Published in final edited form as:

Dev Dyn. 2012 December ; 241(12): 1993–2004. doi:10.1002/dvdy.23882.

Analysis of Post-Embryonic Heart Development and Maturation in the Zebrafish, *Danio rerio*

Corinna Singleman and Nathalia G. Holtzman

Department of Biology, Queens College; City University of New York, 65-30 Kissena Blvd, Flushing NY 11367, USA and The Graduate Center; City University of New York, 365 Fifth Avenue, New York, NY 10016, USA

Abstract

Background—Cardiac maturation is vital for animal survival and must occur throughout the animal's life. Zebrafish are increasingly used to model cardiac disease; however, little is known about how the cardiovascular system matures. We conducted a systematic analysis of cardiac maturation from larvae through to adulthood and assessed cardiac features influenced by genetic and environmental factors.

Results—We identified a novel step in cardiac maturation, termed cardiac rotation, where the larval heart rotates into its final orientation within the thoracic cavity with the atrium placed behind the ventricle. This rotation is followed by linear ventricle growth and an increase in the angle between bulbous arteriosus and the ventricle. The ventricle transitions from a rectangle, to a triangle and ultimately a circle that is significantly enveloped by the atrium. In addition, trabeculae are similarly patterned in the zebrafish and humans with both muscular fingerlike projections and muscle bands that span the cardiac chamber. Interestingly, partial loss of atrial contraction in *myosin heavy chain 6* (*myh6/wea*^{hu423/+}) mutants result in the adult maintaining a larval cardiac form.

Conclusions—These findings serve as a foundation for the study of defects in cardiovascular development from both genetic and environmental factors.

Keywords

Heart development; cardiac maturation; zebrafish; *Danio rerio*; ventricle; atrium; trabeculation; bulbous arteriosus; congenital heart disease; *myh6*; *weak atrium*

INTRODUCTION

Development and maturation of the cardiovascular system is a crucial and ongoing process that continues throughout the life of an animal. The cardiovascular system is required to distribute nutrients, gases, hormones and blood cells throughout the body. During embryonic development, the system must function before being completely formed and then must respond to the changing demands of the body such as increase in body size, exertion and

perhaps cardiac injury (Icardo, 1996; Antkiewicz et al., 2006; Chinchilla and Franco, 2006). The cardiovascular system has a very dynamic structure through out life. No doubt, cardiac function is key to organismal survival and proper function is aided by proper cardiac form, physiology and adaptability.

Clearly, species-specific differences in cardiac form exist. Specifically, the number of chambers and their relative positions vary; however, many of the underlying cellular and molecular processes that drive cardiac development are similarly controlled (reviewed in Chico et al., 2008 and Bakkers, 2011). While each model organism has its strengths, zebrafish are particularly well suited to the study of morphogenesis and organogenesis (Lohr and Yost, 2000; Glickman and Yelon, 2002; Schoenebeck and Yelon, 2007). Zebrafish have a prototypic heart with only one ventricle and one atrium. The initial formation of the zebrafish heart has been the focus of many studies because of the embryos' optical clarity, accessibility to the externally fertilized embryos and because of the ease with which development can be perturbed. In addition, zebrafish can survive into larval stages without a functioning cardiovascular system because oxygen diffusion from the environment is sufficient to meet the early needs of the growing embryo and larvae (Manasek, 1970; Manasek and Monroe, 1972). This feature allows the study of early cardiac maturation in the absence of proper cardiac function, a phenotype that would be lethal in mice. As a consequence of its value for studying embryonic development, the zebrafish genome has been sequenced and methods for forward and reverse genetics exploited such that laboratories have begun to extend their studies from embryos to larvae and through adulthood. These studies include analysis of external development (Parichy et al., 2009), neurogenesis through adulthood (Marcus et al., 1999; Adolf et al., 2006; Gorelick et al., 2008; Zupanc, 2008), olfactory development (Koide et al., 2009; Paskin et al.), behavior (Norton and Bally-Cuif, 2010), pigment (reviewed in Kelsh et al., 2009), and cancer (reviewed in Mione and Trede, 2010). Significant work has begun to elucidate the mechanisms underlying adult cardiac wound healing and regeneration (reviewed in Major and Poss, 2007). Studies examining aspects of cardiac development and maturation in larval and juvenile zebrafish are limited (Antkiewicz et al., 2006; Hill et al., 2009; Martin and Bartman, 2009; Liu et al.; Peshkovsky et al.).

The zebrafish heart has four distinct components: the inflow tract called the sinus venosus; two cardiac chambers, the atrium and ventricle; and the outflow tract, called the bulbous arteriosus. The heart is the first functional organ in the developing embryo. It forms through an elegant series of cell migrations (Holtzman et al., 2007; Baker et al., 2008; Rohr et al., 2008; Smith et al., 2008) and cell fate decisions leading to the formation of an asymmetrically placed contractile linear heart tube composed of an inner endocardium and an outer muscular myocardium. Heart tube morphogenesis and its regulation has been the focus of many recent studies. Development of cardiac asymmetry requires two distinct processes, during initial formation of the heart tube, the heart jogs to the left then cardiac looping places the atrium to the left of the ventricle. As this simple linear heart tube pumps blood, region specific cell shape changes and oriented cell divisions drive cardiac chamber ballooning, further transforming the heart (Auman et al., 2007). In parallel with chamber development, the myocardial wall compacts and thickens, trabeculae form and thicken (Hu et al., 2000; Liu et al., 2010; Peshkovsky et al., 2011), an epicardium forms (Manner et al.,

2005; Serluca, 2008; Liu and Stainier, 2010) and the cardiac valves develop (Martin and Bartman, 2009). Ultimately, the cardiac pacemaker develops (Arrenberg et al., 2010) and begins to respond to cues from other parts of the body to properly regulate the rate of contraction to meet the needs of the growing and active body.

Small changes in cardiac form can significantly alter cardiac function. Similarly, small changes in cardiac function, such as partial or complete loss of contraction of the atrium or ventricle, can alter cardiac form (Berdougo et al., 2003; Auman et al., 2007; Lin et al., 2012). Features, including overall chamber morphology, ventricle length and width, bulbous arteriosus length, outflow tract angle, chamber volume and trabeculation density and thickness of the compact myocardium, have all been shown to play a role in heart disease (Di Donato et al., 2006; Lang et al., 2006; Hicken et al., 2011). In zebrafish, hypoxia, anemia, environmental toxins such as crude oil, as well as several mutations that disrupt cardiac contractions, lead to disruption of cardiac form during many life stages (Hicken et al., 2011; Ding et al., 2011). These cardiomyopathies can result from small defects early in life or in adults through cardiac remodeling. This body of work clearly demonstrates changes in cardiac form both internally and externally; however, each study uses significantly different measures of fish maturation (age/size) and measures of cardiac growth (wet weight/length and width) making it difficult to compare results across publications. While efforts have begun to elucidate the molecular regulation required to specify cardiac fate and direct initial cardiac morphogenesis, little work has examined cardiac growth and maturation. Considerable changes in cardiac form and function clearly take place between completion of an embryonic heart and the cardiac form at death. Failure of cardiac maturation or adaptive response by the cardiovascular system can have dire consequences, yet the mechanisms underlying these processes are poorly understood.

Despite the increasing interest in the growth and maturation of zebrafish and its organ systems, there has been very limited systematic analysis of cardiac growth and development from larval stages to adulthood. Here we address this deficit and provide a stage-by-stage analysis of cardiac form in postembryonic zebrafish. We have developed a measurement standard for cardiac development that extends upon the zebrafish external morphology series by Parichy et al. (2009) and previous observations of cardiac maturation (Hu et al., 2000; Hu et al., 2001). Cardiac characteristics, including external and internal organ morphologies, are described. We also identify a cardiac rotation that places the heart in its final location within the pericardial cavity. Our findings will serve as a standardized series for cardiac maturation and provide a basis for future research into factors that disrupt or modulate cardiac form and/or function. To demonstrate the value of this approach, we identify a zebrafish mutation causing partial loss of atrial contractility due to a dominant mutation in the atrial specific *myosin heavy chain 6/weak atrium* (*myh6/wea*)^{hu423}. The contractility defect in *myh6*^{hu423/+} heterozygous mutants results in failure to undergo cardiac maturation as measured by external parameters. Clearly defining the stages and characteristics of the normal zebrafish cardiac maturation will allow for further experimentation on the zebrafish heart and the ability to better use this model to follow cardiac maturation changes in comparison to human disease states.

RESULTS

Zebrafish Maturation

Zebrafish growth, like human growth, is not exclusively tied to age, resulting in a range of fish lengths at every age. While fish age is a convenient variable for collecting cohorts, zebrafish growth and maturation are influenced by factors other than time, including tank density, food availability and other environmental and genetic factors (Gamperl and Farrell, 2004). Thus size, here in the form of standard length (SL), which is the distance from the snout to the base of the tail fin (Fig. 1A), is the better indicator of overall maturation. Based on the finding of Parichy et al., (2009), zebrafish with an SL of 3.4–12 mm are considered larval (Fig. 1D), 12–18 mm are considered juvenile (Fig. 1E) and 18 mm or larger are adults (Fig. 1F). Interestingly, in zebrafish with an SL of less than 11.0 mm, environmental and genetic factors can affect the relationship between the SL and other traits. In the case of cardiac maturation, we find that SL correlates directly with ventricular length (VL, Fig. 1C), thus SL and VL are used as maturation standards through this paper. While whole fish and heart wet weight have previously been assessed during development in zebrafish (Hu et al., 2000), this often requires that fish hearts are weighed as a group. Therefore we chose to examine fish and heart length as these parameters allow analysis of individual hearts and are often used for chamber quantification in fish and other species (Lang et al., 2006).

As the fish age, we find considerable variation in SL even in our standardized growth conditions such that by 90 days post fertilization (dpf), we observe SL ranging from 10–29 mm (Fig. 1B). In general, a rapid growth rate of 0.32 mm/day is observed up to 60 dpf followed by slower but significant growth throughout adulthood with a rate of 0.059 mm/day between 60–180 dpf. Fish continue to grow throughout their lives and from 180 days to 3 years of age they have a sustained though slow growth rate of 0.005 mm/day (Fig. 1B).

Terminal Cardiac Rotation

Two distinct cardiac rotation events have been described during embryonic heart development. The first occurs as the heart tube elongates to the left. During cardiac jogging the right-sided myocardial cells move to a ventral position within the heart tube. Concurrently, the left-sided myocardial cells move to occupy a dorsal position within the heart tube (Baker et al., 2008; de Campos-Baptista et al., 2008; Rohr et al., 2008; Smith et al., 2008). A second cardiac rotation occurs during cardiac looping and returns the cells to original sides at about 36 hours post-fertilization (hpf) (Baker et al., 2008). Between days 2 and 4 the two chambers of the heart sit adjacent to each other with the ventricle on the right and the atrium on the left however this is not the terminal cardiac orientation (Fig. 2A,B). We have identified a third cardiac rotation, which, by SL 3.6 (approximately 5 dpf), moves the atrium from a left-sided position deeper into the thoracic cavity repositioning the chamber behind the ventricle. At the same time the ventricle moves from its right-sided position to a more medial location. Careful examination of atrial and ventricular position between SL 3.2 (96 hpf) and SL 3.4 (104 hpf) shows a progressive relocation of the heart within the pericardial cavity (Fig. 2B; supplemental Movie 1–4). Repositioning of chambers

to their final orientation within the pericardial cavity also marks the transition from embryonic to larval stage.

At SL 3.6 the heart is located in its terminal position and the cardiac chambers are clearly morphologically and physiologically distinct. Interestingly, the cardiac chambers appear to balloon out from the rudiment of the primary heart tube such that the tube is still visible (arrows in Fig 2D) when viewed dorsally as late as SL 4.3 (Fig. 2C,D). By late larval stages (> SL6.0) evidence of the primary heart tube has vanished and only distinct chambers are present.

Cardiac Growth

Zebrafish continue to grow throughout their entire lives (Fig. 1B). This increase in fish size is accompanied by an increased cardiovascular demand, so it is not surprising that we find heart growth correlates linearly with fish growth (Fig. 1C). The ventricle length (VL) varies from 2.67–7.78% of the body length, with a life long average of 4.28% (± 0.008) of SL. This is in contrast to ventricle wet weight which decreases from 10.5% of the total body wet weight in the embryo to 1.8% of the adult (Hu et al., 2000). Ventricle size variance does not correlate with any measured parameters such as fish gender or age. Interestingly, just as the fish seem to show rapid whole body growth during the first 60 days of development, the ventricle length increases at a rate of 125.0 $\mu\text{m}/\text{day}$ during the first 60 days of development and then slows to a rate of 17.8 $\mu\text{m}/\text{day}$ between 60–180 dpf. After 180 days the ventricular growth rate slows to 0.7 $\mu\text{m}/\text{day}$.

Direct observation of the external morphology of dissected hearts suggests a progressive change in many features of the heart including an increase in the opacity of the heart as the tissue increases in density (Fig. 1G-I). We quantified a number of these observable traits. As the ventricle grows longer, it increases linearly in ventricle width (VW; Fig. 3A) to maintain a constant ratio of 0.83 (± 0.11 ; Fig. 3C) also known as a measure of circularity where a true circle has a value of 1. The bulbous arteriosus also lengthens in concert with the lengthening of the ventricle (Fig. 3A,D) growing rapidly during the first 60 dpf at a rate of 5.9 $\mu\text{m}/\text{day}$ and then as the ventricle growth slows, the bulbous arteriosus growth slows to a rate of 1.4 $\mu\text{m}/\text{day}$. The bulbous arteriosus remains about half the length (57% on average) of the VL or 1.97% (± 0.0024) of the SL throughout development (Fig. 3D).

In embryos, the heart-tube is linear with a small bulbous arteriosus/ventricle angle (outflow tract angle; OT angle) of 0° (Fig. 3G). During larval stages the OT angle increases rapidly to 30° and then stabilizes such that in juveniles and adults this angle is on average 35° . This increase in OT angle likely reflects a change in the position of the heart within the pericardial cavity, while the movement of the bulbous arteriosus is limited by its connection to the vasculature (Supplemental Movie 5). As the fish grows, the other abdominal organs, particularly the liver, begin to occupy more space, and limit the posterior displacement of the heart. Interestingly, at any given developmental stage, the OT angle can vary as much as 30° . This variation reflects the dynamic nature of the OT angle during each cardiac contraction (Supplemental Movie 5). During ventricular ejection, the ventricle is fully contracted and the OT angle is at a minimum (Fig. 3E). After atrial systole, the ventricle is maximally filled and the OT angle is also at a maximum (Fig. 3F). At least 20° of the

variation observed in BV angle reflects the dynamic nature of this angle during each cardiac contraction.

External Cardiac Morphology

Simple observation of external cardiac morphology reveals that the whole heart is covered directly with a thin epithelium, the epicardium, which is sparsely pigmented, while the heart chambers have no inherent color (Fig. 4). Direct observation of over 200 samples reveals changes in its three-dimensional structure as the heart matures. Quantitative measures of external atrial morphology or whole heart morphology are limited by the extreme variation observed in the atrium. This is mainly due to the balloon like nature of this chamber. Even under optimized conditions, some clearly definable changes occur in atrial coverage of the ventricle. The atrium sits caudal to the ventricle and is deep in the thoracic cavity (Fig. 2A). As the atrium envelops the ventricle, it appears triangular when viewed from the left and right lateral as well as the dorsal side. The atrium is in fact a horseshoe, covering the dorsal side (Fig. 4B), large portions of the left lateral ventricle (Fig. 4A) and some of the right lateral ventricle (Fig. 4C).

The atrium obscures direct observation of the ventricle. Thus, we removed the atrium from the heart after careful documentation of whole heart morphology. The ventricle, when viewed laterally, appears as a rectangle in the larvae (Fig. 5A,E). In juveniles, the apex becomes pointed, forming a triangular ventricle (Fig. 5B,F), which ultimately becomes more circular in adults (Fig. 5C,G). This circular morphology is quantified in the circularity index value of 0.83 ± 0.11 and is sustained during adulthood. The developing ventricle was also imaged dorsally, the larval ventricle is oval (Fig. 5I,M) becoming more triangular in the juvenile (Fig. 5J,N). As the fish reach adulthood, the ventricle expands further creating a projection of one side of the triangular ventricle (Fig. 5K,O). Interestingly, this projection is always on the opposite side of the atrioventricular valve (Fig. 5I-K, * in figure). Note that the atrioventricular valve moves posteriorly and to the left during cardiac maturation. The pear-shaped bulbous arteriosus sits on top of the wider portion of the ventricle, is superior and points rostrally towards the gills while the outflow tract tapers to become the ventral aorta (Fig. 3A).

Internal Cardiac Morphology

The heart pumps blood by forceful contractions of the ventricle that push blood through the body. The high forces of these contractions are in part due to the presence and organization of the trabeculae, branch like structures of myocardial tissue. Trabeculae are also required to prevent over distension of the cardiac muscle, regulate valve function and direct heart morphology. Serial sections of *Tg(myl7:GFP)^{twu34}* (Huang et al., 2003) expressing GFP in the myocardium provide a complete view of the internal organization of the myocardium during cardiac maturation. A thin layer of muscle, called the compact myocardium, surrounds the ventricle. Early in development, the compact myocardium is 2 cell layers thick (Mably et al., 2003). As the heart grows the compact myocardium in the ventricle increases in thickness to 3–4 cells (Fig. 6P).

Trabeculae can first be observed as thickenings in the embryonic myocardium (Liu et al., 2010; Peshkovsky et al., 2011). These thickenings progressively mature and partially detach from the myocardial wall ultimately creating an elaborate network of muscle fibers. During larval (Fig. 6A-D) and juvenile stages (Fig. 6E-H,N), a meshwork of trabeculae forms perpendicular to the compact layer of the ventricular wall. A major tri-tipped trabecula forms (Fig. 6A-L, yellow and *) that is attached to the base of the AV and BV valves (Fig. 6M); however, it does not attach to the ends of the valve leaflets to prevent valve prolapse as the papillary muscle does in mammals, birds and reptiles (Fig. 6M). A second major muscle, a transverse band, spans the central lumen near the ventricular apex (Fig. 6 A-L, blue and arrow head) and may prevent over dilation and constriction of the apex of the ventricle as well as aid in expulsion of the blood from the ventricle. This muscle band superficially resembles the septomarginal trabecula found in reptilian and mammalian hearts in that it spans the ventricle (reviewed in Sedmera, 2011). As these two dominant trabeculae form, they likely also direct the external morphology of the heart, resulting in the projected triangular morphology of the adult ventricle.

Significantly less muscularization is observed in the atrium, but clear bands of pectinate muscles are present in increasing amounts during maturation (Fig. 6 N-O), similar to what has previously been described for zebrafish (Hu et al., 2001). The balloon like nature of the atrium, with its large open lumen, can be seen in serial sections of the heart.

Quantitative analysis of the *Tg(myl7:GFP)^{twu34}* positive cardiac sections allowed the total volume of each chamber to be determined. We have shown that as the fish grows, the ventricle expands linearly in length and width (Figs. 1C, 3C). Here, we show that as VL increases, the ventricle volume grows exponentially (Fig. 7A) at a rate just below that of a sphere. Interestingly, the atrium has almost the same volume as the ventricle and increases in volume at a rate similar to the exponential rate of the ventricle (Fig. 7A). While the chambers have similar total volume, their interior structure is very different (Fig. 6). The atrium has significantly less trabeculation than the ventricle as a measure of total trabeculation (Fig. 7B) and as a percentage of volume (Fig. 7C) with approximately 73% of the ventricle occupied by trabeculae compared to only 33% of the atrium (Fig. 7C) throughout maturation. This difference in chamber morphology likely reflects a difference in chamber function.

Disruption of Cardiac Morphology

Many factors influence the form of the heart during its development and maturation. Here we have provided a standardized series for cardiac maturation to provide a common method for analyzing and characterizing defects in cardiac form during zebrafish maturation. To further demonstrate the value of the staging series we examined the adult *myh6^{hu423/+}* fish. Zebrafish embryos heterozygous for this dominant mutation of atrial specific myosin heavy chain have partial atrial contractility, yet some are viable to adulthood. Contraction of the atria in *myh6^{hu423/+}* mutants is restricted to the region near the atrial-ventricular junction and occasionally to a row of cells at the sinus venosus. Cells within the main body of the atrium generally fail to contract. While the mutation specifically affects contractility in the atrium, ventricular morphology is also altered. Examination of the overall size of the fish

does not indicate a defect in fish growth or maturation due to this reduced contractility. Cardiac rotation also occurs normally in these fish. The VL in *myh6^{hu423/+}* is less than 5% larger than their wildtype siblings. Interestingly, there are three quantifiable differences between the wildtype and *myh6^{hu423/+}* hearts. The BAL is significantly larger when contractility is compromised. The BAL in wildtype fish is 57% of the VL while in *myh6^{hu423/+}* the bulbous arteriosus is significantly longer, with an average length of 68% of the VL. The ventricle is also less circular than that seen in wildtype. Specifically, wildtype ventricles have a spherical index 0.83 while *myh6^{hu423/+}* mutant hearts have a spherical index of 0.69. Lastly, the OT angle in wildtype adults is stable at 35° while *myh6^{hu423/+}* hearts are much more linear with an average OT angle of 24.6°.

Examination of the overall morphology clearly indicates changes in the overall cardiac form of adult *myh6^{hu423/+}* fish. The increase in the length of the bulbous arteriosus and the OT angle are evident in Fig. 4D-F'. In addition, the atrium placement on the heart is also very different. The atrium reaches much higher up over the surface of the bulbous arteriosus (Fig. 4 D, D') and when viewed dorsally, it extends over both the left and right side of the heart (Fig. 4 E, E'). Lastly, the atrium fails to reach the most ventral portions of the ventricle as if the entire atrium is shifted towards the bulbous arteriosus (Fig. 4 F, F'). Examination of ventricular morphology demonstrates the loss of circularity (Fig. 5D, L). The ventricle is very oval in shape, much more reminiscent of wildtype larval morphology; even the AV valve fails to move posteriorly and to the left as would normally occur in wildtypes. Taken together this data suggests that the hearts in the *myh6^{hu423/+}* fail to undergo proper cardiac maturation, perhaps stalled in their developmental progression.

DISCUSSION

Recent interest in the development of heart valves and trabeculae (Martin and Bartman, 2009; Liu et al., 2010; Peshkovsky et al., 2011) highlight the importance of understanding cardiac maturation beyond embryonic stages. While a significant body of work explores early embryonic development of the zebrafish heart, and others have described the adult zebrafish heart, very limited work has explored the maturation of the heart throughout fish life. This study aims to significantly expand upon the characterization of cardiac form from cardiogenesis to adulthood. This detailed morphological analysis of cardiac maturation provides an essential framework for utilizing zebrafish to study the origins and progression of congenital heart conditions and heart disease.

During embryonic development, the embryonic processes of cardiac jogging and cardiac looping to reposition the heart are well described (Baker et al., 2008; de Campos-Baptista et al., 2008; Rohr et al., 2008; Smith et al., 2008). Remarkably, we have identified a third and terminal repositioning of the heart within the thoracic cavity at the transition between embryonic and larval stages. Here we demonstrate this third event which repositions the heart 90 degrees such that the ventricle moves from the right side to a ventral position and the atrium repositions from the left side to deep within the thoracic cavity (Fig. 2). This position is maintained throughout the remainder of the fish's life. Terminal rotation mirrors cardiac-looping in the chick embryo in many ways (Manner, 2000; Ramasubramanian et al., 2008; Kidokoro et al., 2008), though the mechanism driving zebrafish terminal rotation is as

yet unexplored. Cardiac-looping in chick is likely regulated by a number of morphogenetic mechanisms including biomechanical restriction by the splanchnopleure, differential force on the atrium from the inflow tract, and regionalized cardiomyocyte cell shape changes (Manner, 2000; Kidokoro et al., 2008; Latacha et al., 2005; Ramasubramanian et al., 2008). Directed cell migration and cell shape changes are known to direct cardiac jogging and cardiac looping respectively in zebrafish (Auman et al., 2007; Baker et al., 2008). It is likely that these mechanisms work in concert to drive zebrafish terminal rotation.

Zebrafish heart growth and maturation parallels the growth of the fish as a whole. Thus size, measured as the standard length (SL), is used to define both heart and fish maturity. SL is best used to describe post embryonic stages; fish younger than 72 hours, protruding mouth stage, are defined as embryos. Larval fish are defined by a SL of 3.4–12 mm (Parichy et al., 2009) transitioning to juvenile stage at approximately 50 dpf which is likely triggered by a thyroid hormone burst (Brown, 1997; Fig. 1). This is a simple measure of external maturity that can be used on individual fish and accurately reflects cardiac maturity, as we see the most significant changes in cardiac morphology during the larval period. Juvenile fish have all the external morphological features of the adult but are not yet sexually mature. The juvenile heart is also both internally and externally similar in form to the adult heart, though some additional changes in overall form are observed. In general, zebrafish reach sexual maturity at 90 dpf, a size of >18 mm, and are then considered adults (Parichy et al., 2009). We see no further significant changes in cardiac morphology, either internally or externally, once fish reach adulthood.

As the fish doubles in SL, the ventricle and bulbous arteriosus lengths also double, indicating a linear relationship between fish length and ventricle length (Fig. 3). Interestingly, in the *myh6*^{hu423/+}, which have reduced atrial contractility, the bulbous arteriosus length more than doubles. Previous work has demonstrated a log-log ventricle to body weight ratio in zebrafish and other animals (Hu et al. 2000; Loch et al., 2009). However, because of the small size of zebrafish hearts, accurate determination of the ventricle length is a more reliable parameter for individual comparison. Ventricle length has been used in clinical analysis because it can be measured during echocardiography. Ventricle length-to-width ratio has also been used by others as a measure of cardiac fitness (Claireaux et al., 2005) and changes in this ratio serve as an indicator of cardiac disease. Zebrafish ventricles have a spherical index of 0.83, confirming the visual observation that while the hearts are mostly spherical, they are also somewhat elongated (ovoid) in morphology. Changes in the spherical index reflect changes in cardiac fitness and disease state. *myh6*^{hu423/+} hearts have a reduced spherical index (0.69) indicating that the hearts in these fish are more oval in comparison to wildtype hearts.

Study of the external shape of the ventricle (Fig. 5) elucidated clear changes over time as the larval heart, which is rectangular, matured into a triangular juvenile form and finally to a more spherical adult ventricle. These drastic changes in shape are likely due to the increase in blood capacity and force of blood being pumped through the growing body. Reduction of atrial contractility results in morphological changes in the ventricle. While the ventricle in *myh6*^{+/hu423} mutant hearts continues to grow as in wildtype fish, its external morphology does not progress resulting in a very oval adult ventricle. Analysis of the internal ventricular

morphology shows the presence of both disorganized and highly organized trabeculae (Fig. 6). Two distinct trabecular bands form perpendicular to each other and become more defined as the heart matures, a tri-tipped muscle and a transverse band. The tri-tipped muscle attaches at the base of AV valve leaflets of which the adult zebrafish has four (Hu et al., 2001) while the transverse band spans the ventricle near its apex. These structures likely define and help the heart maintain its form, both by preventing over distention and over contraction of the ventricle. They may also help drive the direction of blood flow during cardiac contractions as they form the margins of the ventricular lumen.

While the atrium envelops the ventricle and has an irregular shape, making quantification of the external morphology ineffective, direct observation of overall cardiac morphology is still useful (Fig. 4). Examination of the overall cardiac morphology in *myh6^{hu423/+}* clearly demonstrates changes as a result of reduced atrial contractility. Through analysis of serial sections, we have gained some insight into the atrial anatomy. Interestingly, while the whole chamber size of the atrium and ventricle are similar in zebrafish, the inter-luminal volume is very different in the two cardiac chambers, with the atrium having significantly larger volume than the ventricle. This is in contrast to humans where the atria are about 1/3 the size of the ventricle but have similar internal volume. In zebrafish, like in humans, clear pectinate muscles are observed in the atrium starting during larval development and become more prevalent and refined by adulthood. However, pectinate muscles in human atria are much more defined and are organized into parallel bands of muscles unlike those observed in zebrafish. Nonetheless, they are likely to play similar roles in the function of the atria.

Environmental toxins, drugs, disease and cardiac injury are known to affect the heart in many ways. Changes in the thickness of the compact myocardium, and changes in the outflow tract (bulbous arteriosus) angle in a number of fish species have been associated with exposure to environmental toxins (Gamperl and Farrell, 2004; Claireaux et al., 2005). Here we show that many features of cardiac morphology can be disrupted by genetic reduction of atrial contractility. Further, trabecular volume and patterning are disrupted in many cardiomyopathies. Understanding the cellular, molecular, genetic and epigenetic factors that influence heart development and maturation is key to creating methods of treatment and prevention for these often devastating conditions. Insight into normal cardiac maturation can also serve as a reference for comparison of the mechanisms underlying the progression of early childhood conditions, a very poorly understood area of research. This study serves to set the framework for such studies in zebrafish.

EXPERIMENTAL PROCEDURES

Zebrafish

Zebrafish were maintained as described in Westerfield (2000) using an IACUC approved protocol. Zebrafish were kept through adulthood at a 10–12 fish density per tank in a flow through water (Aquatic Innovation LLC, MA) system at a constant temperature of 28°C. To minimize the variability among our cohorts and yet still represent real lab conditions, two separate fish broods were raised 3 months apart in controlled conditions. These two different sibling sets were examined to account for any husbandry differences but no differences were identified so all data was pooled. Fish were collected at larva through adult stages: 15

(n=36), 30 (n=38), 45 (n=16), 60 (n=31), 90 (n=33), 180 (n=40), 730 (n=28) dpf. Zebrafish were collected without bias for external maturation markers, fixed in 4% paraformaldehyde for 24 hours and photographed. Fish were dissected after fixation because un-fixed atrial morphology is extremely variable and the tissue is very delicate and prone to tearing (Singleman and Holtzman, 2011). To facilitate dissection and visualization of the heart *Tg(myl7:GFP)^{twu34}* (Huang et al., 2003) or *Tg(myl7:GFP)^{twu34};Tg(-5.1myl7:nDsRed2)^{f2}* (Rottbauer et al., 2002) were used except for the immunofluorescence experiments in which wildtype (AB) fish were used.

We have identified a new allele of *myosin heavy chain 6/weak atrium* (*myh6*)^{hu423}, gift from Freek van Eeden. This mutant fails to complement the *myh6*^{m58} allele. cDNA for *myh6* was isolated from *myh6*^{hu423/hu423} mutants as in Berdugo et al., 2003 and reveal a T to A substitution at position 2087. This substitution changes an asparagine (polar) to a lysine (basic) at codon 695 located within the myosin head at the base of the myosin head. Interestingly, this allele is dominant and viable; *myh6*^{hu423/+} mutant carriers are viable to adulthood and when crossed with wild type mates, 46.6% of the offspring have partial loss of atrial contractility (83/178). In-crosses of *myh6*^{hu423/+} mutants result in 28.6% phenotypically wild type embryos, 48.6% partial loss of atrial contractility and 22.8% have complete loss of atrial contractility and are embryonic lethal (178:302:142 respectively). Together this data indicates that *myh6*^{hu423} is a dominant allele of *myosin heavy chain 6/weak atrium*.

Mutant analysis was conducted on 10 adults identified as *myh6*^{hu423/+}, from a *myh6*^{hu423/+} out cross, by their partial atrial contraction phenotype at 3 days post fertilization.

Immunofluorescence

Wholmount immunofluorescence experiments were performed using MF20 and S46 antibodies as previously described (Berdugo et al., 2003). Secondary antibodies, goat anti-mouse IgG2b-TRITC, and goat anti-mouse IgG1-FITC were used at 1:250 (Southern Biotech). Embryos were imaged using a Leica SP5 Confocal with its associated programming (LAS AF Version 2.0.2). 3D maximal projections of confocal image stacks were generated using Imaris (Bitplane) as were the Supplemental Movies 1–4. Images shown are representative of at least 10 samples.

Morphological Analysis

Carefully staged zebrafish, were measured for SL using digital calipers and were photographed to provide a record of external maturation. The hearts were dissected (Singleman and Holtzman, 2011) and photographed in multiple orientations. Fish and hearts were photographed using a stereomicroscope (Zeiss SteREO Discovery.V12) and camera (Zeiss AxioCam MRc) with its associated programming (Zeiss AxioVision). Photographs of dissected hearts were analyzed using ImageJ (NIH).

The VL was measured from the apex of the ventricle to the junction of the ventricle with the bulbous arteriosus (Fig. 1I and 3A). VW was then measured by creating a perpendicular line at the midpoint of the VL extending to the edges of the chamber (Fig. 3A). The BAL was

measured from the junction of the ventricle with the bulbous arteriosus to the point where the bulbous arteriosus stops tapering and becomes the cylindrical ventral aorta (Fig. 3A). Extending the line of the VL past the junction of the ventricle and bulbous arteriosus, then connecting it with the BAL, formed an angle that was measured as the OT angle (Fig. 3B, E and F). Movies of cardiac contractions were taken with a Panasonic FX30 on a Zeiss Stemi 2000 and edited in Quicktime. Individual frames were extracted and the OT angle was calculated for each frame as described above.

Gelatin Sectioning

Hearts were embedded in gelatin solution and sectioned using a vibratome (Vibratome 3000, Leica) (Ulrich et al., 2003). Gelatin was trimmed to orient the heart for sagittal sectioning. Sections were typically cut at 40µm thickness. Gelatin blocks and sections were stored in 1X PBS. The sections were mounted on slides in glycerol and photographed using a 10x dry objective on an Apotome (Zeiss AxioImager.M2) and camera (Zeiss AxioCam MRm) with its associated program (Axiovision Rel 4.8) to visualize GFP in *Tg(myl7:GFP)^{twu34}* (Huang et al., 2003) or DsRed in *Tg(-5.1myl7:nDsRed2)^{f2}* (Rottbauer et al., 2002) within the cardiac muscle. To visualize the cardiac valves we used *Tg(fli1a:EGFP)^{y1}* (Lawson and Weinstein, 2002). Images of very large samples were stitched together in Photoshop CS3 (Adobe).

The percent of muscular tissue was analyzed from these photographs using ImageJ (NIH). For each image of the serial sections, the ventricle was outlined and the percent area of the picture was measured using program functions to measure area, and area percent while utilizing the threshold tool. Volume of a single heart was determined by summing the area of each section of that heart and multiplying by 40µm, the thickness of each section. All graphs were made in excel (Microsoft) and all images were processed in Photoshop and Indesign CS3 and CS5 (Adobe).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We wish to thank Marie Birne and Arellys Uribe for managing the fish facility, Areti Tsiola for technical support, Jaymie Estevez, Dorit Ziv and Farhana Oza for assistance with experiments, and Deborah Yelon for fish and ongoing support and PoKay Ma, Kimara Targoff and Sana Khan for helpful discussion and the anonymous reviewers for helpful comments. Thanks to Freek van Eeden for the *myh6^{hu423}* mutant fish. Some of the experiments were done on equipment from the Core Facilities for Imaging, Cellular and Molecular Biology at Queens College; CUNY. This study was supported by Howards Hughes Medical Institute Summer Program for Undergraduate Students (to C.S.), NIH R03 HD055399, PSC-CUNY PSCREG-40-1135 and PSCREG-39-61492 and Queens College Research Enhancement funds (to N.G.H).

REFERENCES

- Adolf B, Chapouton P, Lam CS, Topp S, Tannhauser B, Strahle U, Gotz M, Bally-Cuif L. Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. *Dev Biol.* 2006; 295:278–293. [PubMed: 16828638]
- Antkiewicz DS, Peterson RE, Heideman W. Blocking expression of AHR2 and ARNT1 in zebrafish larvae protects against cardiac toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Sci.* 2006; 94:175–182. [PubMed: 16936225]

- Arrenberg AB, Stainier DY, Baier H, Huisken J. Optogenetic control of cardiac function. *Science*. 2010; 330:971–974. [PubMed: 21071670]
- Auman HJ, Coleman H, Riley HE, Olale F, Tsai HJ, Yelon D. Functional modulation of cardiac form through regionally confined cell shape changes. *PLoS Biol*. 2007; 5:e53. [PubMed: 17311471]
- Baker K, Holtzman NG, Burdine RD. Direct and indirect roles for Nodal signaling in two axis conversions during asymmetric morphogenesis of the zebrafish heart. *Proc Natl Acad Sci U S A*. 2008; 105:13924–13929. [PubMed: 18784369]
- Bakkers J. Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovasc Res*. 2011; 91:279–288. [PubMed: 21602174]
- Berdougo E, Coleman H, Lee DH, Stainier DY, Yelon D. Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development*. 2003; 130:6121–6129. [PubMed: 14573521]
- Chico TJ, Ingham PW, Crossman DC. Modeling cardiovascular disease in the zebrafish. *Trends Cardiovasc Med*. 2008; 18:150–155. [PubMed: 18555188]
- Chinchilla A, Franco D. Regulatory mechanisms of cardiac development and repair. *Cardiovasc Hematol Disord Drug Targets*. 2006; 6:101–112. [PubMed: 16787195]
- Claireaux G, McKenzie DJ, Genge AG, Chatelier A, Aubin J, Farrell AP. Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. *J Exp Biol*. 2005; 208:1775–1784. [PubMed: 15879059]
- de Campos-Baptista MI, Holtzman NG, Yelon D, Schier AF. Nodal signaling promotes the speed and directional movement of cardiomyocytes in zebrafish. *Dev Dyn*. 2008; 237:3624–3633. [PubMed: 18985714]
- Di Donato M, Dabic P, Castelvechio S, Santambrogio C, Brankovic J, Collarini L, Joussef T, Frigiola A, Buckberg G, Menicanti L. Left ventricular geometry in normal and post-anterior myocardial infarction patients: sphericity index and 'new' conicity index comparisons. *Eur J Cardiothorac Surg*. 2006; 29(Suppl 1):S225–S230. [PubMed: 16564696]
- Gamperl AK, Farrell AP. Cardiac plasticity in fishes: environmental influences and intraspecific differences. *J Exp Biol*. 2004; 207:2539–2550. [PubMed: 15201287]
- Glickman NS, Yelon D. Cardiac development in zebrafish: coordination of form and function. *Semin Cell Dev Biol*. 2002; 13:507–513. [PubMed: 12468254]
- Gorelick DA, Watson W, Halpern ME. Androgen receptor gene expression in the developing and adult zebrafish brain. *Dev Dyn*. 2008; 237:2987–2995. [PubMed: 18816841]
- Hicken CE, Linbo TL, Baldwin DH, Willis ML, Myers MS, Holland L, Larsen M, Stekoll MS, Rice SD, Collier TK, Scholz NL, Incardona JP. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. *Proc Natl Acad Sci U S A*. 2011; 108:7086–7090. [PubMed: 21482755]
- Hill AJ, Heiden TC, Heideman W, Peterson RE. Potential roles of Arnt2 in zebrafish larval development. *Zebrafish*. 2009; 6:79–91. [PubMed: 19374551]
- Holtzman NG, Schoenebeck JJ, Tsai HJ, Yelon D. Endocardium is necessary for cardiomyocyte movement during heart tube assembly. *Development*. 2007; 134:2379–2386. [PubMed: 17537802]
- Hu N, Sedmera D, Yost HJ, Clark EB. Structure and function of the developing zebrafish heart. *Anat Rec*. 2000; 260:148–157. [PubMed: 10993952]
- Hu N, Yost HJ, Clark EB. Cardiac morphology and blood pressure in the adult zebrafish. *Anat Rec*. 2001; 264:1–12. [PubMed: 11505366]
- Huang CJ, Tu CT, Hsiao CD, Hsieh FJ, Tsai HJ. Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev Dyn*. 2003; 228:30–40. [PubMed: 12950077]
- Icardo JM. Developmental biology of the vertebrate heart. *J Exp Zool*. 1996; 275:144–161. [PubMed: 8676095]
- Kelsh RN, Harris ML, Colanesi S, Erickson CA. Stripes and belly-spots -- a review of pigment cell morphogenesis in vertebrates. *Semin Cell Dev Biol*. 2009; 20:90–104. [PubMed: 18977309]
- Koide T, Miyasaka N, Morimoto K, Asakawa K, Urasaki A, Kawakami K, Yoshihara Y. Olfactory neural circuitry for attraction to amino acids revealed by transposon-mediated gene trap approach in zebrafish. *Proc Natl Acad Sci U S A*. 2009; 106:9884–9889. [PubMed: 19497864]

- Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman MJ, Seward J, Shanewise J, Solomon S, Spencer KT, St John Sutton M, Stewart W. Recommendations for chamber quantification. *Eur J Echocardiogr.* 2006; 7:79–108. [PubMed: 16458610]
- Lin YF, Swinburne I, Yelon D. Multiple influences of blood flow on cardiomyocyte hypertrophy in the embryonic zebrafish heart. *Dev Biol.* 2012; 362:242–253. [PubMed: 22192888]
- Liu J, Bressan M, Hassel D, Huisken J, Staudt D, Kikuchi K, Poss KD, Mikawa T, Stainier DY. A dual role for ErbB2 signaling in cardiac trabeculation. *Development.* 2010; 137:3867–3875. [PubMed: 20978078]
- Liu J, Stainier DY. Tbx5 and Bmp signaling are essential for proepicardium specification in zebrafish. *Circ Res.* 2010; 106:1818–1828. [PubMed: 20413782]
- Lohr JL, Yost HJ. Vertebrate model systems in the study of early heart development: *Xenopus* and zebrafish. *Am J Med Genet.* 2000; 97:248–257. [PubMed: 11376436]
- Mably JD, Mohideen MAPK, Burns CG, Chen J-N, Fishman MC. heart of glass regulates the concentric growth of the heart in zebrafish. *Current biology : CB.* 2003; 13:2138–2147. [PubMed: 14680629]
- Major RJ, Poss KD. Zebrafish Heart Regeneration as a Model for Cardiac Tissue Repair. *Drug Discov Today Dis Models.* 2007; 4:219–225. [PubMed: 19081827]
- Manasek FJ. Histogenesis of the embryonic myocardium. *Am J Cardiol.* 1970; 25:149–168. [PubMed: 5413194]
- Manasek FJ, Monroe RG. Early cardiac morphogenesis is independent of function. *Dev Biol.* 1972; 27:584–588. [PubMed: 5029499]
- Manner J, Schlueter J, Brand T. Experimental analyses of the function of the proepicardium using a new microsurgical procedure to induce loss-of-proepicardial-function in chick embryos. *Dev Dyn.* 2005; 233:1454–1463. [PubMed: 15977171]
- Marcus RC, Delaney CL, Easter SS Jr. Neurogenesis in the visual system of embryonic and adult zebrafish (*Danio rerio*). *off. Vis Neurosci.* 1999; 16:417–424. [PubMed: 10349963]
- Martin RT, Bartman T. Analysis of heart valve development in larval zebrafish. *Dev Dyn.* 2009; 238:1796–1802. [PubMed: 19449301]
- Mione MC, Trede NS. The zebrafish as a model for cancer. *Dis Model Mech.* 2010; 3:517–523. [PubMed: 20354112]
- Norton W, Bally-Cuif L. Adult zebrafish as a model organism for behavioural genetics. *BMC Neurosci.* 2010; 11:90. [PubMed: 20678210]
- Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. *Dev Dyn.* 2009; 238:2975–3015. [PubMed: 19891001]
- Paskin TR, Iqbal TR, Byrd-Jacobs CA. Olfactory bulb recovery following reversible deafferentation with repeated detergent application in the adult zebrafish. *Neuroscience.* 2011; 196:276–284. [PubMed: 21933699]
- Peshkovsky C, Totong R, Yelon D. Dependence of cardiac trabeculation on neuregulin signaling and blood flow in zebrafish. *Dev Dyn.* 2011; 240:446–456. [PubMed: 21246662]
- Rohr S, Otten C, Abdelilah-Seyfried S. Asymmetric involution of the myocardial field drives heart tube formation in zebrafish. *Circ Res.* 2008; 102:e12–e19. [PubMed: 18202314]
- Rottbauer W, Saurin AJ, Lickert H, Shen X, Burns CG, Wo ZG, Kemler R, Kingston R, Wu C, Fishman M. Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell.* 2002; 111:661–672. [PubMed: 12464178]
- Schoenebeck JJ, Yelon D. Illuminating cardiac development: Advances in imaging add new dimensions to the utility of zebrafish genetics. *Semin Cell Dev Biol.* 2007; 18:27–35. [PubMed: 17241801]
- Serluca FC. Development of the proepicardial organ in the zebrafish. *Dev Biol.* 2008; 315:18–27. [PubMed: 18206866]
- Singleman C, Holtzman NG. Heart dissection in larval, juvenile and adult zebrafish, *Danio rerio*. *J Vis Exp. Sep.* 2011; 30(55):e3165.

- Smith KA, Chocron S, von der Hardt S, de Pater E, Soufan A, Bussmann J, Schulte-Merker S, Hammerschmidt M, Bakkers J. Rotation and asymmetric development of the zebrafish heart requires directed migration of cardiac progenitor cells. *Dev Cell*. 2008; 14:287–297. [PubMed: 18267096]
- Ulrich F, Concha ML, Heid PJ, Voss E, Witzel S, Roehl H, Tada M, Wilson SW, Adams RJ, Soll DR, Heisenberg CP. *Slb/Wnt11* controls hypoblast cell migration and morphogenesis at the onset of zebrafish gastrulation. *Development*. 2003; 130:5375–5384. [PubMed: 13129848]
- Zupanc GK. Adult neurogenesis and neuronal regeneration in the brain of teleost fish. *J Physiol Paris*. 2008; 102:357–373. [PubMed: 18984045]

Bullet points

- A third cardiac rotation, occurring at standard length 3.4 mm (104 hours post fertilization) places the heart in its final orientation.
- Cardiac maturation significantly modifies cardiac form during larval stages but continues throughout the juvenile stages to adulthood.
- Ventricle size directly correlates with fish length.
- Two dominant cardiac trabeculations regulate cardiac form and function.
- Partial loss of atrial contraction seen in *myh6/wea^{hu423/+}* results in maintenance of juvenile cardiac form throughout adulthood.

Figure 1. Standard Length and Cardiac Maturation

(A) The standard length (SL) of the fish as measured from snout to the base of the tail is a more meaningful measure of maturation state than zebrafish age. Zebrafish show a wide range of SL by age (B). The solid line represents mean growth while upper and lower dashed lines represent 95th and 5th percentiles size range respectively. As the zebrafish grows, its cardiac needs grow proportionally, as seen by the linear relationship between SL and ventricle length (VL), measured from the ventricle apex to the bulboventricular junction (C) as indicated with the yellow line in (I). Maturation of zebrafish from larva (D) to juvenile (E) and finally in adulthood (F) demonstrates the changes in external features. Cardiac maturation from larva (G) to juvenile (H) and finally in adulthood (I) show not only an increase in heart size, but also changes in cardiac form. BA= bulbus arteriosus, V=ventricle and A= atrium. Scale bar= 100 μ m.

Figure 2. Terminal Cardiac Rotation

At the transitions between embryonic and larval stages, the atrium moves from the left side of the ventricle to a dorsal location, behind the ventricle. (A) Schematic ventral view of embryonic chamber repositioning. The ventricle (magenta) and atrium (light green) are side by side and then the ventricle moves to be more ventral to the atrium. (B) Ventral view of muscle marker MF20 antibody (magenta) and the atrial specific marker S46 antibody (green) at SL 3.2 (84 hpf) embryo show chambers next to each other. Beginning at SL 3.3 (100 hpf), cardiac rotation initiates. By SL 3.4 (104 hpf) cardiac rotation is complete with

the SL 3.6 (120 hpf) heart now in the final orientation. Movies clearly demonstrating the relative three-dimensional organization of the heart at each stage is available as Supplementary Movie 1–4. (C) While two distinct cardiac chambers are evident in this left lateral view of *Tg(myl7:GFP)^{twu34}* labeled heart, the rudiment of the primary heart tube from which the cardiac chambers balloon is visible on the dorsal aspect of the heart (arrows) (D) at SL 4.3. V=ventricle, A= atrium, JM=Jaw Muscles, L=Left, R=Right. Scale bars =100µm.

Figure 3. Quantitative Cardiac Analysis

To better understand cardiac maturation, we carried out a series of quantitative analyses. A) and B) show measurements taken for each external cardiac feature. For better characterization of the ventricular morphology the atrium in (B) has been removed in (A). Bulbous arteriosus length (BAL- purple), ventricle length (VL-yellow) and ventricle width (VW- red) are labeled. The outflow tract angle (OT angle) is indicated in green (B, E and F). (C) VW increases linearly with VL. (D) BA length increases linearly with VL, staying about half the length of the ventricle. The OT angle changes throughout each cardiac contraction by as much as 30°. E and F are images extracted from a Supplemental Movie 5 demonstrate a minimal OT angle of 29° during ventricular ejection (E) and a maximal OT angle of 46° following atrial systole (F). (G) During embryonic and larval development that ends at a VL of 0.5 mm, the OT angle, increases quickly followed by a period of very slow change though adulthood. BA= bulbous arteriosus, V=ventricle, A= atrium, a= anterior, p=posterior, d=dorsal and v= ventral orientation. Scale bar = 100µm.

Figure 4. Atrial morphology and its disruption in *wea* mutants

The atrium is a U shaped structure that covers the ventricle, shown here in dorsal, left and right lateral views in wildtype (A-C) and *myh6^{hu423/+}* mutants (D-F), paired with a schematic image (A'-C') and (D'-F'). A, A') The left side of the ventricle is partially covered by the atrium. B, B') The dorsal aspect of the ventricle is predominantly covered by the atrium while the right side of the ventricle (C, C') has very limited coverage of the atrium. The atrium in *myh6^{hu423/+}* mutants is morphologically distinct from wildtype. D, D') The left side of the ventricle is partially covered by the atrium and the bulbous arteriosus. E, E') The dorsal aspect of the ventricle is completely covered by the atrium while the right side is barely covered by the atrium (F, F'). A= Atrium (green), V= Ventricle (red), BA= Bulbous arteriosus (orange).

Figure 5. Ventricule morphology maturation and its disruption in *wea* mutants

Ventricule morphology is observed after removal of the atrium in wildtype (A-C and I-K) and in *wea* mutants (D and L). A-D are right lateral images of dissected ventricles. (A) Larval ventricles have a rectangular form (E), (B) juvenile heart are triangular (F) while (C) adult heart have a round shaped ventricle (G). E-H are tracings of the ventricle (V- red) and bulbous arteriosus (BA-orange) with representative ventricle morphology in the lower right-hand corner. The ventricle in *wea* mutant adults (D) fails to mature and appears oval (H). I-L show dorsal images of the same ventricles as in A-D. From a dorsal view larval heart (I)

have an oval form (M). (J) juvenile hearts have a triangular shape (N) and (K) adult hearts have an extension (arrow) of the ventricle opposite of the atrioventricular valve (O). The immature oval morphology of the *wea* ventricle is also evident when viewed dorsally (L and P). M-P tracings of the ventricle and bulbous with standard shapes in the lower right-hand corner. The (*) indicates the atrioventricular valve location.

Figure 6. Internal chamber organization

The ventricular myocardium is highly trabeculated by the early larval stages and becomes progressively more organized during maturation (A-L). A-L are serial sections of *Tg(myl7:GFP)^{twu34}* hearts during different stages of maturation. A-C are larval, E-G are juvenile and I-K are representative adult hearts. During maturation, two clear muscle groups become apparent. A transverse band (arrowhead) can be seen in A, E, and J and is depicted in blue on the diagram in D, H and L. A tri-tipped muscle (*) in B, F and J and depicted in yellow on the diagram in D, H and L. (M) The tri-tipped muscle (false-colored magenta with *Tg(-5.1myl7:nDsRed2)^{f2}*) can be seen attached the base of the AV and BV valves (labeled green with *Tg(fli1a:EGFP)^{y1}*). The atrium is significantly less trabeculated than the ventricle even during larval and adult stages however pectinate muscles are observed (arrows in N, and O). P demonstrates the presence of a clear ventricular compact myocardium (CM) adjacent to the trabeculae (Tra) that is three cell layers thick in this cross section of an adult heart (arrows) with myocardial nuclei false-colored magenta using *Tg(-5.1myl7:nDsRed2)^{f2}* and the myocardial cells green with *Tg(myl7:GFP)^{twu34}*.

Figure 7. Quantitative analysis of chamber trabeculation

As the ventricle grows in length, the volume increases exponentially (A). The atrial volume increases almost identically to the ventricle (A). The amount of trabeculation in the ventricle is significantly higher than in the atrium, seen as a total amount of trabeculation (B) and as a percentage of total volume (C). Solid gray line, circles: ventricle measurements; Dashed black line, diamonds: atrial measurements.