRNA changes only associated with pacemaker rate resetting' in the fish acclimated for > 3 weeks *(Fig. 6, Fig. 7)*.

DISCUSSION

Before our study was undertaken, it was assumed that thermal acclimation was a typical homogenous process within species, and differences observed in pacemaker resetting response to thermal acclimation were species-specific (Matikainen and Vornanen, 1992). However, unexpected results from our study demonstrated that pacemaker resetting does not always occur in rainbow trout. While Experiment 1 showed intrinsic heart rate resetting in response both warm and cold acclimation, Experiment 2 did not show intrinsic heart rate resetting, even after 10 weeks acclimation to either 4°C or 12°C. This means that the lack of pacemaker resetting responses in fishes cannot be attributed solely to species differences, as suggested previously by Matikainen and Vornanen, 1992. What might turn off pacemaker resetting awaits further study, but the present results suggest seasonal acclimatisation or previous life experiences could be a factor. Indeed differences in both intrinsic heart rate, and in the electrophysiology of cardiac myocytes have been previously observed in a number of fish species, although not in salmonids (Filatova et al., 2019; Harri and Talo, 1975; Matikainen and Vornanen, 1992). A severe hypoxic stress event is one experience that could affect intrinsic heart rate resetting in rainbow trout (Sutcliffe, 2018) . Even so, our two experimental groups were from different brood years with different life experiences and sampled at different times of year, all of which could contribute to the variation in the pacemaker resetting response to thermal acclimation seen here.

The second novel discovery from our study was the speed at which intrinsic heart rate resetting can occur, which we quantified by reciprocally transferring rainbow trout to either 4°C or 12°C. In the fish with a pacemaker resetting response (Experiment 1), intrinsic heart rate was significantly different after just 1 h of warm acclimation to 12°C, and after just 8 h of cold acclimation to 4°C. The changes in heart rate were in the direction expected, adding confidence to our novel finding rapid intrinsic heart rate resetting. Furthermore, the change in intrinsic heart rate was faster with warm acclimation, as expected, and most likely as a result of faster reaction rates at higher temperatures. Similarly, changes in CTmax and CTmin in sheepshead minnow were more rapid for warm *vs* cold acclimation (Fangue et al., 2014).

Few studies have explicitly examined the timescale to reset intrinsic heart rate with temperature acclimation; they typically examine heart rate after many days or weeks. For example, heart rate resetting was observed after 4-10 days in American bullfrogs and wood frogs (Lotshaw, 1977), after 2 weeks in the common frog (Harri and Talo, 1975), after 3 weeks in perch (Talo and Tirri, 1991), after 4 weeks in rainbow trout (Aho and Vornanen, 2001) and after 2 months in goldfish (Hiroko et al., 1985; Tsukuda, 1990). Only one study (Ekstrom et al., 2016) examined the time course of pacemaker resetting between 1 day and 6 weeks after transfer from 9°C to 16°C and in rainbow trout. However, they observed of no intrinsic heart rate resetting. While they concluded that pacemaker rate resetting takes longer than 6 weeks, our study offers two alternative explanations that likely depend the specific population or group of rainbow trout: a) intrinsic heart rate may not reset, like our Experiment 2; or b) the resetting had been completed by their first measurement at 24 h, like our Experiment 1. Whatever the explanation, our study demonstrates that pacemaker resetting can be significantly faster rate than previously assumed, within hours rather than weeks, and even with cold acclimation.

Pacemaker resetting is not the only cardiac response to thermal acclimation. While not the focus of our study, cardiac thermal acclimation responses identified in numerous other studies include decreases in the stiffness of the ventricle tissues, in the weight of the ventricle relative to body size, in cardiac force, in the density of cardiac β-adrenergic receptors and in the cardiac sensitivity to β-adrenergic stimulation (Aho and Vornanen, 1999; Aho and Vornanen, 2001; Altimiras and Axelsson, 2004; Anttila et al., 2013; Brett, 1971; Casselman et al., 2012; Chen et al., 2013; Chen et al., 2015; Costa et al., 2002; Drost et al., 2014; Eliason et al., 2011; Eliason et al., 2013; Farrell, 1991; Fry, 1947; Gilbert et al., 2019; Graham and Farrell, 1989; Keen et al., 1993; Keen et al., 2016; Keen et al., 2017; Klaiman et al., 2011; Klaiman et al., 2014; Shiels and Farrell, 1997; Steinhausen et al., 2008; Verhille et al., 2013; Wood et al., 1979). Whether these responses are homogenous with thermal acclimation or even whether they occurred in our study are unknown. However, we did observe some mRNA expression changes in atrial and ventricle tissue that might be related to the previous studies that

have examined mRNA expression in atrial and in ventricle tissue during warm acclimation to identify what mRNA expression changes might be causing these responses. For example, similar to Hassinen et al. (2007) and by Korajoki and Vornanen (2012 and 2009), fish with an intrinsic heart rate resetting response had no change in *$K_{ir}2.1$* and *$K_{ir}2.2$* expression, a downregulation in *SERCA2* in atrial and ventricle tissue, and a downregulation of *calsequestrin* in only atrial tissue during warm acclimation. However, other previously observed differences in mRNA expression with warm acclimation that we did not not observe, *i.e. ERG*, *Cx43* and *collagen 1α1* (Fenna, 2013; Hassinen et al., 2008). Whether this difference reflects a different experimental temperatures among studies (4°C and 12°C in our study vs 4°C and 18-19°C previously), or some other variable is unclear. For fish that did not reset intrinsic heart rate, downregulation in SERCA2 was not observed, downregulation of calsequestrin was observed only in the ventricle and an upregulation of collagen α1a was observed in the atrium. While previous studies report other cardiac changes during thermal acclimation, heart rate was not measured in parallel with mRNA expression, something that we feel should be done to confirm resetting.

We identifies a number of novel mRNA expression changes in the atrium and ventricles of the fish that reset intrinsic heart rate, as well as in those that did not. Resetting of intrinsic heart rate with acclimation was associated with downregulation of *NKA α1c* and *RYR3* was observed in atrium and ventricle, a downregulation of *NKA α1a* in the atrium, a downregulation of *NCX* in the ventricle, and an upregulation in *NKA α1a*, *Nav1.6*, *HCN1*, *HC*N3 and *HCN4a1/2* in the ventricle of the. Of these changes only the downregulation in the ventricle of *RYR3* and upregulation in the ventricle of *HCN3* was observed also in the fish that did not reset intrinsic heart rate. Other changes observed in the atrium of fish that didn't reset intrinsic heart rate were downregulation of *HCN2a1* and *HCN2a2,* and upregulation of *HCN3*, and *TGF-β1*, as well as an upregulation of *HCN 2b1* in the ventricle. What role these novel changes play in thermal acclimation is unclear as in this study we did not confirm any differences in cardiac thermal acclimation responses between the two groups of fish other than in intrinsic heart rate resetting.

SAN, not ventricle or atrial, tissues is responsible for resetting pacemaker rate. Therefore, mRNA expression differences between SAN tissue and other cardiac tissues such as the atrium likely reflect a role in pacemaking. However, since the SAN occupies a small region of the heart and cannot be easily defined by eye, we conservatively dissected the SAN tissue to reduce the risk of accidently missing SAN tissue, but this meant that a minimal portion of atrial tissue was included. Despite this potential contamination, mRNA expression patterns seen in the SAN were not necessarily seen in atrium, and vice versa. For example, *HCN1*, *collagen 1α1*, and *$Ca_v1.3$* were differentially expressed between the atrial and SAN tissues. Therefore, the mRNA expression ascribed solely the SAN tissue is

done with a good measure of confidence and, if anything, any expression differences identified would potentially be underestimated.

Our is the first in fishes to examine the mRNA expression of components of the calcium and the molecular clock in the SAN. Both clocks are hypothesised to drive spontaneous depolarisation of pacemaker cells in the SAN in mammals, and evidence exists that may (Hassinen et al., 2017; Marchant and Farrell, 2019) or may not be the case in fishes (Wilson and Farrell, 2013). Therefore, the differentially higher expression of *HCN1*, a molecular clock component, observed in SAN compared with atrial tissues is of significant interest. This is similar to observations in mammals (Marionneau et al., 2005) where HCN1 is the fastest activating HCN isoform and is activated at the highest voltage (Wahl-Schott and Biel, 2009). If rainbow trout HCN1 has similar properties, then its significantly increased expression in the SAN could mean that the molecular clock is responsible for the SAN's rapid rate of depolarisation. This, along with physiological experiments demonstrating that HCN antagonists do slow down heart rate *in vivo* in rainbow trout (Altimiras and Axelsson, 2004; Keen and Gamperl, 2012), and calcium antagonists have only small effects on heart rate (Haverinen and Vornanen, 2007), lends support to a membrane clock theory of spontaneous depolarisation in rainbow trout. However, a high *HCN1* mRNA expression does not necessarily correlate with a functional role in the spontaneous depolarisation of pacemaker cells. Also, it was the HCN4 isoform that dominated in all cardiac tissues from rainbow trout. Furthermore, in brown trout, cardiac pacemaker cells have a high HCN expression, but no I_f current, suggesting a membrane clock may not function in the spontaneous depolarisation of pacemaker cells in this species (Hassinen et al., 2017). Thus, further work is needed to resolve the mechanism responsible for spontaneous depolarisation in salmonids, and further studies of mRNA expression may not be the best way forward.

Key to understanding what drives pacemaker rate, is understanding what causes it to reset. Despite this, changes in mRNA expression with temperature acclimation have not been previously examined in SAN of any fish. If *HCN1*, and other molecular clock components set the pacemaker rate, we would expect changes in expression of these isoforms in SAN after warm acclimation in fish that reset intrinsic heart rate. However, we observed very few mRNA expression changes that were unique to the SAN. These were restricted to downregulation of *HCN2a2* and *s100*, components of the membrane clock and the calcium clock, respectively. Conversely, mRNA expression in a different calcium clock component, *calsequestrin*, was observed unique to the SAN of fish that did not reset intrinsic heart rate. However, if a mRNA expression change is associated with heart resetting, it will probably would not only be observed in fish with a pacemaker rate resetting response, it would also be unique to those fish. Identifying these changes was greatly aided by comparisons between fish in Experiment 1 and fish in Experiment 2. These would be genes with significantly different mRNA expression between warm- and cold-acclimated fish, only in fish with a pacemaker rate resetting

response, along with a greater fold change in mRNA expression changes with warm acclimation. Of these changes the genes most likely to be responsible are those which occurred in the SAN such as *NKA α1c*, *S100* and *SERCA2*. However, no mRNA expression changes unique to the fish that reset intrinsic heart rate were significant after FDR adjustment. Therefore, we did not identify any strong links between mRNA expression and intrinsic heart rate resetting.

This leads back to the rapid pacemaker resetting response observed *in vitro.* None of the mRNA expression that were unique to the fish that reset intrinsic heart rate with >3 weeks acclimation were present in the fish after just 1 h of warm acclimation. While changes in mRNA expression might not be expected to occur so rapidly, this lack of association does suggest that the initial and rapid resetting of pacemaker rate, and perhaps pacemaker resetting in general, is not driven by changes in mRNA expression. Therefore, future studies of pacemaker setting in fishes should consider alternative mechanisms, such as post-transcriptional protein modifications.

In conclusion, pacemaker rate resetting with thermal acclimation occurred much more rapidly than previously shown in any ectotherm, even though we also showed thermal acclimation can occur in rainbow trout without pacemaker rate resetting. The rapid pacemaker rate resetting response, however, was not associated with any significant changes in mRNA expression for key proteins in the SAN, and we did not identify any mRNA expression changes that that would be a strong candidate to be driving the pacemaker resetting response. Nevertheless, differential changes in mRNA expression during thermal acclimation were discovered among SAN, atrial and ventricular, including novel candidate genes in SAN tissue that are associated with warm acclimation but independent of resetting pacemaker rate.

LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degrees Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
18s	18s ribosomal RNA
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
bpm	Beats per minute
cAMP	Cyclic adenosine monophosphate
$Ca_v1.3$	L-type calcium voltage-gated channels subunit α1D

$Ca_v\beta2$	L-type calcium voltage-gated channels subunit β2
CCDC84	Coiled-coil domain containing 84
cDNA	Complementary deoxyribonucleic acid
Col1α1	Collagen type 1a1
Ct	Cycle threshold
CT_{max}	Critical thermal maximum
CT_{min}	Critical thermal minimum
Cx43	Connexin 43
DnaJA2	DnaJ heat shock protein family (Hsp40 member A2)
EF1-α	Elongation factor 1-α
ERG	Ether-à-go-go related gene
$E^{-\Delta Ct}$	Expression normalised to reference genes
$E^{-\Delta\Delta Ct}$	Expression normalised to reference genes and inter-run calibrators
FDR	False discovery rate
g	Gram
h	Hour
HCN1	Hyperpolarisation-activated cyclic nucleotide-gated channel 1
HCN2a1	Hyperpolarisation-activated cyclic nucleotide-gated channel 2a1
HCN2a2	Hyperpolarisation-activated cyclic nucleotide-gated channel 2a2
HCN2b1	Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1
HCN2b1/2	Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1/2
HCN3	Hyperpolarisation-activated cyclic nucleotide-gated channel 3
HCN4a1/2	Hyperpolarisation-activated cyclic nucleotide-gated channel 4a1/2
HCN4b	Hyperpolarisation-activated cyclic nucleotide-gated channel 4b
IRC	Inter-run calibrator
$K_{ir}2.1$	Potassium voltage-gated channel subfamily J member 2
$K_{ir}2.2$	Potassium voltage-gated channel subfamily J member 12
L	Litre
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar

mRNA	Messenger ribonucleic acid
Mrpl40	39s ribosomal protein L40
n	Sample size (number of animals or tissue samples in each group)
$Na_v1.4$	Sodium voltage-gated channel α subunit 4
$Na_v1.5$	Sodium voltage-gated channel α subunit 5
$Na_v1.6$	Sodium voltage-gated channel α subunit 8
NCX	Sodium-calcium exchanger
NKA α1a	Sodium/potassium ATPase subunit α1a
NKA α1b	Sodium/potassium ATPase subunit α1b
NKA α1c	Sodium/potassium ATPase subunit α1c
NKA α3	Sodium/potassium ATPase subunit α3
P-value	Probability value
pg	Picogram
Q_{10}	Temperature coefficients
qRT-PCR	Quantitative real-time polymerase chain reaction
RYR3	Ryanodine receptor 3
s	Second
S100	S100 calcium binding protein
SAN	Sinoatrial node
sem	Standard error of the mean
SEP15	15 kDa selenoprotein
SERCA2	Sarcoplasmic/ endoplasmic reticulum calcium ATPase 2
TGF-β1	Transforming growth factor β1
TRPC1	Transient receptor potential cation channel subfamily C member 1
UBC	University of British Columbia

APPENDIX

Equation A1: $$\frac{E(target)^{-Ct\,(target)}}{(E(CCDC84)^{-Ct(CCDC84)} \times E(SEP15)^{-Ct(SEP15)})^{\frac{1}{2}}}$$

Equation A2: $$\frac{E(target)^{\Delta Ct(sample\,-\,IRC(target))}}{(E(CCDC84)^{-\Delta Ct(sample-IRC\,(CCDC84))} \times E(SEP15)^{-\Delta Ct(sample-IRC\,(SEP15))})^{\frac{1}{2}}}$$

Equation A3: $$\frac{E(target)^{-\Delta Ct_2\,(arithmetic\ mean\ 4^{\circ}C\ -IRC\,(target))}}{(E(CCDC84)^{-\Delta Ct_2(arithmetic\ mean\ 4^{\circ}C-IRC\,(CCDC84))}\times E(SEP15)^{-\Delta Ct_2(arithmetic\ mean\ 4^{\circ}C\ -IRC\,(SEP15))})^{\frac{1}{2}}}$$

Table A1 - All primer pairs used for Fluidigm qRT-PCR analysis.

All primers were designed specifically for this study from the sequence given by the accession number or copied directly from the study, unless indicated otherwise. Primers are displayed in the 5' to 3' direction.

Target	Forward Primer	Reverse Primer	Accession Number/ Source Study
18s (Reference Gene)	CGGTCGGCGTCCAACTT	CAATCTCGCGTGGCTGAA	AF243428
β-actin (Reference Gene)	TGGGGCAGTATGGCTTGTATG	CTGGCACCCTAATCACTCT	Ojima (2007)
CCDC84 (Reference Gene)	GCTCATTTGAGGAGAAGGAGGATG	CTGGCGATGCTG TTCCTGAG	Jeffries et al. (2014)
DnaJA2 (Reference Gene)	TTGTAATGGAGAAGGTGAGG	TGGGCCGCTCTCTTGTATGT	Hassinen et al. (2007)
EF1-α (Reference Gene)	ACCCTCCTCTTGGTCGTTT	TGATGACACCAACAGCAACA	Raida and Buchmann (2007)
External standard (Reference Gene)	GTGCTGACCATCCGAG	GCTTGTCCGGTATAACT	Ellefsen et al. (2008)
Mrpl40 (Reference Gene)	CCCAGTATGAGGCACCTGAAGG	GTTAATGCTGCCACCCTCTCAC	Jeffries et al. (2014)
SEP15 (Reference Gene)	TCACAGCAAACCACATTTTGG	AAGATGCCCAGAGTGACACACA	AY255833
Hyperpolarisation-activated cyclic nucleotide-gated channel 1 (HCN1)	CGCTGAGGATCGTGAGGTTT	TGAGCCGGGAAAGTCTCAGT	AF421883
Hyperpolarisation-activated cyclic nucleotide-gated channel 2a1 (HCN2a1)	ATCGTGGACTTTGTCTCCTCCAT	GATCCCCTTCTCCACGATCA	XM_014141001
Hyperpolarisation-activated cyclic nucleotide-gated channel 2a2 (HCN2a2)	CTGCAGGACTTCCCCTCAGA	CCAGGTGTCATTCACCATCTTG	XM_014191309
Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1 (HCN2b1)	CGCCAGTACCAGGAGAAGTACA	AGCTGGCAGTTTGTGGAAAGA	XM_014148806

Hyperpolarisation-activated cyclic nucleotide-gated channel 2b1/2 (HCN2b1/2)	CCAGTGCAGTGATGCGTATCTT	ACAGCCATCCCAGTGACACA	AY148882
Hyperpolarisation-activated cyclic nucleotide-gated channel 3 (HCN3)	ACGGACGTATGGCTGACTATCA	CCGGAAACATGGCATAGCA	XM_014176849
Hyperpolarisation-activated cyclic nucleotide-gated channel 4a1/2 (HCN4a1/2)	TGGGAGGAGATCTTCCATATGAC	CAGGTTGACGATACGCACCAT	XM_014175391 and XM_014126754.1
Hyperpolarisation-activated cyclic nucleotide-gated channel 4b (HCN4b)	CGGGCGCTGAGAATCGT	CGGAGCAACCTCAACAAACTC	XM_014170228
L-type calcium voltage-gated channels subunit α1D ($Ca_v1.3$)	CGGCAAGTCGCCCAAGT	GCGGAGCGTGCTCGTAGTAG	NM_001124328
L-type calcium voltage-gated channels subunit β2 ($Ca_vβ2$)	TGACATAGATGCCACAGGCTTAGA	GAGCGGAGGTGGACTGGAA	DQ198264
Calsequestrin	CCAACCCTACATCAAATTCTTTGC	TCATTTTCAGGGTCAGCTCCTT	NM_001160499
Sodium-calcium exchanger (NCX)	GCAATGCCGTCAACGTCTT	GGTAGATGGCAGCGATGGA	NM_001124598
Ryanodine receptor 3 (RYR3)	AGGCTTCCTCGGCTTTCAC	TGTCGGAAGTTGGAGATCTTCTT	EF032937
Sarcoplasmic/ endoplasmic reticulum calcium ATPase 2 (SERCA2)	GTGCTCGTCACGATAGAGATGTG	CAGCAGGGACTGGTTCTCTGA	Primers designed off sequences from Korajoki and Vornanen (2012)
S100 calcium binding protein (S100)	GTCAAGACTGGAGGCTCAGAG	GATCAAGCCCCAGAAGTGTTTG	Jeffries et al., 2014
Transient receptor potential cation channel subfamily C member 1 (TRPC1)	GAAGCGGAAGCGTGATGAG	GGTAGCGGTGGACAAGACAAC	NM_001185053
Ether-à-go-go-related gene (ERG)	TCCACGACGCACGAGAAAC	ACACGACTGGTCTGGAAATGAGT	NM_001124676
Potassium voltage-gated channel subfamily J	GCCCCAGAGCCGCTTT	AGAGACATTGATGAACTGCACGTT	DQ435674

member 2 ($K_{ir}2.1$)			
Potassium voltage-gated channel subfamily J member 12 ($K_{ir}2.2$)	CTACGGCTACCGCTGTGTGA	GGACTGGAAGACCACCATGAA	DQ435676
Sodium voltage-gated channel α subunit 4 ($Na_v1.4$)	TGTGCTCCGAGCCCTTAAA	CGCCCACAATGGTTTTCAG	EF203231
Sodium voltage-gated channel α subunit 5 ($Na_v1.5$)	TGCCACCCCTGCTGGTA	CGTGGACTGCTTTCCCAGAT	EF203232
Sodium voltage-gated channel α subunit 8 ($Na_v1.6$)	CGACACCTTCACCTGCAACA	CATTGGTGACGAAGTCGTTCA	EF203233
Sodium/potassium ATPase subunit α1a (NKA α1a)	CCTTGGATGAGCTTAACAGGAAA	GCCCGAACCGAGGATAGAC	AY319391
Sodium/potassium ATPase subunit α1b (NKA α1b)	CCCATGGATTTGCTGGGTAT	CCTCCATGTCGTTCATTATCTTGT	AY319390
Sodium/potassium ATPase subunit α1c (NKA α1c)	TGCCAGAGGTATTGTCATCAACAC	GAGGCGAGGGTAGCAATACG	AY319389
Sodium/potassium ATPase subunit α3 (NKA α3)	GCCAGCAATGGACATATGAACA	ACTGACGAAGAAGGCTGTGTGA	AY319388
a_{1A} adrenoceptor	TGGGCTCTGTCTGTCACAATCT	AGGCGCCGGCTCCTT	NM_001124653
a_{1B} adrenoceptor	ACCGAAGAACCGGGCTATG	CATAAGCGGCAGGTAGAAGGA	NM_001124650
$в_2$ adrenoceptor	GCCTAAGCCCCAAGGACAAG	AGCTCCACGGTCCCAACAT	NM_001124440
ANP precursor	TCTGCTCCTGCTTTGTCAACA	AGGGTATGGTCTGCCCAACA	NM_001124211
Collagen type 1a (Col1α1)	CCCGAGCCATGCCAGAT	CAGATAACTTCGTCGCACATGAC	NM_001124177
Connexin 43 (Cx43)	TGGCAGCACCATCTCCAA	GGGTGTCGTCAGGGAAGTCA	NM_001124563
Transforming growth factor в1 (TGF-в1)	GGGCTGGAAGTGGATCCAT	GGGCCGATGCAGTAGTTAGC	KF870471

ACKNOWLEDGEMENTS

We would like to thank Jonathon Stecyk, University of Alaska, for the kind donation of external standard.

COMPETING INTERESTS

No competing interests compared

AUTHOR CONTRIBUTIONS

R.L.S collected and analysed the heart rate data and designed and tested the qRT-PCR assays at UBC. The Fluidigm qRT-PCR was performed at the Pacific Biological Station, Fisheries and Oceans by S.L, with the assistance of R.L.S, under the supervision of K.M.M. Editorial and technical feedback was received from all authors. The whole study was conducted under the supervision of A.P.F.

FUNDING

This work is supported by the Natural Science and Engineering Research Council (NSERC) Discovery Grant 2015-05059 to A.P.F. A.P.F. holds a Canada Research Chair (Tier I).

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FIGURE LEGENDS

Figure 1 – Changes in intrinsic heart rate over time in response to an acute temperature change, for fish from Experiment 1 (performed in the winter)

Red symbols are for fish acutely transferred from 4°C- to 12°C with intrinsic heart rate measurements being made at 12°C (n=6-9) while blue symbols are for fish acutely transferred from 12°C to 4°C with intrinsic heart rate measurements being made at 4°C (n=4-6).

The data are presented as mean ± SEM. Dissimilar letters are used to indicate statistically significant differences between time points within an experimental group (one-way ANOVA).

Figure 2 – Principal component analysis of mRNA expression of cardiac function genes.

(A) Principal component analysis plot of sinoatrial node, atrial and ventricle mRNA expression of cardiac function genes, in fish acclimated to 4°C for more than 3 weeks from Experiment 1 (n=7). All E-*ΔΔCt* shown in Figure 3 were used in the PCA analysis, and plots were created from PC1 and PC2 which explained 35.9% and 19.4% of variance respectively. 68% confidence limit ellipses were plotted around each tissue type. (B) Principal component analysis plot of mRNA expression of cardiac function genes, in the SAN of fish acclimated to 4°C and 12°C from Experiment 1 (n=7). All E-*ΔΔCt* shown in Figure 6A were used in the PCA analysis, and plots were created from PC1 and PC2 which

explained which explained 24.0% and 22.1% of variance respectively. 68% confidence limit ellipses were plotted around each tissue type. Component Loadings are given in Table S2.

Figure 3 – Sinoatrial node, atrial and ventricle mRNA expression of cardiac function genes in fish acclimated to 4°C for more than 3 weeks from Experiment 1.

Expression ($E^{-\Delta\Delta Ct}$) is normalised first to the geometric mean of the reference genes, *CCDC84* and *SEP15*, and then an inter-run calibrator, and presented as mean ± SEM (n=7). Table shows the statistical comparisons between cardiac tissues for the data displayed in the graph on the right. * is used to indicate statistically significant differences with FDR adjustment (<0.05)

Figure 4 – mRNA expression of cardiac function genes, in the SAN (A), atrium (B), and ventricle (C) of fish acclimated to 4°C and 12°C for more than 3 weeks for fish from Experiment 1.

Expression ($E^{-\Delta\Delta Ct}$) is normalised to the geometric mean of the reference genes, *CCDC84* and *SEP15*, then an inter-run calibrator and presented as mean ± SEM (n=7 except in those examples specified in the methods) * is used to indicate statistically significant differences with FDR adjustment (<0.05), and (*) is used to indicate statistically significant differences without FDR adjustment (<0.05) between 4°C and 12°C acclimations (Student's t test or Mann-Whitney test)

Figure 5 – mRNA expression of cardiac function genes, in the SAN (A), atrium (B) and ventricle (C) of fish acclimated to 4°C and 12°C for more than 3 weeks from Experiment 2.

Expression ($E^{-\Delta\Delta Ct}$) is normalised to the geometric mean of the reference genes, *CCDC84* and *SEP15*, then an inter-run calibrator and presented as mean ± SEM (n=7) * is used to indicate statistically significant differences with FDR adjustment (<0.05), and (*) is used to indicate statistically significant differences without FDR adjustment (<0.05) between 4°C and 12°C acclimations (Student's t test or Mann-Whitney test).

Figure 6 - Venn Diagrams showing mRNA expression changes after more than 3 weeks of warm acclimation that occur only in fish that reset intrinsic heart rate (Experiment 1), that only occur in fish that don't reset intrinsic heart rate (Experiment 2) and in both groups of fish.

This figure combines data from Fig. 4, Fig. 5 and Fig. S4.

Figure 7 – mRNA expression of cardiac function genes, in the SAN (A), atrium (B) and ventricle (C) of fish acclimated to 4°C and 12°C for 1 hour with an intrinsic heart rate resetting response.

Expression ($E^{-\Delta\Delta Ct}$) is normalised to the geometric mean of the reference genes, *CCDC84* and *SEP15*, then an inter-run calibrator and presented as mean ± SEM (n=7) * is used to indicate statistically significant differences with FDR adjustment (<0.05), and (*) is used to indicate statistically significant differences without FDR adjustment (<0.05) between 4°C and 12°C acclimations (Student's t test or Mann-Whitney test).

TABLES

Table 1 - Intrinsic heart rate measurements of fish both with and without a pacemaker intrinsic heart rate resetting response, at a variety of temperature acclimations, time points and temperatures.

Fish Group	Temperature Treatment	Saline Measurement Temperature (°C)	Time at Treatment Temperature	Intrinsic Heart Rate	s.e.m.	N
Pacemaker Rate Reset (Group 1)	4°C	4	6 weeks	36.1	0.9	4
	Acute change from 12°C to 4°C	4	0 h (also >3 weeks (3-4 weeks) acclimation to 12°C measured at 4°C)	33.4	0.7	6
			1 h	31.8	0.9	4
			8 h	37.7	1.2	7
			24 h	37.7	1.2	6
	Acute change from 4°C to 12°C	12	0 h (also >3 weeks (4-6 weeks) acclimation to 4°C measured at 12°C)	56.8	1.2	8
			1 h	50.8	1.5	7
			8 h	52.0	1.0	10
			24 h	50.3	1.5	6
	4°C to 4°C	12	1 h	57.9	1.9	8

Pacemaker Rate not Reset (Group 2)	Acute change from 12°C to 4°C	4	0 h	30.6	1.7	4
			24 h	29.2	0.6	8
	Acute change from 4°C to 12°C	12	0 h	52.7	2.2	5
			24 h	57.5	2.7	8
	4°C	4	10-12 weeks	29.9	1.1	7
	12°C	4	10-12 weeks	29.4	1.1	7
	4°C	12	10-12 weeks	53.3	2.8	8
	12°C	12	10-12 weeks	52.5	2.3	8

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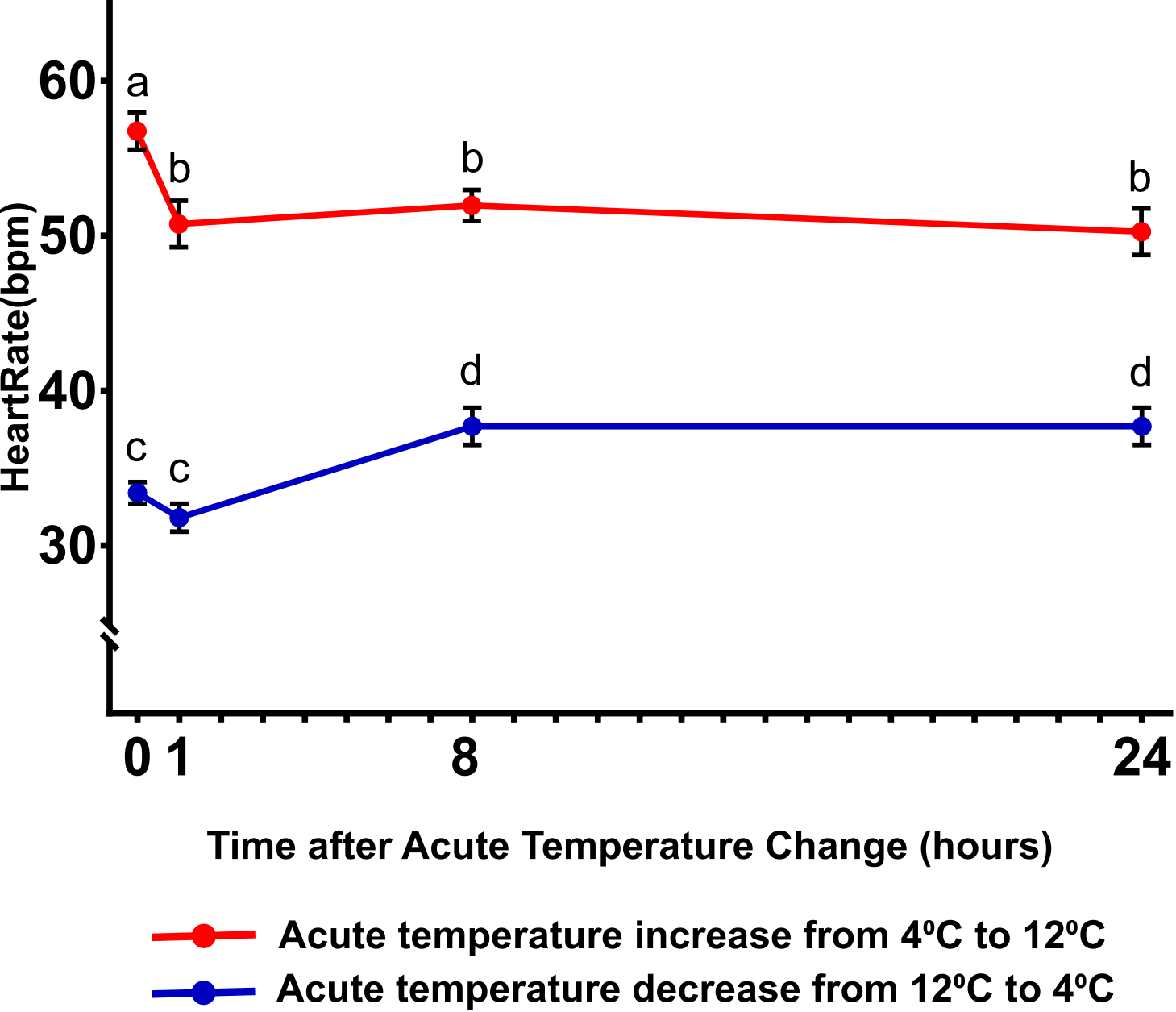
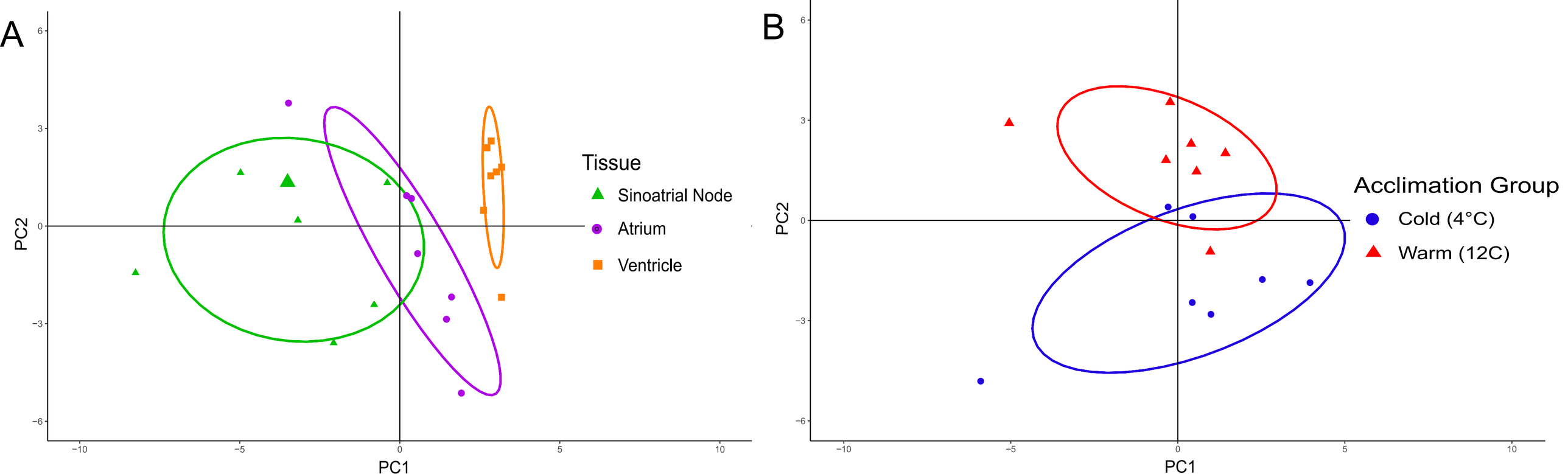
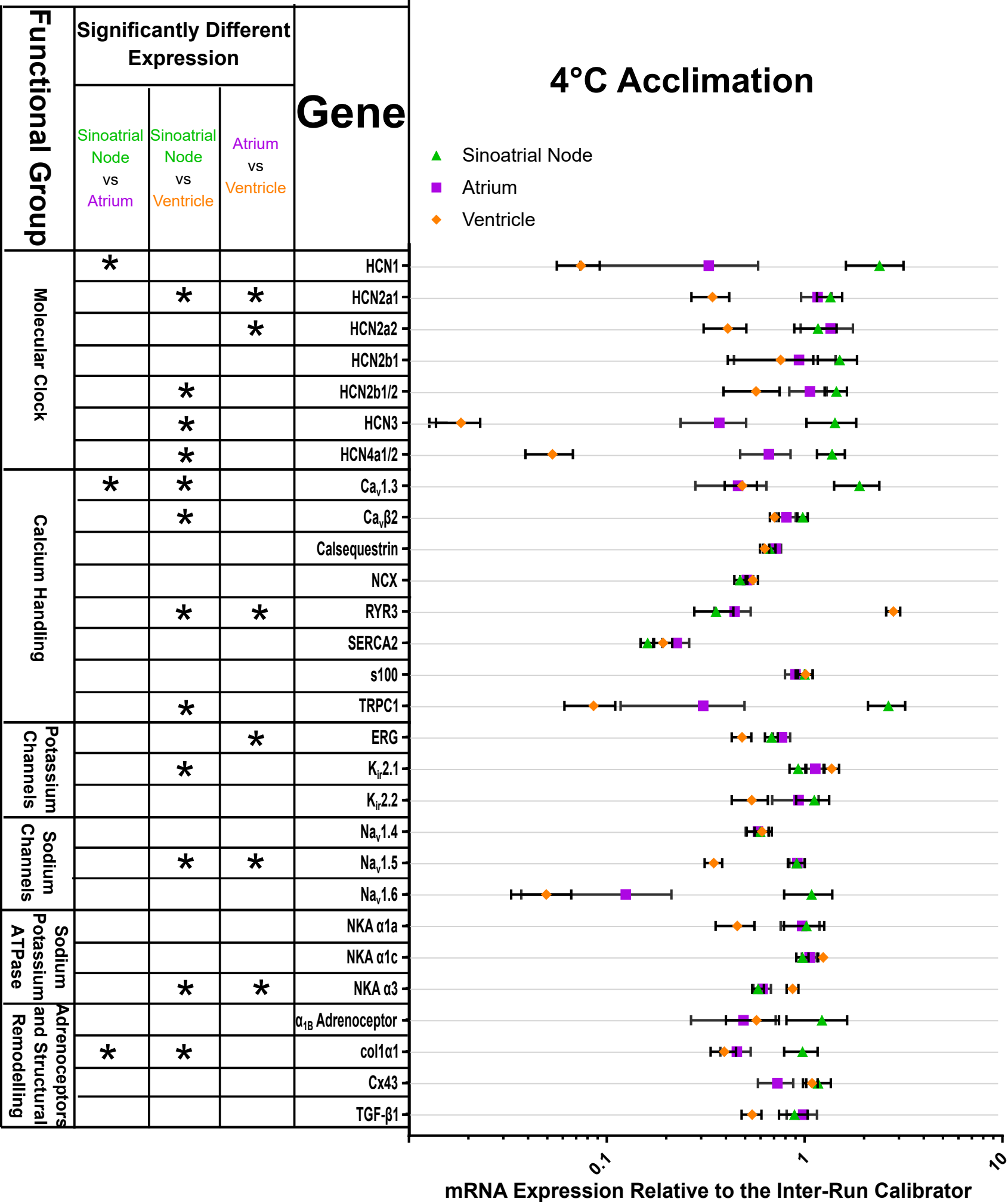
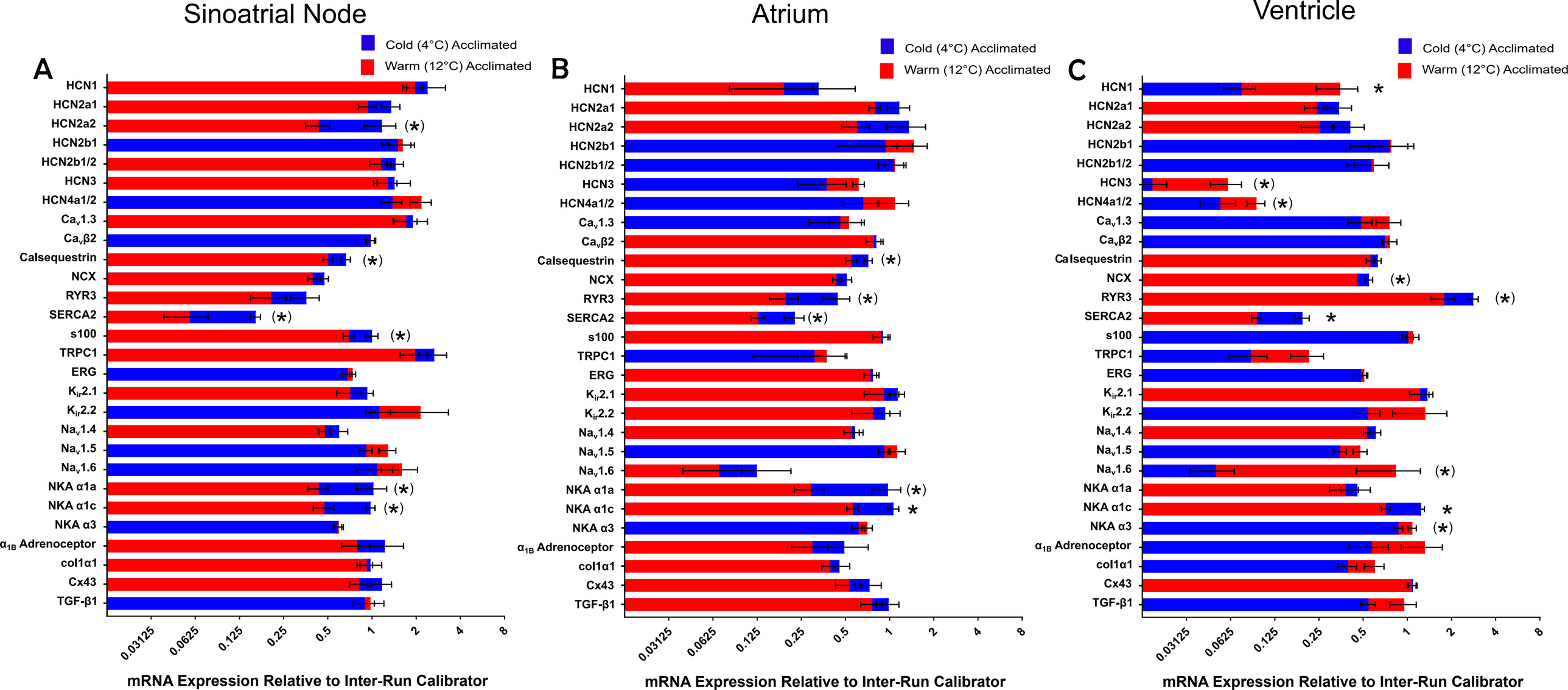

60
50
40
30
HeartRate(bpm)
a
b
b
b
d
d
c
c
0 1
8
24
Time after Acute Temperature Change (hours)
Acute temperature increase from 4ºC to 12ºC
Acute temperature decrease from 12ºC to 4ºC


A
B
PC1
PC2
Tissue
Sinoatrial Node
Atrium
Ventricle
Acclimation Group
Cold (4°C)
Warm (12C)


Functional Group
Significantly Different Expression
Sinoatrial Node vs Atrium
Sinoatrial Node vs Ventricle
Atrium vs Ventricle
Gene
Molecular Clock
HCN1
HCN2a1
HCN2a2
HCN2b1
HCN2b1/2
HCN3
HCN4a1/2
Calcium Handling
Cav1.3
Cavβ2
Calsequestrin
NCX
RYR3
SERCA2
s100
TRPC1
Potassium Channels
ERG
Kir2.1
Kir2.2
Sodium Channels
Nav1.4
Nav1.5
Nav1.6
Sodium Potassium ATPase
NKA α1a
NKA α1c
NKA α3
Adrenoceptors and Structural Remodelling
α1B Adrenoceptor
col1α1
Cx43
TGF-β1
4°C Acclimation
Sinoatrial Node
Atrium
Ventricle
0.1
1
10
mRNA Expression Relative to the Inter-Run Calibrator


Sinoatrial Node
Atrium
Ventricle
A
B
C
Cold (4°C) Acclimated
Warm (12°C) Acclimated
HCN1
HCN2a1
HCN2a2
HCN2b1
HCN2b1/2
HCN3
HCN4a1/2
Cav1.3
Cavβ2
Calsequestrin
NCX
RYR3
SERCA2
s100
TRPC1
ERG
Kir2.1
Kir2.2
Nav1.4
Nav1.5
Nav1.6
NKA α1a
NKA α1c
NKA α3
α1B Adrenoceptor
col1α1
Cx43
TGF-β1
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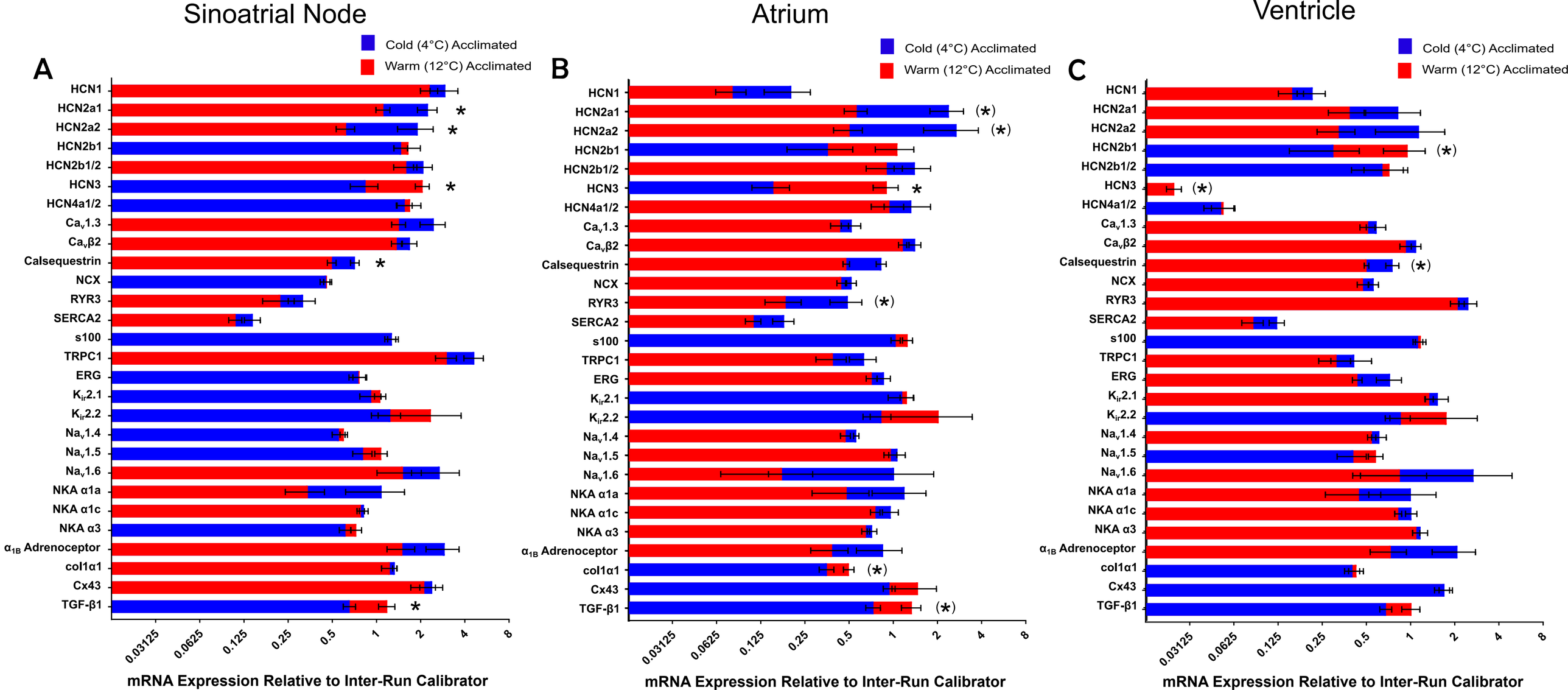
0.0625
0.125
0.25
0.5
1
2
4
8
mRNA Expression Relative to Inter-Run Calibrator


Sinoatrial Node
Atrium
Ventricle
A
B
C
Cold (4°C) Acclimated
Warm (12°C) Acclimated
HCN1
HCN2a1
HCN2a2
HCN2b1
HCN2b1/2
HCN3
HCN4a1/2
Cav1.3
Cavβ2
Calsequestrin
NCX
RYR3
SERCA2
s100
TRPC1
ERG
Kir2.1
Kir2.2
Nav1.4
Nav1.5
Nav1.6
NKA α1a
NKA α1c
NKA α3
α1B Adrenoceptor
col1α1
Cx43
TGF-β1
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0.0625
0.125
0.25
0.5
1
2
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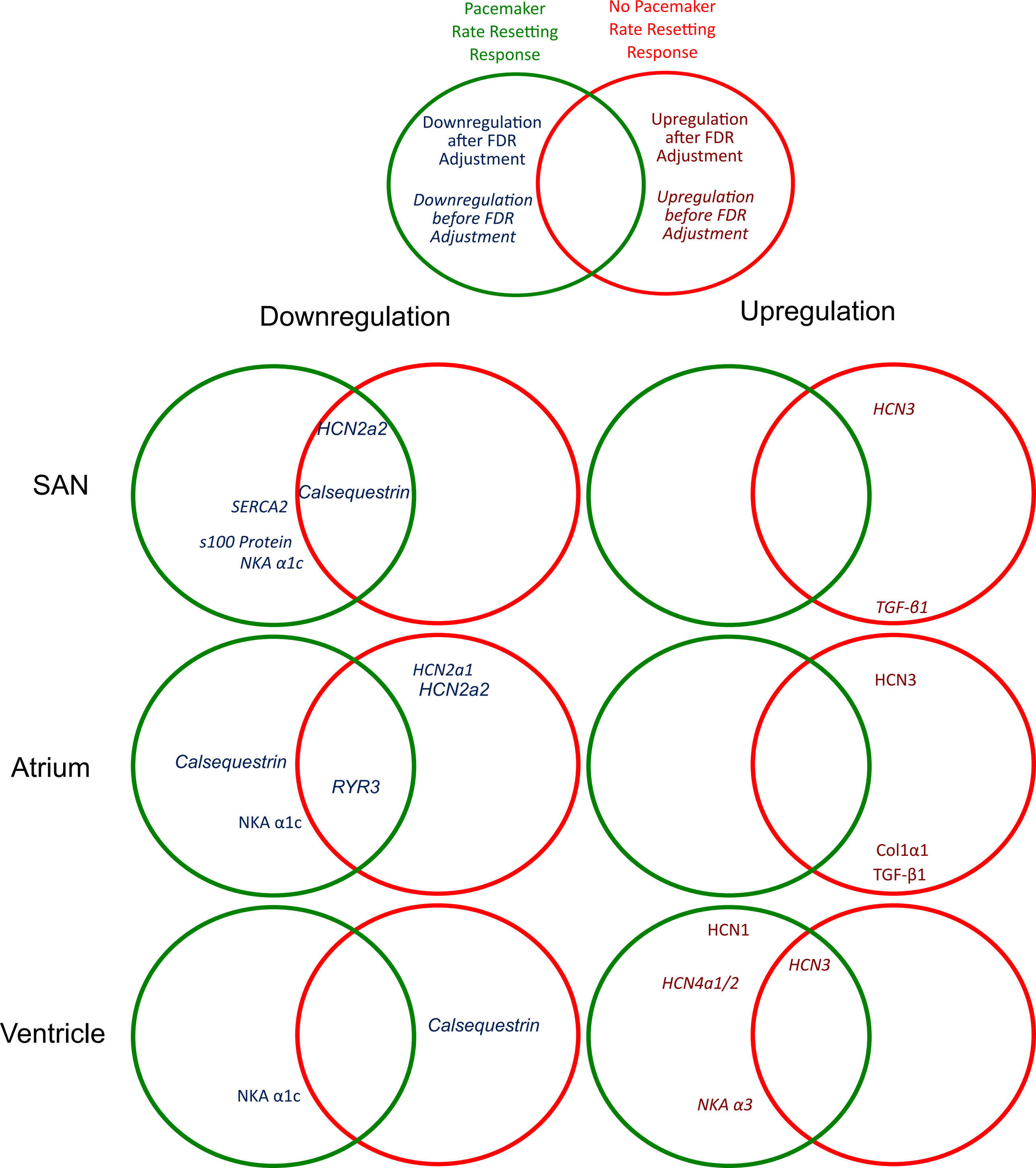
8
mRNA Expression Relative to Inter-Run Calibrator


Pacemaker
Rate Resetting
Response
No Pacemaker
Rate Resetting
Response
Downregulation
after FDR
Adjustment
Downregulation
before FDR
Adjustment
Upregulation
after FDR
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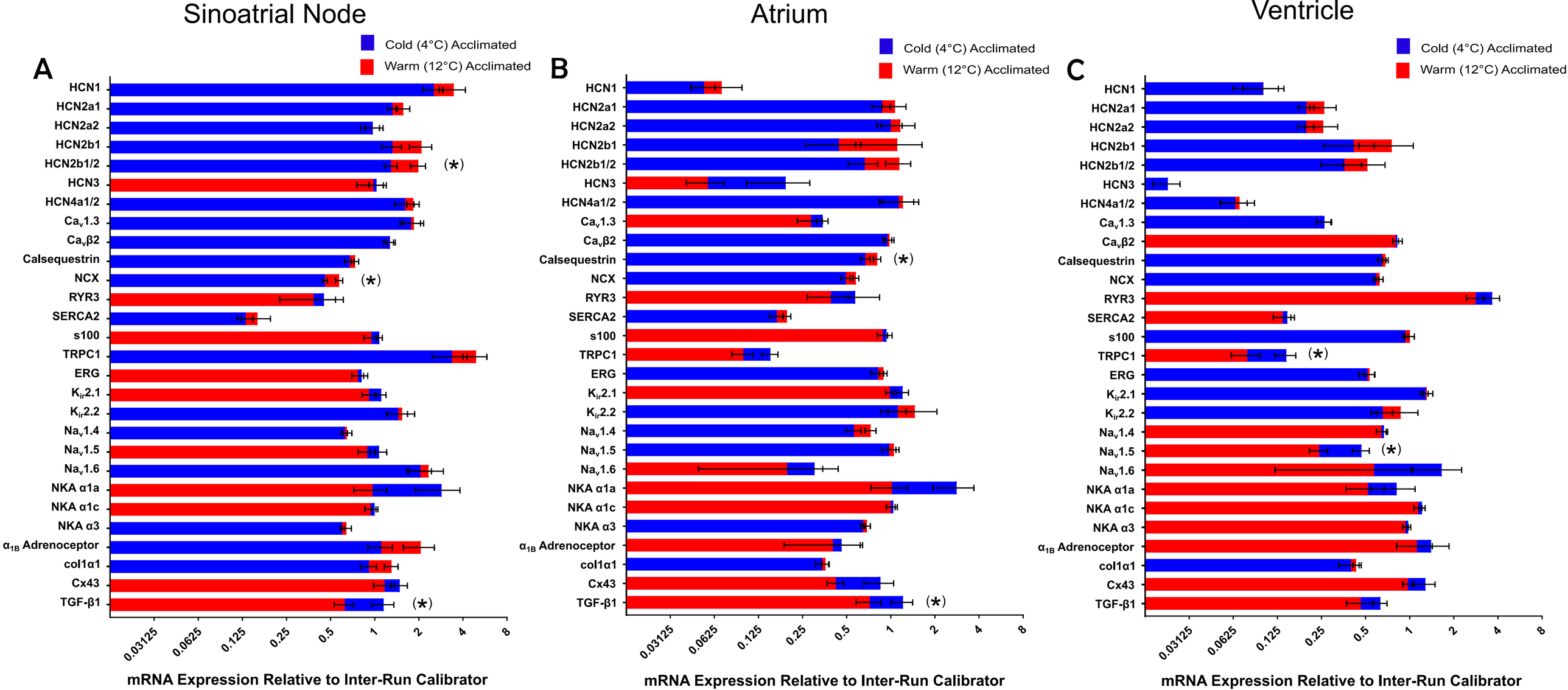
Upregulation
before FDR
Adjustment
Downregulation
Upregulation
SAN
HCN2a2
Calsequestrin
SERCA2
s100 Protein
NKA α1c
HCN3
TGF-β1
Atrium
HCN2a1
HCN2a2
Calsequestrin
RYR3
NKA α1c
HCN3
Col1α1
TGF-β1
Ventricle
Calsequestrin
NKA α1c
HCN1
HCN3
HCN4a1/2
NKA α3


Sinoatrial Node
Atrium
Ventricle
A
B
C
Cold (4°C) Acclimated
Warm (12°C) Acclimated
HCN1
HCN2a1
HCN2a2
HCN2b1
HCN2b1/2
HCN3
HCN4a1/2
Cav1.3
Cavβ2
Calsequestrin
NCX
RYR3
SERCA2
s100
TRPC1
ERG
Kir2.1
Kir2.2
Nav1.4
Nav1.5
Nav1.6
NKA α1a
NKA α1c
NKA α3
α1B Adrenoceptor
col1α1
Cx43
TGF-β1
(*)
0.03125
0.0625
0.125
0.25
0.5
1
2
4
8
mRNA Expression Relative to Inter-Run Calibrator