Zebrafish as a Model of Mammalian Cardiac Function: Optically Mapping the Interplay of Temperature and Rate on Voltage and Calcium Dynamics

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Abstract:

The zebrafish (*Danio rerio*) heart is a viable model of mammalian cardiovascular function due to similarities in heart rate, ultrastructure, and action potential morphology. Zebrafish are able to tolerate a wide range of naturally occurring temperatures through altering chronotropic and inotropic properties of the heart. Optical mapping of cannulated zebrafish hearts can be used to assess the effect of temperature on excitation-contraction (EC) coupling and to explore the mechanisms underlying voltage (V_m) and calcium (Ca^{2+}) transients.

Applicability of zebrafish as a model of mammalian cardiac physiology should be understood in the context of numerous subtleties in structure, ion channel expression, and Ca^{2+} handling. In contrast to mammalian systems, Ca^{2+} release from the sarcoplasmic reticulum (SR) plays a relatively small role in activating the contractile apparatus in teleosts, which may contribute to differences in restitution. The contractile function of the zebrafish heart is closely tied to extracellular Ca^{2+} which enters cardiomyocytes through L-type Ca^{2+} channel (LTCC), T-type Ca^{2+} channel (TTCC), and the sodium-calcium exchanger (NCX).

Novel data found that despite large temperature effects on heart rate, V_m, and Ca^{2+} durations, the relationship between V_m and Ca^{2+} signals was only minimally altered in the face of acute temperature change. This suggests that zebrafish V_m and Ca^{2+} interactive kinetics are largely rate-independent. In comparison to mammalian systems, zebrafish Ca^{2+} cycling is inherently more dependent on transsarcolemmal Ca^{2+} transport and less reliant on SR Ca^{2+} release. However, the compensatory actions of various components of the Ca^{2+} cycling machinery of the zebrafish cardiomyocytes, allow for effective maintenance of EC coupling over a wide range of environmental temperatures.

Keywords: EC coupling, Cardiovascular function, Action potential, Atria, Ventricles, Phase-plot, RH-237, Rhod-2 AM, Electrical stimulation

Introduction

Zebrafish hearts operate over a similar range of heart rates (HRs) and have similar action potential (AP) characteristics to mammalian hearts (Hove-Madsen, Llach et al. 1998, Gillis and Tibbits 2002, Milan, Jones et al. 2006, Arnaout, Ferrer et al. 2007, Leong, Skinner et al. 2010, Nemtsas, Wettwer et al. 2010, Llach, Molina et al. 2011) (Bovo, Dvornikov et al. 2013, Skarsfeldt, Bomholtz et al. 2018). However, the zebrafish system is relatively simple and contains all the essentials for functioning while maintaining the flexibility needed to adjust to myriad environmental stressors, such as temperature (6-38 °C)(Spence, Gerlach et al. 2008).

The availability of transgenic lines and genome editing techniques has made the zebrafish heart a popular and powerful tool to study numerous aspects of the cardiovascular system (Howe, Bradford et al. 2013). Preceding the use of small interfering RNA (siRNA) and short hairpin RNA (shRNA) technology in mammalian models, morpholino oligonucleotides were used as a cost- and time-efficient means to create anti-sense knockdown mutations in zebrafish (Nasevicius and Ekker 2000, Bill, Petzold et al. 2009). Susceptibility to off-target mutations has seen this technique largely replaced by a clustered regularly interspaced palindromic repeats (CRISPRs) approach to perform knock-out and knock-in mutations in zebrafish (Leong, Skinner et al. 2010, Shah, Davey et al. 2015, Zhang, Qin et al. 2017).

Optical mapping can be used to visualize the transients which underlie contractile events, in so doing, this technique provides a relative measurement of the voltage (V_m) and calcium (Ca^{2+}) (Efimov, Nikolski et al. 2004, Herron, Lee et al. 2012). New optogenetic sensors are also available and can now be used in conjunction with optical mapping to provide a stable, high quality signal that can be used to measure these parameters in absolute terms (Looger 2011, Rose, Goltstein et al. 2014, Cohen 2016).

Due to shared characteristics, zebrafish hearts can be used to recapitulate features of human cardiac arrhythmias and clinically relevant channelopathies, such as various forms of long QT syndrome (Sanguinetti and Tristani-Firouzi 2006, Arnaout, Ferrer et al. 2007, Mittelstadt, Hemenway et al. 2008, Bakkers 2011). Advantages must be considered in light of differences which exist; thus understanding the zebrafish and mammalian cardiovascular systems and the underlying molecular correlates is an essential precursor to using zebrafish as a model.

Structure of the zebrafish heart

The zebrafish heart is composed of one atrium and one ventricle connected in series (Hu, Sedmera et al. 2000, Santoriello and Zon 2012). Blood from the systemic circulation enters the sinus venosus and then passively moves into the atrium, where atrial contraction is the primary force propelling blood into the ventricle. Ventricular contraction then pumps the blood to the bulbous arteriosus where pulsatility is dampened prior to entering the gills for oxygenation (Cotter, Han et al. 2008).

Cardiomyocytes are the individual cells of the heart that are electrically and physically coupled to create a functional syncytium and allow for efficient cardiac contraction (Cobb 1974). Intercalated discs and adhesion junctions create a physical coupling between adjacent cells (Bers 2002). The connexin proteins which compose gap junctions are critical for the electrical coupling between cardiomyocytes. The zebrafish genome contains orthologs of known mammalian connexins Cx43 and Cx45 (Essner, Laing et al. 1996); zebrafish and mammalian connexins have a high degree of sequence homology (Christie, Mui et al. 2004, Lafontant, Behzad et al. 2013). While adult mammalian cardiomyocytes are typically rod-like in shape and measure ~120 x 20 μm in dimension (Li, Stevens et al. 2013), zebrafish cardiomyocytes resemble neonatal mammalian cells whereby they are spindle-shaped (~100-180 μm) and thin (~5-15 μm) (Zhang, Llach et al. 2011).

Cardiomyocytes contain parallel myofibrils which are made up of repeating sarcomeric units. Sarcomeres are comprised of interacting thick and thin myofilaments. The thick filament is composed primarily of: α- and β-myosin heavy chains, myosin light chains, myosin binding protein C, and titin. The thin filament is composed of: the double stranded actin helix, coiled-coil tropomyosin dimer, and the troponin (Tn) complex. The sarcomere is similar in structure and resting length between mice (~2 μm) (Iorga, Blaudeck et al. 2007), zebrafish (~1.9 μm) (Iorga, Neacsu et al. 2011), as well as all other species studied to date (Patrick, White et al. 2011).

Pacemaker regions

At the core of the differences from small mammals such as the murines and zebrafish is a slower cycling of Ca^{2+} to support contraction in zebrafish and a lower HR (~120 bpm) (Iorga, Neacsu et al. 2011, Tsai, Wu et al. 2011) compared to frequently-employed mouse models (~600 bpm) (Iorga, Neacsu et al. 2011).

Contraction is subsequent to the electrical events of the cardiac cycle initiated in the sinoatrial (SA) node; a ring-like structure between the sinus venosus and the atrium

(Vornanen 2017). Multiple hyperpolarization-activated cyclic nucleotide-dependent channel (HCN) transcripts, including HCN1, HCN2a, and HCN3 are expressed in brown trout hearts where the funny current (I_f) has much lower current density than seen in zebrafish (Hassinen, Haverinen et al. 2017). These channels associated with pacemaker cells in the zebrafish SA node are likely paralogs of the mammalian (HCN4) which passes the I_f responsible, at least in part, for the rhythmic depolarizations of mammalian pacemaker cells (Tessadori, van Weerd et al. 2012, Stoyek, Croll et al. 2015, Verkerk and Wilders 2015). To date, no other HCN channel variants have been isolated from zebrafish myocytes.

I_f has been recorded in both the atrial and ventricular cardiomyocytes of zebrafish with higher density in the former (Baker, Warren et al. 1997, Warren, Baker et al. 2001). Interestingly, blockage of the rapid delayed rectifier potassium current (I_{kr}) and the slow delayed rectifier potassium current (I_{ks}) causes bradycardia in zebrafish (Langheinrich, Vacun et al. 2003, Tsai, Wu et al. 2011, Stengel, Rivera-Milla et al. 2012). Therefore it is likely that these channels also play a role in the pace-making function of the heart, as reviewed elsewhere (Tessadori, van Weerd et al. 2012).

Ionic currents

AP morphology is determined by a complex sequence of activation and inactivation of channels and transporters carrying sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), and chloride (Cl^-) ions across the sarcolemma. The zebrafish AP bears distinct similarities to the human cardiac cycle arising from the actions of underlying ionic currents. Initially, voltage-gated Na^+ channels depolarize the cell membrane. Voltage-gated Ca^{2+} channels then prolong the plateau phase of the AP. Finally, the combined effect of the delayed rectifying K^+ channels which conduct I_{Ks} and I_{Kr} helps to repolarize the cell membrane back towards the baseline.

The inward rectifier current (I_{K1}) maintains a resting membrane potential of approximately -90 mV in mammals (phase 4), but is thought to be less dense in zebrafish ventricles where the resting membrane potential is closer to -70 mV (Nemtsas, Wettwer et al. 2010, Hassinen, Haverinen et al. 2015). The functional divergence of the orthologs which underlie the channels responsible for this current in zebrafish (mainly Kir2.2a and Kir2.4) and mammals (Kir2.1, Kir2.2, Kir2.3) necessitates careful consideration when studying I_{K1} (Hassinen, Haverinen et al. 2015). Also active during this phase of the AP are the Na^+-Ca^{2+} exchanger (NCX) and the Na^+/K^+ ATPase (Santana, Cheng et al. 2010).

During the upstroke of the AP (phase 0), inward Na^+ current (I_{Na}) depolarizes the membrane from -90 to ~+20 mV in mammals (and + 30 mV in zebrafish) (Asimaki, Kapoor et al. 2014). The zebrafish ventricular I_{Na} shows more negative V_m dependence of inactivation and slower upstroke velocity than the mammalian variant, possibly due to lower current density (Warren, Baker et al. 2001, Nemtsas, Wettwer et al. 2010). Density of the I_{Na}, however, is not different when comparing zebrafish atrial and ventricular myocytes (Haverinen and Vornanen 2006).

$Na_V1.4$ and $Na_V1.5$ are expressed in both mammalian and zebrafish cardiomyocytes (Novak, Taylor et al. 2006). The orthologs (*SCN5Laa* and *SCN5Lab*) underlying the $Na_V1.5$ channel in the zebrafish heart have ~60% sequence homology with the human *SCN5A* gene and produce the corresponding current (Novak, Taylor et al. 2006, Chopra, Stroud et al. 2010). Zebrafish $Na_V1.5$ bears similarities to mammalian skeletal ($Na_V1.4$) and neonatal splice variants of the $Na_V1.5$ channels (Haverinen, Hassinen et al. 2007). The SCN4aa/SCN4ab which underlie $Na_V1.4$ current in the zebrafish show variable expression patterns in the tissues of the developing embryo and is only observed in the cardiomyocytes of adult zebrafish, as has been described in detail elsewhere (Novak, Taylor et al. 2006). Further, the paralog of $Na_V1.5$ expressed in fish is ~1000 times more sensitive to tetrodotoxin (TTX) than mammalian variants (Gershome, Lin et al. 2011, Vornanen, Hassinen et al. 2011). Changes in the TTX binding site (Haverinen, Hassinen et al. 2007) and variable expression of multiple accessory subunits in zebrafish likely underlie this difference (Chopra, Watanabe et al. 2007).

The transient outward current (I_{to}) passed by $K_V1.4$ and $K_V4.3$ functions primarily during the rapid repolarization (phase 1) of the AP in mammalian cells but has not been found to play a major role in the zebrafish heart (Amin, Tan et al. 2010, Nemtsas, Wettwer et al. 2010). Increased T-type Ca^{2+} currents and limited I_{to} are the likely explain the much-reduced phase 1 of repolarization in zebrafish cardiomyocytes (Chopra, Stroud et al. 2010, Verkerk and Remme 2012).

Phase 2 is brought about by I_{Ca} current which cases a plateau in the AP; a feature that is absent in mice but observed in humans and zebrafish (London 2001, Sidi, Busch-Nentwich et al. 2004, Leong, Skinner et al. 2010, Lin, Ribeiro et al. 2014) .

Repolarization (phase 3) of the AP is characterized by opening of the K^+ channels which results in K^+ efflux. I_{Kr} is passed by the product of the human ether-a-go-go related gene (hERG) ($K_V11.1$) and plays a key role in mammalian late repolarization. *KCNH2* underlies this current in the mammalian heart (Leong, Skinner et al. 2010). The zebrafish equivalent of this gene (zERG) shows similar channel properties (Scholz,

Niemer et al. 2009). An ortholog of the mammalian hERG 1 (*KCNH6*), normally found in the brain of mammals is likely the gene which underlies I_{Kr} in zebrafish. However, three other gene products (*KCNH2A*, *KCNH2B*, and *KCNH7*) are also expressed in the zebrafish heart and contribute to I_{Kr} (Leong, Skinner et al. 2010, Vornanen and Hassinen 2016).

Action potential duration (APD) and QT interval are prolonged in zebrafish blockage and knockdown models of the I_{Kr} (Langheinrich, Vacun et al. 2003, Milan, Peterson et al. 2003, Arnaout, Ferrer et al. 2007). Drug-induced QT prolongation does not result in severe arrhythmias in fish species which are more tolerant to ventricular tachyarrhythmias (Langheinrich, Vacun et al. 2003), due at least in part to the smaller size and wall thickness of the heart whereby dispersion of the repolarizing wave, a prerequisite for mammalian arrhythmogenesis is absent (Brunet, Aimond et al. 2004).

Repolarization reserve in mammalian hearts is provided by the slow delayed rectifier potassium current (I_{Ks}) which is passed by the *KCNQ1* channel and the *KCNE1* accessory subunit (Amin, Tan et al. 2010). Mammalian I_{Ks} is also produced by the $K_V1.5$ channel (KCNA5) (Nerbonne 2004), and $K_V2.1$ (London 2001, Salama and London 2007); however these channels have not been identified in zebrafish cardiac tissue to date.

Orthologs of the mammalian variants of the $K_V7.1$ channel are expressed in the zebrafish heart (Vandenberg, Perry et al. 2012), where blockage prolongs the APD (Tsai, Wu et al. 2011). There is some discrepancy between measurements made on whole heart vs. isolated cells leading some to suggest that this current may (Tsai, Wu et al. 2011) or may not (Alday, Alonso et al. 2014) be present in adult zebrafish hearts. *KCNQ1*-encoding mRNA point to the presence of I_{Ks} in zebrafish cardiomyocytes (Wu, Sharma et al. 2014), however blockage of this current is hard to resolve given the relatively large I_{Kr} current (Nemtsas, Wettwer et al. 2010, Alday, Alonso et al. 2014).

We have previously presented data demonstrating that blockage of zERG with dofetilide significantly prolongs the AP in a concentration-dependent manner (Genge, Lin et al. 2016). This provides further evidence for the applicability of zebrafish as a model for whole heart pharmacological screens to corroborate work by others (Langheinrich, Vacun et al. 2003, Mittelstadt, Hemenway et al. 2008).

Excitation-contraction coupling and calcium homeostasis

In mammalian cardiomyocytes, an extensive transverse tubule (t-tubule) network carries extracellular Ca^{2+} deep within the cell where the LTCCs open upon membrane

depolarization to allow for inward Ca^{2+} flow. T-type Ca^{2+} channels (TTCC) ($Ca_V3.1$) feature more prominently in immature mammalian cells and pacemaker regions (Zhang, Cui et al. 2002, Bers 2008, Mesirca, Torrente et al. 2014). These channels contribute to the rapidly (~100 ms) elevation of cytosolic Ca^{2+} in cardiomyocytes (Yue 1987). The time constant for recovery from inactivation of LTCCs is ~100-200 ms (Bers 2008). This is consistent with the recovery time of $I_{Ca,L}$ in zebrafish cardiomyocytes ($T_{1/2}$= 96 ms) (Zhang, Llach et al. 2011). In the cytosol, [Ca^{2+}] fluctuates between diastole (~100 nM) and systole (~1 μM) (Bers 2001) .

Activation of the calcium transient and contraction

Voltage-dependent calcium channels: The LTCC is a protein complex composed of multiple subunits (Takahashi and Momiyama 1993). The largest of these is the 190-250 kDa α_1-subunit that includes the pore, voltage sensor, gate, and many regulatory sites. This subunit is formed by 4 domains (I-IV), each of which includes 6 transmembrane segments (S1-S6) (Hell, Westenbroek et al. 1993). S4 is the voltage sensing domain and the loop between the S5 and S6 segments of each domain determines pore selectivity (Catterall, Perez-Reyes et al. 2005). Both the α_{1C}-subunit (also known as $Ca_V1.2$) expressed in the human heart, as well as the α_{1D}-subunit ($Ca_V1.3$) have been identified in the zebrafish heart (Rottbauer, Baker et al. 2001, Sidi, Busch-Nentwich et al. 2004).

The β-subunit plays a role in voltage-dependence of activation of the channel in a way that is highly dependent on the specific variant expressed and its interaction with the α_1-subunit (Zhou, Horstick et al. 2008). Two orthologous genes express the equivalent of the human β-subunit and are essential for the normal function of zebrafish muscle (Schredelseker, Di Biase et al. 2005). In addition to these components, the $\alpha_2\delta$-subunit also play a key role in controlling trafficking and localization of the channel Given the interaction of these numerous proteins to form the LTCC, this channel can vary significantly depending on the tissue expression pattern in zebrafish in ways that are still under investigation (Zhou, Saint-Amant et al. 2006).

The $Ca_V1.2$ channel passes $I_{Ca,L}$ in human heart tissue and plays a significant role in the plateau phase of the zebrafish cardiac AP and EC coupling (Rottbauer, Baker et al. 2001, Nerbonne and Kass 2005). $Ca_V1.3$ is also expressed, mostly in the nodal and atrial tissue of both the zebrafish and the mammalian heart along with other modifying subunits which are still under investigation (Sidi, Busch-Nentwich et al. 2004, Zhou, Horstick et al. 2008, Mesirca, Torrente et al. 2015). $I_{Ca,L}$ is more prominent in zebrafish ventricles and explains the mammalian-like morphology of the AP plateau (Nemtsas, Wettwer et al. 2010).

The I-V relationship for the $I_{Ca,L}$ is very similar for zebrafish and mammals such as guinea pigs and rabbits (Huang, Hove-Madsen et al. 2008, Zhang, Llach et al. 2011). Calcium-dependent inactivation (CDI) of the zebrafish $I_{Ca,L}$ mirrors that seen in all mammalian $Ca_V1.2$ channels, which is to be expected from numerous shared structural homologies and functional motifs (Yuan, Ginsburg et al. 1996, Zhang, Llach et al. 2011). Moreover, specific sequence variations such as the *island beat* (*isl*) mutation in $Ca_V1.2$ abolish $I_{Ca,L}$ in zebrafish myocytes (Rottbauer, Baker et al. 2001). Even amongst ectotherms, there are variances at basal HRs with an $I_{Ca,L}$ density in zebrafish ventricular myocytes three times that seen in trout and five times that of human cardiomyocytes (Vornanen 1997, Hove-Madsen, Prat-Vidal et al. 2006, Zhang, Llach et al. 2011).

Despite a greater contribution by the LTCC, the TTCC also plays a prominent role in the chambers of the zebrafish heart (Nemtsas, Wettwer et al. 2010, Alday, Alonso et al. 2014). The amount of Ca^{2+} entering through the LTCC/TTCC varies among teleost species and tissue ranging from as little as 2.9 pC (15 amol Ca^{2+}) in trout ventricular myocytes (Hove-Madsen, Llach et al. 1998) or 3.8-4.5 pC (19-23 amol Ca^{2+}) in trout atrial myocytes (Hove-Madsen and Tort 1998) to as much as 7.8 pC (40 amol) in carp (Vornanen 1997) and 12 pC (63 amol) in zebrafish ventricular tissue (Zhang, Llach et al. 2011), giving rise to a total increase in the non-mitochondrial cytosolic [Ca^{2+}] of 17, 22-26, 40 and 71 μM in trout atria, trout ventricular, carp, and zebrafish ventricles respectively. These amounts of Ca^{2+} are insufficient to activate a full Ca^{2+} transient (CaT) in trout while Ca^{2+} entry through LTCC in zebrafish approaches the change in total Ca^{2+} during a normal CaT. This however, does not imply that Ca^{2+} entry through LTCC is the sole contributor to the CaT as elevation of cytosolic Ca^{2+} by other mechanisms such as reverse-mode NCX and Ca^{2+}-induced Ca^{2+} release (CICR) may negatively feed-back on LTCC amplitude and duration by speeding up CDI of the LTCC (Hove-Madsen, Llach et al. 1999).

Calcium entry via the sodium-calcium exchanger (NCX1): The NCX1 normally operates in forward mode to remove 1 Ca^{2+} from the cytosol for 3 Na^+ brought into the cell during the relaxation phase of the heart cycle, contributing to a relatively slow decay in the CaT (~400 ms) (Yue 1987, Bridge and Spitzer 1990, Nicoll, Longoni et al. 1990). Incoming Ca^{2+} is buffered intracellularly such that the rise in cytosolic Ca^{2+} levels and subsequent contraction are delayed behind $I_{Ca,L}$ activation. The human NCX1 and zebrafish (NCX1h) isoforms have high sequence similarity (Langenbacher, Dong et al. 2005).

In mammalian cardiomyocytes, normal (or forward) mode NCX activity is favored by hyperpolarized membrane potentials and/or high cytosolic Ca^{2+} with the voltage-dependence of the CaT closely mirroring the bell shaped voltage-dependence of $I_{Ca,L}$ with a peak near 0 mV (Bers 2001). In zebrafish, peak LTCC current occurs at 0 mV (atrium) and +10 mV (ventricles) (Nemtsas, Wettwer et al. 2010).

In zebrafish, LTCC plays a smaller role in driving the CaT, with reverse-mode NCX contributing to increase the amplitude of the CaT and cell shortening at depolarized membrane potentials (Zhang, Llach et al. 2011). Moreover, teleost myocytes are thought, in general, to contain high cytosolic Na^+ (~15 mM) and are known to have greater NCX activity than mammals (Vornanen 1999), properties that allow for passage of greater amounts of current by reverse-mode NCX given the necessary prerequisites (Leblanc 1990, Levesque, Leblanc et al. 1991). In the fish heart, depolarized membrane potentials (at the AP upstroke) are likely accompanied by NCX-mediated Ca^{2+} entry to compensate for reduced $I_{Ca,L}$ activity as evident by the fact that blocking NCX decreases contractility by up to 50% (Hove-Madsen, Llach et al. 2000, Hove-Madsen, Llach et al. 2003, Birkedal and Shiels 2007).

The high degree of colocalization of $Na_V1.4$ with NCX1 in the rabbit cardiomyocyte, has led to the hypothesis that opening of $Na_V1.4$ causes a local increase in cellular [Na^+] which in turn can trigger reverse-mode NCX activity, particularly in the mammalian neonate heart (Gershome, Lin et al. 2011). It is enticing to speculate that a similar phenomenon occurs in zebrafish myocytes.

Sarcoplasmic Reticulum calcium content: The SR is a specialized endoplasmic reticulum which makes up ~10% of the cell volume in mammals (Bers 2001) and about ~6% in fish (Di Maio and Block 2008). While it is generally agreed that Ca^{2+} release from the SR is the main contributor in activating contraction by a process termed CICR, this contribution varies (50-90%) depending on species, tissue, and pathophysiological conditions in mammalian hearts (Bers 2001). Multiple studies on the contribution of the SR in the lower vertebrate heart report highly variable values, which to a large extent represent variability in the experimental conditions, species, and cardiac tissue used in each study. Conclusions from early studies relied strongly on pharmacological manipulation of the RyR2 and reported relatively small contributions of SR Ca^{2+} release to the activation of contraction ranging from 0 - 50% (Driedzic and Gesser 1988, Hove-Madsen and Gesser 1989, Tibbits, Hove-Madsen et al. 1991, Hove-Madsen 1992, Keen, Farrell et al. 1992). Subsequently, quantitative estimates of SR Ca^{2+} content and uptake rates have shown that teleost myocytes have larger Ca^{2+} storage capacities that in many cases surpass that of mammals. Thus, the first measurements of the caffeine-induced NCX currents in trout ventricular myocytes reported a time integral of 65 pC

corresponding to a total of 674 amol of Ca^{2+} released from the SR or a steady-state SR Ca^{2+} content of 750 µmol Ca^{2+}/L non-mitochondrial cell volume, which is 3-10 times larger than values reported in mammals (Hove-Madsen, Llach et al. 1998). Subsequently, steady-state values of 860-1700 amoles has been reported for trout atrial myocytes at room temperature (Hove-Madsen, Llach et al. 1999) and near 1100 amoles (1120 µmol Ca^{2+}/L non-mitochondrial cell volume) across physiological temperatures ranging from 7 - 21°C (Hove-Madsen and Tort 2001). Values for burbot and carp myocytes vary from 225 - 381 µM for burbot ventricle, 488 to 541 µM for carp ventricle, 304-628 µM for burbot atria and 718 - 831 µM for carp atria (Haverinen and Vornanen 2009).

For zebrafish, there are currently no direct measures of the SR Ca^{2+} content. However, the SR Ca^{2+} storage capacity is sufficient to support spontaneous Ca^{2+} release, i.e. sparks in resting ventricular myocytes (Llach, Molina et al. 2011, Bovo, Dvornikov et al. 2013) that are comparable to spark frequencies in trout cardiomyocytes (Llach, Molina et al. 2011, Llach, Molina et al. 2011) and in human atrial myocytes under similar experimental conditions (Llach, Molina et al. 2011).

Ryanodine receptor (RyR) - the Sarcoplasmic Reticulum calcium release channel:
The RyR2 is a large (~2.2 MDa) homotetrameric protein with a transmembrane region and numerous regulatory sites (Sutko and Airey 1996). Ca^{2+} binding to cytosolic sites on the RyR is the primary activator that causes opening to release SR Ca^{2+} into the cytosol (Meissner 2010). The zebrafish RyR2 channel has 85% sequence homology with its rabbit counterpart (Bovo, Dvornikov et al. 2013). Fish RyR2 have been proposed to have lower Ca^{2+} sensitivity than rat isoforms and thus greater cytosolic levels of Ca^{2+} may be required to induce CICR (Vornanen 2006). In zebrafish, this could be due to RyR2 density, which is ~70% lower than in mammalian myocytes. As discussed previously, the geometrical differences in T-tubule structure could also be a determining factor. Moreover, clearance of the SR Ca^{2+} content was found to reduce the cytosolic CaT to ~40% of control values (Bovo, Dvornikov et al. 2013). Coupling between the LTCC and the RyR which exists in dyads at the boundaries of the t-tubule network allows for CICR and these peripheral coupling sites are more tenuously linked in fish species; this has been proposed to decrease the likelihood of CICR in fish and hence decrease the contribution of CICR to the activation of contraction (Di Maio and Block 2008). This notion would be in accordance with the first reports measuring the impact of inhibition of SR Ca^{2+} release on contraction in paced teleost ventricular preparations that showed minor effects of RyR2 inhibition (Driedzic and Gesser 1988). Significant effects were first reported in trout and only at warm temperatures (Hove-Madsen 1992). It should, however, be noted that the estimations of SR Ca^{2+} release based on pharmacological interventions intending to prevent Ca^{2+} release through the RyR2 are

limited by the ability of other Ca^{2+} delivering mechanisms to compensate for the inhibition of Ca^{2+} release from the SR. To circumvent this limitation, specific electrophysiological protocols were designed to quantify the amount of Ca^{2+} released from the SR and revealed that the amount of SR release in trout atrial myocytes was approximately 33 amoles (or 37 µmol Ca^{2+}/L non-mitochondrial cell volume), equivalent to approximately 40% of the total Ca^{2+} required for the activation of contraction at room temperature (Hove-Madsen, Llach et al. 1999). Estimates of SR Ca^{2+} release yielded similar values (near 35 µmol Ca^{2+}/L non-mitochondrial cell volume) at both 7 and 21°C, demonstrating robust SR Ca^{2+} release over the physiological temperature range for trout atrial myocytes. Moreover, pharmacological dissection of the ability of $I_{Ca,L}$ and reverse-mode NCX revealed that both can trigger CICR with reverse-mode NCX contributing significantly at membrane potentials above +30 mV (Hove-Madsen, Llach et al. 2003).

Interestingly, there is a linear relationship between SR Ca^{2+} content and CICR for SR Ca^{2+} loads ranging from 300 to 1500 µmol Ca^{2+}/L non-mitochondrial cell volume, with a minimal SR Ca^{2+} load of approximately 300 µmol Ca^{2+}/L non-mitochondrial cell volume necessary for a detectable CICR to occur (Hove-Madsen, Llach et al. 1999). These values suggest that the Ca^{2+} sensitivity of CICR is lower for fish than mammalian cardiac myocytes where an SR Ca^{2+} content of 50-200 µmol Ca^{2+}/L non-mitochondrial cell volume is sufficient for normal CICR (Negretti, Varro et al. 1995, Venetucci, Trafford et al. 2006). On the other hand, the much higher SR Ca^{2+} content at steady-state in teleost myocytes (near 1 mmol Ca^{2+}/L non-mitochondrial cell volume) offsets the lower Ca^{2+} sensitivity, to trigger Ca^{2+} release from the SR that reaches as much as 60% of the total CaT in trout atrial myocytes. However, due to this low Ca^{2+} sensitivity of CICR, fractional release only amounts to about 4% in the trout atrial myocytes. Consequently, it takes a large number of beats to deplete the SR Ca^{2+} content in fish hearts, making them a poor model to study acute effects of pharmacological interventions or pathological conditions that impair normal SR Ca^{2+} sequestration, which have immediate and dramatic effects in mammalian myocytes where fractional SR Ca^{2+} release is near 50% (Bassani, Bassani et al. 1994).

Under physiological conditions, Ca^{2+} release from the SR is triggered by $I_{Ca,L}$, giving rise to the CaT and activation of contraction. However, in pathological conditions such as heart failure (Reiken, Gaburjakova et al. 2001) and arrhythmia (Jiang, Wang et al. 2005) (Hove-Madsen, Llach et al. 2004) Ca^{2+} can be released spontaneously from the SR triggering spontaneous membrane depolarizations that have the potential to induce arrhythmic episodes. Local Ca^{2+} release events produced by spontaneous opening of a single RyR2 cluster, termed Ca^{2+} sparks can sum temporally and spatially to form Ca^{2+} waves (Cheng, Lederer et al. 1993) large enough to trigger membrane depolarizations

(Voigt, Li et al. 2012). Therefore, Ca^{2+} sparks are often considered to reflect the propensity of the RyR2s to release Ca^{2+} spontaneously and elevated spark frequencies have been associated with atrial and ventricular arrhythmias in humans (Hove-Madsen, Llach et al. 2004, Jiang, Wang et al. 2005, Voigt, Li et al. 2012), as well as in animal and cellular models of disease (Terentyev, Nori et al. 2006, Cañón, Caballero et al. 2016). Interestingly, Ca^{2+} sparks have also been reported in isolated cardiac myocytes from trout and zebrafish (Llach, Molina et al. 2011, Bovo, Dvornikov et al. 2013) with similar properties and occurring at frequencies similar to those recorded in human atrial myocytes under comparable experimental conditions (Llach, Molina et al. 2011). Moreover, spontaneous Ca^{2+} release in the trout myocytes was also able to trigger spontaneous APs, suggesting that the zebrafish heart and myocytes might be useful models to study mechanisms underlying the induction of arrhythmia provided that further studies confirm the ability of spontaneous Ca^{2+} release to induce ectopic and arrhythmic activity in the perfused zebrafish heart model.

Calcium Removal from the cytosol and relaxation

Sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA): The cardiac paralog of SERCA (SERCA2a) plays a role in relaxation of the cardiac myocyte and maintains the SR Ca^{2+} available for activation of contraction by pumping Ca^{2+} back into the SR lumen between each heartbeat. As mentioned above, the contribution of this phenomenon on a beat-to-beat basis accounts for 50-90% of the total Ca^{2+} cycling in mammals.

With large Ca^{2+} storage capacity and SR Ca^{2+} content at steady-state, teleost myocytes must also accumulate significant amounts of Ca^{2+}. However, the ability of SERCA2a to accumulate physiologically relevant amounts of Ca^{2+} on a beat-to-beat basis has been controversial. Thus, indirect pharmacological approaches using a combination of SERCA2a and RyR2 inhibitors (Shiels, Di Maio et al. 2011) or clearance of the SR Ca^{2+} content with caffeine (Bovo, Dvornikov et al. 2013) found only moderate effects on the amplitude and decay of the CaT in tuna and zebrafish ventricular myocyte, respectively. On the other hand, studies using electrophysiological protocols designed to measure SR Ca^{2+} uptake have reported SR Ca^{2+} uptake rates near 3.1 amol Ca^{2+}/s/pF (or 106 µmol Ca^{2+}/s/L non-mitochondrial cell volume) at 10 mV for both atrial (Hove-Madsen, Llach et al. 1999) and ventricular myocytes (Hove-Madsen, Llach et al. 1998) at physiological temperatures ranging from 7 - 21 °C (Hove-Madsen and Tort 2001). In line with this, SERCA2a inhibition with cyclopiazonic acid gradually reduced the CaT amplitude to less than 20% of the control in trout atrial myocytes (Llach, Molina et al. 2011). Similarly, the force of contraction was reduced by 35-50% in paced zebrafish atrial and ventricular slices by depleting the SR Ca^{2+} content with ryanodine (Haustein,

Hannes et al. 2015). Several studies have underlined the essential role played by SERCA2a in maintaining Ca^{2+} homeostasis in the zebrafish heart (Ebert, Hume et al. 2005, Langenbacher, Dong et al. 2005).

SERCA2a activity is highly regulated by phospholamban (PLB) with the dephosphorylated form strongly inhibiting the pump. β-adrenergic stimulation and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) mediated phosphorylation of PLB removes this inhibition (Mattiazzi and Kranias 2014) to allow for greater subsequent uptake of Ca^{2+} into the SR and larger CICR events. This mechanism of regulation is also observed in trout atrial (Llach, Huang et al. 2004) and zebrafish ventricular myocytes (Bovo, Dvornikov et al. 2013).

Calcium removal by the sodium-calcium exchanger: The cardiac NCX1 is considered the primary mechanism for Ca^{2+} removal from the cytosol in fish cardiomyocytes since NCX activity alone was sufficient to remove cytosolic Ca^{2+} allowing for relaxation when SERCA2a was inhibited (Hove-Madsen and Tort 2001), while inhibiting NCX greatly prolonged relaxation (Hove-Madsen, Llach et al. 2003). It should be kept in mind that SR Ca^{2+} uptake has to match SR Ca^{2+} release and Ca^{2+} extrusion by NCX has to match sarcolemmal Ca^{2+} entry (LTCC and reverse mode NCX) in order to maintain the Ca^{2+} homeostasis at steady-state. Indeed, SR can take up Ca^{2+} even at resting membrane potentials of -80 mV in trout atrial and ventricular myocytes and increases strongly at more depolarized potentials (Hove-Madsen and Tort 1998, Hove-Madsen and Tort 2001) supporting the notion the SERCA2a can compete with the NCX in removing Ca^{2+} from the cytosol, especially during the peak and the plateau phase of the AP. Moreover, if SR Ca^{2+} uptake has to match release at steady-state, SR Ca^{2+} uptake should account for nearly 50% of the total CaT and NCX for the rest in trout atrial and ventricular myocytes (Hove-Madsen, Llach et al. 1998, Hove-Madsen and Tort 2001). On the other hand, forward mode NCX must match the large LTCC plus Ca^{2+} entering via reverse mode NCX during the upstroke and the peak of the AP in the zebrafish heart (Zhang, Llach et al. 2011), making the relative contributions of SERCA and NCX to Ca^{2+} removal from the cytosol in the zebrafish heart somewhat different from mammalian and human cardiomyocytes.

Cardiac contractile myofilaments

Myofibrils are the smallest functional units of the contractile apparatus. In mammalian tissue, myofilaments are arranged throughout the cardiomyocyte in many layers, showing high organization and a striated pattern resulting from alternating actin and myosin within the sarcomeres (le Guennec, Mosca et al. 2008). In contrast, fish

myofilaments are peripherally located below the sarcolemma and may form only a single layer due to the smaller diameter of the cells (Dvornikov, Dewan et al. 2014).

The t-tubule network which is formed by invaginations of the sarcolemma is an integral component of cardiomyocytes and a hallmark of cell maturation; the absence of these structures limits the extent by which Ca^{2+} channels can become juxtaposed with SR release channels (Chugun, Taniguchi et al. 2003, Birkedal, Christopher et al. 2009). As in mammalian cardiomyocytes, fish myocytes possess small invaginations (caveolae) that increase surface area to volume ratio and decrease diffusion distances to some extent (Brette, Luxan et al. 2008). Moreover, these cells are long and slender (Tibbits, Hove-Madsen et al. 1991); more similar to birds, amphibians, and reptiles, therefore the distance between the cell periphery and the myofilaments is more limited and extensive t-tubular invaginations may be unnecessary to co-ordinate the rise of the CaT. These differences necessitate additional considerations when using fish species to study cardiac diseases such as Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT), atrial/ventricular fibrillation, and long/short QT syndrome (Di Maio and Block 2008).

Frank-Starling mechanism

Humans increase cardiac output through increases in both stroke volume (SV) and HR. Some fish species are capable of limited changes in HR (Farrell and Milligan 1986) (Shiels and White 2008) but are able to double or triple SV to bring about necessary increases in cardiac output (Genge, Lin et al. 2016, Lee, Genge et al. 2016, Farrell and Smith 2017).

Length-dependent activation (LDA) is the molecular underpinning of the absolutely critical Frank-Starling relationship of the heart whereby increased venous return (and end diastolic volume) results in greater contractile strength and SV. LDA in a key property which human (Allen and Kurihara 1982) and zebrafish hearts (Dvornikov, Dewan et al. 2014) have in common. The increased Ca^{2+} sensitivity of troponin C (TnC) at longer SL is thought to underlie the force-SL relationship seen in mammals (Kentish, ter Keurs et al. 1986, Granzier, Akster et al. 1991). This relationship is conserved in zebrafish (Dvornikov, Dewan et al. 2014). Despite comparable resting sarcomere length (SL), fish may be able to increase active tension at lengths beyond which mammals are able and thus pump a greater range of volumes (Shiels, Calaghan et al. 2006).

Cold water fish such as trout normally exhibit greater sensitivity at all SLs when compared to rats (Churcott, Moyes et al. 1994, Gillis and Tibbits 2002, Patrick, Hoskins et al. 2010) at a given temperature. In contrast, zebrafish cardiomyocytes exhibit a Ca^{2+}

sensitivity that is comparable to the mouse myocardium (Iorga, Neacsu et al. 2011). Sensitivity of the contractile apparatus to Ca^{2+} is modulated by numerous factors. Amongst these factors, SL is critical such that increased SL results in an increase in force production independently of Ca^{2+} release from the SR in all species measured to date, including trout (Shiels, Calaghan et al. 2006, Patrick, Hoskins et al. 2010) and the mammalian heart (Janssen and de Tombe 1997, de Tombe, Mateja et al. 2010).

The mechanisms by which LDA act are disputed, with the proponents of the lattice spacing theory hypothesizing that increased length corresponds to decreased cross sectional area to allow for greater contact between thick and thin filaments. This model is controversial in mammals and has not yet been explored in fish species. Recent studies highlight myosin head orientation rather than proximity to actin as the governing factor in LDA (Kumar, Govindan et al. 2015).

Passive tension which is generated primarily by elastic components of the contractile apparatus such as titin also plays a role in LDA (Granzier and Labeit 2002, Moss and Fitzsimons 2002). Phosphorylation (Driever, Solnica-Krezel et al. 1996) and differential expression (Seeley, Huang et al. 2007, Zou, Tran et al. 2015) of titin isoforms may modulate passive stiffness in zebrafish as it seems to in mammals. These isoforms are tissue, species, and developmental stage dependent (Fukuda and Granzier 2005, On, Marshall et al. 2008). It has been shown that teleosts have more passive tension compared to rat myocytes (Patrick, Hoskins et al. 2010).

Contraction

Elevation in cytosolic Ca^{2+} accompanies systole and increases the probability of Ca^{2+} binding to site II of TnC within the heterotrimeric Tn complex. Ca^{2+} binding to cTnC exposes a hydrophobic pocket within the molecule which allow the switch peptide of cardiac troponin I (cTnI) to bind to it. This interaction sets off a series of conformational changes in the thin filament, exposing the actin binding sites which are then able to interact with myosin heads allowing for force production (Holroyde, Robertson et al. 1980). This sequence of events is maintained in fish species (Gillis and Tibbits 2002).

Decreasing temperature reduces the Ca^{2+} sensitivity of the cardiac thin filament in all species examined to date and increases the cytosolic concentration of this ion necessary to produce a given force output, this effect is seen in cardiac tissue to a much greater extent than skeletal muscle (Shattock and Bers 1987, Harrison and Bers 1989). Altering the affinity of cTnC for Ca^{2+} can change the properties of the thin filament and has implications for the force production capabilities of the heart (Harrison and Bers 1990, Liu, Wohlfart et al. 1990, Wang, Huang et al. 1997).

cTnC sequence is one factor that determines Ca^{2+} sensitivity (Gillis, Moyes et al. 2003) and specific amino acid substitutions can offset the decrease in Ca^{2+} sensitivity which occurs in the cold (Gillis and Tibbits 2002). Environmental temperatures necessitate that most fish myofilaments are more Ca^{2+} sensitive than mammals at a given temperature (Churcott, Moyes et al. 1994). Interestingly, changing only four residues in cTnC (NIQD) can increase the sensitivity of mammalian cardiomyocytes to trout-like level (Gillis, Moyes et al. 2003, Gillis, Liang et al. 2005).

We have shown that sensitivity is tuned through variable expression of protein isoforms to allow for adaptation to environmental constraints (Stevens, Rayani et al. 2016). A slow skeletal (ssTnC) and a cardiac (cTnC) troponin C paralog are expressed in the zebrafish heart with the former having lower Ca^{2+} sensitivity overall; cTnC is therefore expressed to a greater extent with acclimation to lower temperatures (Genge, Davidson et al. 2013). This occurs in the absence of overt structural perturbation and likely involves interactions between the highly interrelated proteins of the cardiac Tn complex (Stevens, Rayani et al. 2016).

This high degree of interplay is highlighted by the ability of other members of the complex, especially cTnI to modulate Ca^{2+} sensitivity of TnC (OGAWA 1985). Three paralogs of TnI exist in mammals (Shaffer and Gillis 2010) and up to seven exist in teleosts (Alderman, Klaiman et al. 2012). Functional divergence of the gene products would be expected as redundant copies of a gene would normally be lost (Hughes 1994). Sequence variance, specifically in key regions such as the switch arm modifies Ca^{2+} sensitivity and creates the need to maintain multiple differentially expressed genes of the “same protein” (Wilke and Drummond 2010, Genge, Stevens et al. 2016).

Acute temperature perturbation of the zebrafish heart

The effects of acute and chronic temperature change on teleost heart remodeling has been reviewed elsewhere, with information on changes on the *in vivo* function of the heart outlined (Vornanen, Shiels et al. 2002). The focus of our study is the functional changes in an *ex vivo* preparation in the absence of nervous system and endocrine regulation, allowing for greater control over the rate and environmental conditions.

Prolongation of the AP plateau is associated with reduced inward rectifying potassium current (Vornanen and Tuomennoro 1999). Mammalian I_{kr} is more temperature sensitive (Kiyosue, Arita et al. 1993, Vandenberg, Varghese et al. 2006) than the LTCC (Puglisi, Yuan et al. 1999), and likely more highly expressed than I_{ks} (Alday, Alonso et al. 2014). Thus reduced repolarizing I_{kr} current is likely to be the main factor in AP

prolongation in the face of reduced temperature. Given the higher expression of this channel in the atria (Nemtsas, Wettwer et al. 2010), the prolongation of the AP resulting from acute temperature reduction is expected to be more pronounced in this chamber of the zebrafish heart.

Given that Ca^{2+} buffers such as cTnC are highly temperature dependent (Gillis and Tibbits 2002), lower temperatures acutely induce lesser binding of Ca^{2+} and higher free concentrations of the ion in the cytosol leading to changes in SR loading, NCX activity, and membrane hyperpolarization. The trout NCX is relatively temperature insensitive (Q_{10} = 1.5) (Xue, Hryshko et al. 1999), this combined with elevated Na^{+} and prolonged AP would be expected to combine to facilitate removal of Ca^{2+} from the cell.

The RyR is locked in the open state in mammals in the cold (Sitsapesan, Montgomery et al. 1991), while burbot RyR are able to maintain function to near freezing temperatures (Tiitu and Vornanen 2002), and leakage of Ca^{2+} from the SR is reduced at lower temperatures in trout species (Hove-Madsen, Llach et al. 1998)

The role of the LTCC in acute temperature-induced changes on the AP merits discussion (Cavalié, McDonald et al. 1985). Despite the temperature dependence of $I_{Ca,L}$ amplitude in trout, the combination of slowed inactivation and prolonged AP duration likely causes unaltered $I_{Ca,L}$ in the face of temperature reduction (Shiels, Vornanen et al. 2000).

The teleost LTCC, RyR, and NCX combine to create functional flexibility allowing fish to maintain cardiac contractility well below physiological mammalian body temperatures. While the LTCC is slightly less temperature-dependent in mammals, the NCX and RyR are less temperature dependent in fish species such as trout. The culmination of regulating Ca^{2+} is control of contractility. While fish myofibrillar ATPase is highly temperature dependent (Aho and Vornanen 1999) and cold induced changes in the myosin ATPase would be expected to reduced contractility, inherently higher than mammalian sensitivities would offset these negative chronotropic effects as discussed above (Harrison and Bers 1990, Churcott, Moyes et al. 1994, Gillis, Marshall et al. 2000).

The significance of acute temperature changes on zebrafish heart function is not well understood. Given that AP characteristics are not always predictive of Ca^{2+} handling, simultaneous visualization of V_m and Ca^{2+} transients can provide significant perspective into the relationship between HR and EC coupling. With this in mind, optically mapping the zebrafish heart can shed insight into the similarities and differences between the

zebrafish and the mammalian cardiovascular systems. And this is what we sought to examine in detail.

A further consideration is the effect of altering temperature on intracellular pH and the confounding effect this factor has on electrophysiological parameters which underlie the heart cycle. The alpha-stat hypothesis states that the ionization of imidazole (the primary intracellular buffer) (Cameron 1984) and $[OH^-]/[H^+]$ balance remains constant in the face of altered temperature (Reeves 1972). While the variance of pH with temperature is not well in zebrafish, in our previous experiments on isolated hearts, the pH of the solution varied from 7.10 to 7.32 when reducing the temperature by 10 °C from 28 °C. Within this range, the HR, APD, and AV-delay did not change significantly (Lin, Ribeiro et al. 2014).

The information reviewed above discusses in detail the electrophysiological underpinnings of EC coupling in teleost species. Most of these areas that have been explored in zebrafish and other species are still under active investigation. Optical mapping has changed due to both technological and computational advances since its first inception over a decade ago in seminal work by Efimov et al. (Efimov, Nikolski et al. 2004). The improvements in system capabilities allow for removal of cross-talk between multiple dyes to simultaneously visualize stable V_m and Ca^{2+} signals in relatively long duration recordings, as will be described below. These recordings can be analyzed relative to each other by removing the temporal component. In so doing, the major confounding variable we have been faced with, namely rate change in the face of drug application is removed and V_m and Ca^{2+} transients which underlie the cardiac cycle can be studied independent of the time course of contraction. The applicability of this tool to a wide range of future studies is apparent. Reinstatement of the V_m-Ca^{2+} phase map and the use of distinct markers within the cycle is applicable as a tool to explore the balance of the numerous transporters and channels which underlie zebrafish EC coupling, which despite its similarities to the mammalian system needs more complete characterization as a prerequisite to its use as a model of the cardiovascular system.

Material and Methods

Hearts were isolated from adult zebrafish, ~30-35 mm in length, using the previously described method (Lin, Ribeiro et al. 2014) in which the pericardial space was accessed from the caudal aspect by removing a triangular wedge of tissue between the gills and pectoral fins after euthanasia by ice-bath immersion. Checking the heart prior to excision for rate and rhythm allowed for determination of any potential damage to the structure (Lin, Craig et al. 2015). Complete excision of the heart, while technically challenging yielded a highly stable setup which can be monitored for up to 24 hours without decrements in rate and rhythm and in the absence of external perfusion or oxygenation (Lin, Ribeiro et al. 2014). Cannulation of the heart through the bulbous arteriosus allowed for manipulation and prevented movement of the heart during solution changes (Genge, Lin et al. 2016).

Hearts were immersed in a Calcium Tyrode's solution (in mM): 117 NaCl, 5.7 KCl, 4.4 $NaHCO_3$, 1.5 NaH_2PO_4, 1.7 $MgCl_2$, 10 Na-HEPES ($C_8H_{17}N_2O_4S$), 5 glucose, 5 creatine, 5 Na-pyruvic acid, 1.8 $CaCl_2$, pH 7.3 and the bulbus arteriosus cannulated using a 34 gauge needle (Harvard Apparatus, Holliston, MA, USA). After cannulation, hearts were immersed in 8 µM of the potentiometric dye RH-237 (Molecular Probes, Eugene, OR, USA) for 15 minutes followed by immersion in 15 µM of the contractile inhibitor blebbistatin (Sigma-Aldrich, St. Louis, MO, USA) until quiescent or 30-60 minutes. Hearts were immersed in 10 µM of the Ca^{2+}-indicator Rhod-2 AM for 15 minutes before transferring to an imaging chamber containing 15 µM blebbistatin to allow for de-esterification of Rhod-2 AM (**Figure 1**). Temperature of the aluminum imaging chamber was controlled using a thermoelectric Peltier device (HP-199-1.4-0.8P, TE Technology, Traverse City, MI) and a PID temperature controller (TC-36-25 RS232; TE Technology, Traverse City, MI).

Hearts were cannulated, labelled (RH-237, Rhod-2) and contraction-inhibited (blebbistatin) at ambient/room temperature (~23^{o}C) and then loaded into an imaging chamber held initially at 28^{o}C. Previous work found isolated ZF hearts were able to maintain stable activity for many hours without external or internal perfusion (Ref). Since the inhibitory effects of blebbistatin on contraction can be partially released by movement of the heart, both super-perfusion of the bath solution and retrograde perfusion through the cannulation needle are able to restore cardiac contraction, creating severe movement artefacts. Therefore, for these relatively short experiments, a perfusion system was not used.

V_m and Ca^{2+} images of the heart were acquired on two separate GE680 cameras (Allied Vision Technologies GmbH, Germany), with 640x480 pixel resolution at 205 frames per second through a custom optical pathway as previously described. A >200 mW green

532 nm DPSS laser (LCS-0532, Laserglow Technologies, Toronto, ON, Canada) provided excitation and was directed onto the heart using a 560 nm long-pass dichroic (XF2017, 560DRLP, Omega Optical). Images were collected using a 75 mm objective lens and a 100 mm camera lens, providing 1.33x optical magnification. V_m and Ca^{2+} signals were separated by a 630 nm long pass dichroic mirror (XF2021, 630DRLP, Omega Optical Inc., Brattleboro, VT, USA) and further refined with by a 565-600 nm band pass filter (XF3412, 580QM30, Omega Optical) for the Ca^{2+} signal and a 700 nm long pass filter (XF3095, 700ALP, Omega Optical) for the V_m signal.

Initial recordings of the heart were made at 28^{o}C, after verifying voltage and calcium labeling characteristics and the absence of ventricular movement. Atria were found to be especially sensitive to handling conditions and even the very small perturbations encountered during the transfer from loading chambers to the imaging chamber were sufficient to restore some degree of motion in many hearts. The amount of motion necessary to cause movement artefact in the optical mapping recordings is extremely small and a swing of one or two pixels is sufficient to cause severe degradation of the atrial signal. Additional incubation time in the imaging solution, which contains 15 µM blebbistatin, will occasionally settle unrestrained atria. However, in most hearts, atria movement typically increased as the experiment progressed due to photo-release caused by laser exposure. Since the ventricular signal is unaffected by small amounts of atrial movement, some experiments were conducted as 'ventricular-only' experiments by disregarding the atrial compartment. For this reason, not all experiments contribute an atrial recording which is reflected by a lower number of atrial data points.

All hearts had spontaneously sinus activity, however, some rhythm abnormalities were present in some hearts, notably at the lowest HRs. Hearts with rates greater than 20 bpm were utilized as these provide a sufficient number of transients for the application of cycle averaging techniques. Those with the slowest intrinsic rate (significant bradycardia) presented the lowest interference to field-stimulation protocols, allowing the largest dynamic range. Electrical stimulation for constant-rate and variable-rate protocols were software-controlled through a USB-6501 USB Digital I/O Device (National Instruments, Austin, TX, USA) connected to a NPN Darlington transistor and a 24-volt variable power supply, using platinum electrodes (Sigma-Aldrich, St. Louis, MO, USA). The stimulation electrodes were separated by ~8 mm and 6.5 volts provided good rate capture.

At 28^{o}C, the average ZF excised heart rate was 151 bpm, however, considerable variability was found. Previously, the Q_{10} of heart rate was found to be ~0.4, such that individual heart rates are expected to decrease by 20% and 40% by cooling to 23^{o}C and 18^{o}C respectively. For hearts with very slow spontaneous activity at 28^{o}C (i.e. near the

20 bpm cut-off), subsequent cooling may result in sub-threshold heart rates which causes the number of effective experiments to drop accordingly at lower temperatures. Although these lower heart rate values may not be found commonly in vivo, their inclusion as ex vivo data was used to detect the presence of non-linear rate effects at these lower heart rates. Secondary to cooling effects, photorelease of blebbistatin in longer experiments with more imaging time can create movement artefacts in later recordings, which also contributes to lower experiment numbers at lower temperatures. Increasing the concentration of blebbistatin can be used to improve retention, albeit at the risk of non-specific cardiac effects.

Although the focus of this study was on spontaneous activity, hearts with low spontaneous activity were found to be particularly amenable for field stimulation experiments since field stimulation can only be used to increase heart rate.

For each heart, rectangular regions of interest (ROIs) were used to define atrial and ventricular areas. Illumination of the zebrafish heart by the diode laser results in minor photobleaching and reduces the signal intensity during image acquisition. To compensate for these effects, a polynomial function was used to estimate the decaying baseline and was subtracted from the intensity trace to stabilize the baseline intensity. Individual cardiac cycles were extracted by locating the peak of each AP. Each AP or CaT was independently normalized by the pre-upstroke intensity value and by the peak intensity value such that the intensity range scales varies from 0 -> 1. Multiple V_m or Ca^{2+} cycles were then averaged to produce representative recordings for each heart. The AP and CaT duration were defined as the time between 50% of the upstroke to 50% of the downstroke.

Results

Average temperature effects

To quantify the effects of temperature, average cycles were calculated from each recording (**Figure 1)**. For each atrial recording, the average APD_{50} was 39, 65 and 93 ms at 28ºC, 23ºC and 18ºC, respectively. Average atrial $CaTD_{50}$ values were determined to be 56, 90 and 125 ms, respectively. T-tests indicated that these temperature induced changes to APD_{50} and $CaTD_{50}$ were all statistically different ($p<0.001$) (**Table 1**).

The ratio between $CaTD_{50}$ and APD_{50} ($CaTD_{50}/APD_{50}$) describes the length of the CaT as a proportion of the AP and these ratios were 1.44, 1.39 and 1.34 at 28ºC, 23ºC, and 18ºC, respectively. The 7% difference between the $CaTD_{50}/APD_{50}$ ratio at 28ºC (1.44) and at 18ºC (1.34) is equivalent to a 6.5 ms difference and suggests that $CaTD_{50}$ and APD_{50} undergo similar degrees of elongation in response to temperature (**Table 1**).

In the ventricle, the average APD_{50} were 104, 161 and 229 ms and the average $CaTD_{50}$ were 112, 177 and 249 ms at 28ºC, 23ºC and 18ºC, respectively and these temperature-induced changes were all statistically different ($p<0.001$) (**Table 1**). The $CaTD_{50}/APD_{50}$ ratios at these temperatures are 1.08, 1.10 and 1.13 and there was a 5% difference, equivalent to a 12 ms difference between 28ºC (1.08) and 18ºC (1.13).

The data suggest that, within the atria, the $CaTD_{50}$ is about 40% longer than the APD_{50} and since both factors exhibit similar temperature responses, that this ratio is maintained across the tested temperature range. Within the ventricle, the $CaTD_{50}$ is about 10% longer than the APD_{50} and this ratio is also maintained across the temperature range.

These $CaTD_{50}$ and APD_{50} results suggest that the V_m-Ca^{2+} relationships are unique to each cardiac compartment and that those relationships are largely maintained over a wide temperature range. However, this apparent V_m-Ca^{2+} relationship may simply reflect how these parameters were initially defined and perhaps a different relationship would be inferred using $CaTD_{90}$ and APD_{90}.

Voltage-calcium phase plots:

An alternative to quantifying the occurrence of V_m and Ca^{2+} events occurred is to quantify their relationship. Taking advantage of simultaneous V_m and Ca^{2+} imaging, Ca^{2+} responses can be investigated as a 'function' of the matching Vm response. The

average Vm and CaT at each temperature, together with the Vm-Ca^{2+} phase-plot are shown in **Figure 2**. Phase-plots allow the relationship between Vm and Ca^{2+} to be summarized, without relying on duration parameters, as they have no temporal component. This property allows phase-maps to be used to compare between different conditions and results.

To facilitate comparisons between different phase-plots, 5 registration points were selected: 1) Ca^{2+} level at 50% of the V_m upstroke, 2) the Ca^{2+} level at the peak of the AP, 3) the Vm level at the peak of the CaT, 4) the Ca^{2+} level at half-repolarization (APD_{50}) and 5) the Vm level at half-relaxation ($CaTD_{50}$). Average values were calculated for each registration point and t-tests were used to compare 28ºC vs. 23ºC, 23ºC vs. 28ºC, and between 28ºC vs. 18ºC (**Supplementary Table 1**).

Atrial Phase Plots at different temperatures:

It was known that temperature has significant effects on atrial $CaTD_{50}$ and APD_{50} values and durations at 23ºC and at 18ºC are ~50% and 100% longer than values at 28ºC. However, most differences in the phase-relationship were not statistically different, except for some notable exceptions. The largest statistically significant difference was found in the amount of the Ca^{2+} signal present at the peak of the AP. At 28ºC, the Ca^{2+} level at the AP peak was 84% versus 69% (23ºC) and 61% (18ºC). The amount of the Ca^{2+} signal present during the upstroke of the AP was also significantly different, between 28ºC (7%) and 18ºC (4%). However, the physiological effect of this 3% may be limited. Nevertheless, although there were very large differences in atrial $CaTD_{50}$ and APD_{50} values at different temperatures, the relationship described by the phase-plots did not indicate strong temperature dependence (**Figure 2**).

Ventricular Phase Plots:

In the ventricle, $CaTD_{50}$ and APD_{50} values undergo similar changes as do the atrial $CaTD_{50}$ and APD_{50} values with respect to temperature so that ventricular values are always about 2.4-fold longer than the atrial values. However, the phase-relationship between ventricular results at different temperatures were not statistically significant at $p<0.001$. At $p<0.05$, numerous statistically significant differences could be reported: The Ca^{2+} level at 50% upstroke was 5% (28ºC) versus 3% (18ºC, $p = 0.0464$). The Ca^{2+} level at the Vm peak was 76% (28ºC) versus 71% (23ºC, $p=0.0365$). The Ca^{2+} level at APD_{50} of 54% (28ºC) was different from 59% (23ºC, $p = 0.006$) as well as different from 61% (18ºC, $p=0.0025$). Vm level at $CaTD_{50}$ of 19% (28ºC) was different from 13% (23ºC, $p = .0129$) and from 10% (18ºC, $p = 0.003$). Although, many statistically

significant changes can be reported at $p<0.05$, the magnitude of the changes themselves are limited (**Figure 3**).

Predicting calcium transients from voltage-calcium phase plots:

Phase-plots are a representation of the Vm-Ca^{2+} relationship; as such they can be used to predict the Ca^{2+} level for a given Vm input. By taking the average AP at 28ºC, the equivalent Ca^{2+} transient represented by the Vm-Ca^{2+} phase-plots can be determined. The predicted transient is limited to areas in which there is a unique Ca^{2+} response for a particular Vm level. At the end of the AP, the remaining Ca^{2+} transient relaxes at diastolic membrane potentials such that the Ca^{2+} signal changes without a change in Vm and in these regions, phase-plots are unable to predict Ca^{2+}. However, within the prediction window, the predicted CaT has a 1-to-1 relationship with the phase-plots results. In the predicted atrial CaT (**figure 4A**), the 23% different between *"Ca^{2+} level at the peak of the AP"* at 28ºC versus 18ºC is represented by a slight left shift in upstroke of the red line (28ºC) relative to the blue line (18ºC). In the predicted ventricular CaT (**figure 4B**), the statistically significant differences of 5% are equivalent to a ~10ms difference in duration.

Comparing atrial and ventricular phase plots:

Phase-plots for a given chamber of the heart are internally consistent at different temperatures, but atrial and ventricular phase-plots are markedly different. Atrial and ventricular properties were compared to investigate whether APD_{50} and $CaTD_{50}$ values and the respective phase-plots are sufficiently sensitive to detect differences in atrial and ventricular dynamics. Atrial and ventricular phase-plots are overlaid in **Figure 4C** and the corresponding statistics are presented in **Supplementary Table 1**. Atrial and ventricular APD_{50} and $CaTD_{50}$ values were statistically different at all temperatures. The Vm level at the peak of the CaT was 75% in the atria and 85% in the ventricle and this 10% difference that was consistent at all three temperatures. The Ca^{2+} level at 50% repolarization was 75% in the atria and 60% in the ventricle, which is consistent with the proportionally longer atrial CaT.

Effects of temperature on heart rate:

In the above results, the effects of HR at a given temperature as well as the effect of temperature on HR were not considered. Therefore, although the results consistently indicate that Vm and Ca^{2+} maintain a similar relationship at different temperatures, they do not reveal whether the relationship between Vm and Ca^{2+} varies with HR.

Experimentally, hearts were initially imaged at 28^{o}C and then cooled to 23 ^{o}C and then 18^{o}C. At 28^{o}C, the average HR at 120 bpm and maximum observed HR was 240 bpm. At 23^{o}C and at 18^{o}C, the average rate was 82 bpm (max = 173 bpm) and 69 bpm (max = 129), respectively. Hearts with rates lower than 20 bpm at 28^{o}C were excluded from analysis and since cooling reduces spontaneous beating rate and results in some attrition. Atrial recordings were especially sensitive to motion artifact, however, both atrial and ventricular compartments saw increased motion artifacts with increasing laser exposure, resulting in exclusion of some hearts.

Atrial rate effects

Using the average HR at each temperature, the effects of rate at one temperature were investigated by dividing individual results into 'fast' and 'slow' categories. In **Supplementary Figure 1**, the individual results are colour-coded, red for fast and blue for slow. To detect rate-dependent changes, the fast and slow results were averaged separately and shown in **Figure 5**. No statistically significant rate-dependent effects were detected in APD_{50} and $CaTD_{50}$ values, which might indicate limited restitution effects in the atria. It is nevertheless possible that the Vm and Ca^{2+} relationship is rate-dependent, however, comparing 'fast' and 'slow' phase-plot results did not find any statistically significant rate effects at any of the three temperatures (**Supplementary Table 3**).

Ventricular rate effects.

The comparison of slow and fast results in the ventricle found statistically significant changes in APD_{50} and $CaTD_{50}$ values at all three temperatures, indicating the presence of rate-dependent restitution (**Supplementary Figures 2 and 6**). Rate-dependent differences were also found at 28^{o}C for the amount of Ca^{2+} at APD_{50} (50% versus 57%, $p < 0.001$). The Vm level at $CaTD_{50}$ may also be rate dependent at 28^{o}C (24% versus 16%, $p = 0.0016$) and at 23^{o}C (19% versus 9%, $p = 0.0109$). Apart from these noted exceptions, the other phase-plot parameters did not indicate statistically significant rate effects (**Supplementary Table 4**).

Atrial rate-dependence

Determining the presence of a rate dependent factor by comparing 'slow' versus 'fast' cycles allows the results from multiple hearts to be aggregated, increasing the ability to resolve small changes. An alternative to that approach is to consider the rate-dependence of individual results by fitting the results to a linear equation at each temperature (**figure 7**). While comparing 'slow' versus 'fast' cycles, apparent rate-

dependence is interpreted from the test statistic. However, with linear fits the rate dependence of a given parameter is determined by both the steepness of the slope (**m**) as well as by square of the Pearson correlation coefficient ($\mathbf{r^2}$) which indicates the goodness of fit (**Supplementary Table 5**). In the atria, fitting APD_{50} values (28ºC) with respect to rate gave a slope value of -0.045 ms per bpm and r^2 of 0.08. For this slope value, a 100 bpm difference would be expected to result in a 4.5 ms difference in APD_{50}. However, because the r^2 value is so low, rate would not be considered a good predicator of atrial APD_{50} at 28ºC. In fact, for all three temperatures, r^2 values of the rate-dependence of APD_{50} and $CaTD_{50}$ were less than 0.23. Changes in the relationship between Ca^{2+} and Vm can still occur in the absence of changes to APD_{50} and $CaTD_{50}$. However, in the atria, the maximum r^2 value in the phase-plot registration points was 0.17. Therefore, both linear fits and slow/fast comparisons indicate that there are no rate-dependent changes present in the atrial data.

Ventricular rate-dependence

In the ventricular results, at 28ºC, APD_{50} and $CaTD_{50}$ had r^2 values of less than 0.28 and slopes of less than 0.16 ms/bpm. Since the maximum r^2 in the phase-plot registration points was at 28ºC was 0.22, the rate dependence as suggested in the slow/fast comparisons was not apparent in the linear fits (**Figure 7**). However, at 23ºC, APD_{50} and $CaTD_{50}$ had r^2 values of 0.64 and 0.60 respectively. Slope values of APD_{50} and $CaTD_{50}$ were -0.50 ms / bpm and -0.64 ms / bpm and a 100 bpm change would predict a difference of 50 ms and 64 ms respectively. At 18ºC, both APD_{50} and $CaTD_{50}$ had r^2 values of 0.81 and slope factors of -1.12 and -1.08 respectively, indicating that temperature dependence is stronger at cooler temperatures. Analysis of the phase-plots results generally found no evidence of phase-related changes except for some results at 23ºC. The "Ca^{2+} level at APD_{50}" and the "Vm level at $CaTD_{50}$" had r^2 values of 0.30 and 0.50 and slope factors of -0.14% per bpm and -0.19% per bpm respectively. At 18ºC, the phase-plot registration values at 18ºC had a maximum r^2 value of 0.05. Therefore, although APD_{50} and $CaTD_{50}$ are rate-dependent at 23 ºC and at 18ºC, the Vm- Ca^{2+} phase-relationship is largely consistent at all temperatures and rates.

Variable-rate stimulation (18ºC)

The effects of rate on APD_{50} and $CaTD_{50}$ can also be tested using variable rate stimulation protocols, which require less recording time than a series of recordings made with constant-rate stimulation. However, since the heart is not necessarily at steady state, restitution responses may not be fully formed. Applying a variable-rate simulation protocol to a heart at 18ºC, was used to increase the rate from 70 bpm to 130 bpm in 10 bpm increments (**Figure 8**). This resulted in atrial APD_{50} and $CaTD_{50}$

shortening ($CaTD_{50}$ = -0.894203x + 237.204, APD_{50} = -0.196867x + 113.406) while the ventricle was refractory at these high rates. Restitution of atrial $CaTD_{50}$ in this recording was higher than the population-based rate-dependent changes however the phase relationship did not change with the increased rate.

High constant-rate stimulation

At high rates of stimulation, insufficient time for relaxation of the CaT causes diastolic Ca^{2+} levels to increase and the amplitude of the CaT to decrease. In the atria, these responses are most easily observed at 18ºC, due to the increased length of the Ca^{2+} transients. Although these changes can also be observed at 28ºC, the high rates required (>300 bpm), limit the number of camera images per cardiac cycle to about 40 frames at 205 fps. In **Figure 9**, HR was increased from 140 bpm to 225 bpm, allowing the membrane to fully repolarize between beats but not for full recovery of the CaT (**Figure 9A**). While it should be noted that 140 bpm at 18ºC is considerably higher than average, at these beating frequencies, the increased rate did not produce additional APD_{50} or $CaTD_{50}$ restitution (**Figure 9D**). In **Figure 9C**, a steeper decline of the late CaT was observed at higher rates, however, because this change occurs after the membrane is fully repolarized, there is no apparent change in the phase relationship.
High stimulation rates also similarly affect ventricular CaT (**Figure 10**). Since the duration of the ventricular CaT at 28ºC is similar to the duration of the atrial CaT at 18ºC, cooling is not required to increase the number of samples per cardiac cycle. However, because of the shallow restitution properties at 28ºC, rate dependent changes in the ventricle can be difficult to resolve if there is a high intrinsic HR, which reduces the dynamic range relative to the maximum HR. Cooling the heart to 23ºC reduces the intrinsic HR and increases the dynamic range while also increasing the restitution effects. Cooling of the heart to 18ºC causes further deceases in HR and increased restitution. It should be noted however, that cooling to 18ºC also reduces the maximum HR.

At 23ºC, increasing the heart rate from 80 bpm to 175 bpm decreases APD_{50} from 154 ms to 133 ms and decreases $CaTD_{50}$ from 184 ms to 143 ms; both statistically significant changes. Notably, this increase in rate also affects the Vm-Ca^{2+} phase relationship statistically significant changes occur in the Vm level at the peak of the CaT (83% to 87%), Ca^{2+} level at APD_{50} (73% to 61%) and Vm level at $CaTD_{50}$ (1% to 7%).

Elevated Baselines due to high stimulation rates

At high HR, it is possible to truncate the end of the action potential or CaT waveforms. Truncation can result in an elevated baseline that changes the shape and apparent

durations when waveform normalization (which is required for the duration and phase calculations) is applied. To simulate these effects, control Vm and Ca^{2+} waveforms were calculated from the 'slow' cycles in **Figure 10**. These control cycles were then artificially truncated, elevating the baseline value in 5% increments up to 50%, followed by re-normalization. The resultant cycles are shown in **Figure 11B** while the reanalyzed APD_{50} and $CaTD_{50}$ values are plotted as a function of the elevated baseline values in **Figure 11C**. As cycle truncation or elevated baseline values are increased, APD_{50} and $CaTD_{50}$ values decrease.

In the experimental data, there was a 20 ms change in APD_{50} with elevated rate. In the simulated data, an elevated baseline value of 25% was required to create a similar change in APD_{50}. In the experimental data, there was a 40 ms change in $CaTD_{50}$ and required an elevated baseline value of 50% to re-create this result. In the original data, there was minimal elevation of the Vm baseline and a ~10% elevation in the Ca^{2+} baseline. Therefore, the elevated Ca^{2+} baseline is expected to artificially shorten the apparent $CaTD_{50}$ by ~10 ms.

The effects of cycle truncation on phase-plots were also investigated. In **Figure 11D**, truncated and re-normalized Vm cycles are plotted against the control Ca^{2+} cycle, effectively shortening the AP waveform. As a result of AP shortening the repolarization phase of the phase-plot is shifted upwards. When truncated and re-normalized Ca^{2+} cycles are plotted against the control Vm cycle (**Figure 11E**), the CaT is being shortened and the repolarization phase of the phase-plot is shifted downwards. Plotting truncated Vm against truncated Ca^{2+} cycles results in complicated effects (**Figure 11F**), altering both depolarization and repolarization kinetics.

Discussion

Our studies on isolated zebrafish hearts indicate that the ventricular APD is ~2.4-fold longer than the atrial AP and that this relationship is maintained at 28ºC, 23ºC, and 18ºC. The relationship between Vm and Ca^{2+} dynamics is distinct within each compartment of the heart and these relationships are maintained at the tested temperatures. Within the atria, the CaT is ~40% longer than the AP and much of the CaT relaxation occurs at diastolic membrane potentials. In the ventricle, the CaT is ~10% longer than the AP, underscoring a distinct Vm-Ca^{2+} relationship in each compartment.

At lower temperatures, average HR is lower causing longer atrial and ventricular dynamics. However, the relationship between atrial and ventricular durations remains consistent and is a reflection of the proportional effects of temperature on elongation. Both atrial and ventricular dynamics are ~50% and ~100% longer at 23ºC and 18ºC, relative to their respective durations at 28ºC. Furthermore, the proportional relationship between the length of the AP and the length of the CaT is also maintained at these lower temperatures.

These findings suggest that the relationship between Vm and Ca^{2+} is temperature invariant. However, comparing Vm-Ca^{2+} relationships using duration values can be difficult since a fixed reference point is required and validation of that reference point quickly becomes circular. Phase-plots, display the Ca^{2+} signal as a function of the Vm signal, effectively using each as a reference for the other. In this way, the Vm signal is being used in as a quasi-internal control in describing the Vm-Ca^{2+} relationship. However, phase-plots are unable to describe how the Vm signal itself is affected by temperature or by rate.

In this report, the duration of the AP and the CaT were described using the time between 50% of the upstroke to 50% of repolarization/relaxation. Therefore, APD_{50} and $CaTD_{50}$ values are significantly shorter than APD_{90} and $CaTD_{90}$. These shorter measures of duration were selected to avoid certain areas, particularly of the CaT, that have shallow rates of change, where gradual slope cause very large timing differences for relatively small intensity changes, causing values to fluctuate considerably from beat-to-beat. While APD_{90} and $CaTD_{90}$ values may more accurately reflect the overall duration, but are less adept at representing relative changes due to greater variability.

The relationship between APD_{50} and $CaTD_{50}$ in the atria at different temperatures suggested that the Vm-Ca^{2+} relationship might also be the same at different temperatures. The Vm- Ca^{2+} phase relationship in the atria at different temperatures

was remarkably consistent although there is some evidence of an earlier Ca^{2+} rise given the higher proportional Ca^{2+} levels at the peak of the AP.

The consistency of the atrial phase relationship may be related to the limited rate-dependent effects observed in APD_{50} and $CaTD_{50}$. Statistical analysis of atrial functioning at rates faster and at rates slower than the average HR did not find significant differences and linear fits had limited goodness of fit. Together, these results would indicate that the variability between the atrial results is greater than the variability introduced by the differences in HR, resulting in no statistically significant changes in APD_{50}, $CaTD_{50}$, and phase-plot values with respect to rate.

The lack of large rate-dependent changes in the atria may be a reflection of the recovery times available at normal HRs. At 28^{o}C and 120 bpm, the cycle interval is 500 ms while the estimated APD_{90} and $CaTD_{90}$ would be less than 100 ms and 200 ms respectively and perhaps with such long periods of recovery additional shortening is not required or warranted. At 28^{o}C, there are also limited rate-dependent changes in the ventricle as well. In the ventricle, estimated APD_{90} and $CaTD_{90}$ would be less than 150 ms and 250 ms, respectively, leaving roughly 250 ms for recovery before the next cycle. The length of ventricular dynamics at 28^{o}C is similar to the length of atrial dynamics at 18^{o}C and neither had significant changes with respect to rate.

In the absence of rate-dependent shortening, phase-related changes may not be expected. However, rate-dependent shortening can be observed without apparent changes in phase. In the atrium, with cooling to 18^{o}C and external stimulation, APD_{50} and $CaTD_{50}$ shorten without statistically significant changes in phase. In the ventricle, rate effects on APD_{50} and $CaTD_{50}$ are readily apparent at 23^{o}C and at 18^{o}C, again without statistically significant changes in phase.

The relationship described by the phase-plot is effectively limited to the duration of the AP but is independent of whether APD_{50} or APD_{90} values are used to describe that duration. The phase-plot results indicate that, at 50% relaxation of the CaT, repolarization is nearly complete in the atria and about half of the atrial CaT is not described by the phase relationship. When the CaT extends past the end of the AP, as in the case of variable rate (**Figure 7**); $CaTD_{50}$ can shorten significantly without altering the phase relationship. It is possible then that there are systematic, rate-dependent changes, occurring in the late CaT. However, there is no indication from the individual atrial results of such a response (**Supplemental Figure 1**). Therefore, the duration of the atrial dynamics is temperature dependent but the relationship between Vm and Ca^{2+} is independent on both temperature and rate.

In the ventricle, only a small part of the CaT extends past the end of the AP and the CaT is well described by the phase-plot. Yet, at 23ºC and 18ºC, there is significant $CaTD_{50}$ shortening at higher rates with no detectable change in phase. Because both APD_{50} and $CaTD_{50}$ are undergoing similar changes, the relative relationship is largely maintained. In the ventricle, there is temperature and rate related changes in the AP and CaTDs but the phase-relationship is maintained.

At high stimulation rates, there can be incomplete relaxation of the CaT, resulting in elevated diastolic Ca^{2+} levels. The rise in diastolic Ca^{2+} is associated with shorter APD_{50} and $CaTD_{50}$, presumably due to restitution. However, truncation of the CaT can also cause shortening of $CaTD_{50}$ as well as phase-related changes, from the forced baseline elevation. In order to recapitulate the degree of $CaTD_{50}$ shortening encountered at high stimulation rates (**Figure 10**), a 50% increase in baseline intensity was required (**Figure 11**). This would imply a stimulation frequency of 300 bpm; almost double the actual frequency used (175 bpm). Therefore, although truncation of the CaT can give the appearance of restitution, the actual error caused by this effect is limited.

In summary, zebrafish atrial and ventricular dynamics undergo significant changes in APD and CaTDs in response to changes in temperature. Over normal HRs, atrial dynamics at 28ºC, 23ºC, and 18ºC are rate-independent as are ventricular dynamics at 28ºC and no phase-related changes with respect to temperature or rate were found. Ventricular dynamics at 23ºC and 18ºC are rate-dependent in terms of duration and undergo relatively large changes. However, these duration changes occur in concert, such that the Vm-Ca^{2+} relationship is rate and temperature independent.

The consistency of the Vm-Ca^{2+} relationships may reflect the Ca^{2+} flux in zebrafish EC coupling. The relatively low proportion of CICR in the zebrafish may result in a tightly coupled Vm-Ca^{2+} relationship in the relative absence of SR Ca^{2+} cycling (Driedzic and Gesser 1988, Hove-Madsen and Gesser 1989, Tibbits, Hove-Madsen et al. 1991, Hove-Madsen 1992, Keen, Farrell et al. 1992, Llach, Molina et al. 2011, Bovo, Dvornikov et al. 2013). Transsarcolemmal Ca^{2+} flux is mediate through a combination of L-type/T-type Ca^{2+} currents and reverse-mode NCX activity (Nemtsas, Wettwer et al. 2010, Zhang, Llach et al. 2011, Alday, Alonso et al. 2014). Ca^{2+} currents in zebrafish ventricular myocytes have a bell-shaped IV relationship with the peak current occurring at +10 mV (Zhang, Llach et al. 2011). Although $I_{Ca,L}$ is reduced at higher membrane potentials, contractility at higher test potentials is maintained by Ca^{2+} transport through reverse-mode NCX activity, which is thermodynamically favored by depolarized membrane potentials (Bers 2000).

Native atrial and ventricular APs are reported to have similar resting V_m (~-70 mV) and AP amplitudes (100 mV) (Nemtsas, Wettwer et al. 2010). Phase-plot analysis found that, at the peak of the atrial CaT, there is consistently 75% of the V_m signal remaining at all studied temperatures. If simplistically combined with the V_m recordings, the V_m at peak Ca^{2+} would be estimated at 0 mV in the atria. Likewise, for the ventricle, 85% V_m at peak Ca^{2+} would estimate to be +12 mV. Therefore, the consistency found in these optical mapping results may be a reflection of the V_m-dependence of contractility in the zebrafish heart.

The presence of a functional SA node in the isolated heart both hinders and benefits experimental design in this animal model. As each heart has its own spontaneous rate, individual V_m and Ca^{2+} recordings can contain an unknown degree of rate-dependent restitution and a ‘fast’ heart may coincidentally have the same APD as a ‘slow’ heart. This problem is further compounded by the ability of many cardioreactive drugs to simultaneously affect heart rate as well as having direct myocardial effects.

The results presented herein strongly indicate that a change in rate alone, is not expected to result in significant differences to the atrial Vm and Ca^{2+} durations. Such that, the ability of a drug to affect atrial dynamics is most consistent with a direct myocardial effect, rather than an indirect rate effect. However with ventricular dynamics, especially at cooler temperatures, drug effects must be rate-corrected using the measured restitution responses.

The relationship between V_m and Ca^{2+} dynamics (phase and relative duration) were maintained within each compartment at 28, 23, and 18^{o}C and was not found to be strongly rate-dependent, which will facilitate the comparisons between different laboratories and their individual experimental conditions. The consistency of the phase-relationship at a variety of temperatures and at different rates indicates that the Ca^{2+} dynamics are tightly regulated by the V_m dynamics: both elongation of the AP as caused by cooling and reduction of APD by rate-dependent restitution, are mirrored by similar changes in the Ca^{2+} transient. The mechanisms of this tight regulation, in the context of chamber specific V_m-Ca^{2+} dynamics, will be closely associated to the mechanisms of arrhythmogenicity and the regulation of cardiac contractility in the zebrafish heart.

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Figure Captions

Figure 1 – Rate independent effects of acute temperature on zebrafish atrial and ventricular voltage and calcium

In all the following figures, the blue line denotes the 18°C condition, the yellow line denotes the 23°C condition, and the red line denotes the 28°C temperature condition. **A)** Averaged Vm transient associated with the atria at all three acute temperatures; **B)** Average Vm transient associated with the ventricle all three acute temperatures; **C)** Averaged Ca^{2+} transient associated with the atria at all three acute temperatures; **D)** Average Ca^{2+} transient associated with the ventricle all three acute temperatures.

Figure 2 – Atrial voltage and calcium transients and phase map generation

A) Average atrial Vm and Ca^{2+} transient at 28°C; **B)** Average atrial Vm and Ca^{2+} transient at 23°C; **C)** Average atrial Vm and Ca^{2+} transient at 18°C; **D)** Phase-map between the atrial Vm and Ca^{2+} transient at 28°C is show; **E)** Phase-map between the atrial Vm and Ca^{2+} transient at 23°C is show; **F)** Phase-map between the atrial Vm and Ca^{2+} transient at 18°C is show; **D-F)** The highlighted points: show the Ca^{2+} level at 50% of the Vm upstroke (red); the Ca^{2+} level at the peak of the AP (yellow); the Vm level at the peak of the Ca^{2+} transient (green); the Ca^{2+} level at half-repolarization (APD_{50}) (light blue); the Vm level at half-relaxation ($CaTD_{50}$) (dark blue).

Figure 3 – Ventricular voltage and calcium transients and phase map generation

A) Average ventricular Vm and Ca^{2+} transient at 28°C; **B**) Average ventricular Vm and Ca^{2+} transient at 23°C; **C**) Average ventricular Vm and Ca^{2+} transient at 18°C; **D)** Phase-map between the ventricular Vm and Ca^{2+} transient at 28°C is show; **E)** Phase-map between the ventricular Vm and Ca^{2+} transient at 23°C is show; **F)** Phase-map between the ventricular Vm and Ca^{2+} transient at 18°C is show; **D-F)** The highlighted points: show the Ca^{2+} level at 50% of the Vm upstroke (red); the Ca^{2+} level at the peak of the AP (yellow); the Vm level at the peak of the Ca^{2+} transient (green); the Ca^{2+} level at half-repolarization (APD_{50}) (light blue); the Vm level at half-relaxation ($CaTD_{50}$) (dark blue)

Figure 4 – predicting calcium transients from phase plots

The “prediction window” is highlighted with the red line depicting 28^{o}C, the yellow line depicting 23^{o}C, and the blue line depicting 18^{o}C. A) The predicted atrial Vm transients is shown as well as the calcium transient within the prediction window; B) The predicted ventricular Vm transients is shown as well as the calcium transient within the prediction window; C) The phase relationship of the atria and ventricles is compared, with lighter colours used for the atria and darker colours for the ventricles.

Figure 5 – Effects of temperature on zebrafish atrial voltage and calcium transients

For the atria, the individual Vm (left), Ca^{2+} (middle), and phase-plot (right) associated with each acute temperature (28, 23, and 18°C – from top to bottom) show the faster than average HRs in red and the slower than average HRs in blue. There is very little discernable rate response in the atria at any temperature. The temperature can be see to change durations for different HRs but the phase-plots show a high degree of overlap consistent with unaltered relationship between the Ca^{2+}-Vm.

Figure 6 – Effects of temperature on average fast and slow zebrafish hearts - atrial voltage and calcium transients

For the atria, the individual Vm (left), Ca^{2+} (middle), and phase-plot (right) associated with each acute temperature (28, 23, and 18°C – from top to bottom) show the faster than average HRs in red and the slower than average HRs in blue. There is very little discernable rate response in the atria at any temperature. The temperature can be see to change durations for different HRs but the phase-plots show a high degree of overlap consistent with an unaltered Ca^{2+}-Vm relationship.

Figure 7 – Effects of temperature on zebrafish ventricular voltage and calcium transients

For the ventricles, the individual Vm (left), Ca^{2+} (middle), and phase-plot (right) associated with each acute temperature (28, 23, and 18°C – from top to bottom) show the faster than average HRs in red and the slower than average HRs in blue. There is very little discernable rate response in the ventricles at any temperature. The temperature can be see to change durations for different HRs but the phase-plots show a high degree of overlap consistent with unaltered relationship between the Ca^{2+}-Vm.

Figure 8 – Effects of temperature on average fast and slow zebrafish hearts – ventricular voltage and calcium transients

For the ventricles, the individual Vm (left), Ca^{2+} (middle), and phase-plot (right) associated with each acute temperature (28, 23, and 18°C – from top to bottom) show the faster than average HRs in red and the slower than average HRs in blue. There is very little discernable rate response in the ventricles at any temperature. The temperature can be see to change durations for different HRs but the phase-plots show a high degree of overlap consistent with unaltered relationship between the Ca^{2+}-Vm.

Figure 9 – Effects of heart rate on zebrafish atrial and ventricular, voltage and calcium transient durations

Blue circles from hearts exposed to 18 °C, yellow “Xs” represents traces obtained from hearts exposed to 23 °C, and red crosses represent hearts exposed to 28 °C**. A)** The

average APD50 in the atria is plotted against the HR. The change in APD50 is most pronounced at the lowest temperatures which cause the greatest prolongation in APD50 with decreasing rate; **B)** The APD50 of the ventricles is plotted against the HR. At each temperature, the effect of rate on APD50 is more pronounced than that seen in the atria; **C)** The CaT50 duration is plotted against the HR in the atria. The greatest rate dependence on CaT duration is evident at the lowest temperature as apparent from the slope of each line; **D)** The effect of rate on CaT50 duration in the ventricles is shown with the most pronounced rate effect seen at the 18 °C temperature condition.

Figure 10 – Effects of variable rate stimulation protocol on zebrafish atrial voltage, calcium, and phase response

This heart had a spontaneous rate of ~35 bpm and was kept at 18 ° C throughout the protocol where a ramp increase from 50 bpm to 130 bpm was instigated. **A)** Variable rate stimulation protocol is applied to the atria with the fastest rates shown in red and the slowest shown in blue. Stimulation protocols can be used to set hearts to a baseline rate and normalize inter-heart variability; **B)** Despite the change in duration with rate, there is no discernable effect on the Ca2+-Vm relationship between rates; **C)** Both the Vm (light colours) and Ca2+ (dark colours) transients are shown at each rate. A Prolongation in both the APD50 and the CaT50 duration is observed with decreasing rate; **D)** The effect of increased rate on the APD_{50} (bottom line) and $CaTD_{50}$ (top line) is plotted, the effect on later are more significant than the former. ($CaTD_{50}$ = -0.894203x + 237.204, APD_{50} = -0.196867x + 113.406).

Figure 11 – Effects of rapid constant rate stimulation on zebrafish atrial voltage, calcium, and phase relationship

A constant rate stimulation protocol (225 bpm) is applied to rapidly beating atrium (140 bpm), the heart was held at 18 °C for the duration of the protocol. The blue traces show the cycles pre- and post-stimulation and the red shows the stimulation induced cycles at above baseline rates; **A)** The Ca2+ and Vm transients are shown for pre- and post-stimulation, before and after a stimulation protocol. The elevation in diastolic Ca2+ is distinctly apparent during stimulation along with a reduction in peak Ca2+ values which both return to normal pre-stimulation levels after termination of the protocol; **B)** The phase relationship between atrial Ca2+ and Vm is shown for all the cycles with and without stimulation and very little difference is observed between these traces; **C)** the Vm (dotted line) and Ca2+ (solid line) are shown for the pre-/post-stimulation and during stimulation. The only discernable difference is at the end of the repolarization where the stimulation seems to cause a more rapid return to baseline after the APD80 point; **D)** The voltage (bottom) and calcium (top) lines show the effect of rate on duration.

Figure 12 – Effects of rapid constant rate stimulation on zebrafish ventricular voltage, calcium, and phase relationship

A constant rate stimulation protocol (225 bpm) is applied to rapidly beating ventricle (70 bpm). The heart was held at 18 °C for the duration of the protocol. The blue traces show the cycles pre- and post-stimulation and the red shows the stimulation induced cycles at above baseline rates; **A)** Application of the simulation protocol causes an increase in the diastolic Ca2+ levels. The baseline Vm does not change significantly with the protocol except for the more rapid rate observed in the trace; **B)** The phase relationship between atrial Ca2+ and Vm is shown for all the cycles with and without stimulation and very little difference is observed between these traces; **C)** the Vm (dotted line) and Ca2+ (solid line) are shown for the pre-/post-stimulation and during stimulation. The phase relationship in the ventricle shows a conserved relationship between the Ca2+ and Vm transients in the depolarisation phase of the AP but a significant difference is notable in the repolarization phase where faster rates alter the phase relationship between Ca2+ and Vm; **D)** The voltage (bottom) and calcium (top) lines show the effect of rate on duration.

Figure 13 – Effect of artificially truncating ventricular voltage and calcium transients

A) Control voltage and calcium transients from the slow heart ventricles were artificially truncated to elevate the baseline value in 5% increments up to 50% and renormalized;
B) Results of the artificial truncation are shown; C) Reanalyzed APD_{50} and $CaTD_{50}$ values are plotted as a function of the elevated baseline values; D) truncated and re-normalized voltage cycles are plotted against the control calcium cycle; E) Truncated and re-normalized calcium cycles are plotted against the control voltage cycle; F) Truncated voltage cycles against truncated calcium cycles –upstroke and relaxation kinetics are altered.

Table 1– Rate-independent effects of acute temperature on zebrafish atrial and ventricular voltage and calcium transient duration

The peak of each AP was taken to represent 0% repolarization and the time from 100% repolarization back to baseline Vm signal was used to calculate an APD_{50} where 50% repolarization has occurred for each Vm transient. The average APD_{50} and average $CaTD_{50}$ ± the standard deviation associated at 18°C, 23°C, and 28°C is provided. The same peak detection and baseline value assessment was used for the duration associated with the Ca^{2+} signal to calculated a $CaTD_{50}$ value for a given chamber and temperature. These values were independent of the rate exhibited by each heart at a given acute temperature. The $CaTD_{50}/APD_{50}$ provides a measure of the degree of elongation in the two variables between the temperature conditions. The conditions were all statistically different ($p<0.001$).

Table 1.5 – Phase map data point comparisons

For each chamber of the heart and at each temperature, five points from the phase relationship was selected: 1) Ca^{2+} level at 50% of the Vm upstroke, 2) the Ca^{2+} level at the peak of the AP, 3) the Vm level at the peak of the CaT, 4) the Ca^{2+} level at half-repolarization (APD_{50}) and 5) the Vm level at half-relaxation ($CaTD_{50}$) The p-value associated with the comparison of each temperature pair, for each of these data points is also presented.