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Cardiac and somatic parameters in zebrafish: tools for the evaluation of cardiovascular function

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Abstract Cardiovascular diseases are a worldwide public health problem. To date, extensive research has been conducted to elucidate the pathophysiological mechanisms that trigger cardiovascular diseases and to evaluate therapeutic options. Animal models are widely used to achieve these goals, and zebrafish have emerged as a low-cost model that produces rapid results. Currently, a large body of research is devoted to the cardiovascular development and diverse cardiovascular disorders of zebrafish embryos and larvae. However, less research has been conducted on adult zebrafish specimens. In this study, we evaluated a method to obtain and to evaluate morphometric parameters (of both the entire animal and the heart) of adult zebrafish. We used these data to calculate additional parameters, such as body mass index, condition factor and cardiac somatic index. This method and its results can be used as reference for future studies that aim to evaluate the pathophysiological aspects of the zebrafish cardiovascular system.

Keywords Zebrafish · Cardiovascular · Heart · Ventricle

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Introduction

Cardiovascular diseases are a major global public health problem worldwide and lead to high morbidity and mortality and negative socioeconomic impacts. Because of this reason, basic and clinical research in this field is both extensive and rigorous. The research goals are broad and range from understanding the pathophysiological mechanisms that trigger these diseases to proposing preventative and therapeutic treatment options. However, there are multiple limitations of clinical research, including high economic costs, complex logistics and bioethical considerations. Due to these disadvantages to clinical investigations, animal models are used as an alternative for studies in this field. There are many animal models used for preclinical studies, including both invertebrates and vertebrates. Some of the most commonly used animal models are insects (*Drosophila melanogaster*), worms (*Caenorhabditis elegans*), fish (*Danio rerio*), amphibians (*Xenopus laevis*), rodents (rats, mice, and rabbits) and birds (Zaffran and Frasch 2002). Using these animal models, experimental cardiovascular studies have addressed embryological, developmental, functional, morphological, pathophysiological, pharmacological and toxicological questions (Hasenfuss 1998; Russell and Proctor 2006). Many published studies highlight the common genetic, molecular, structural and functional patterns of many of the species studied. These common patterns underlie cardiovascular

differentiation and developmental processes and suggest that the development of the cardiovascular system and its signaling pathways has been highly conserved over evolutionary history (Zaffran and Frasch 2002). For many species, these common signaling pathways depend on the expression of specific genes that code for well-defined molecules that determine and regulate the development of cardiovascular structures. The key genes for cardiovascular development can regulate one another; these genes include GATA, CMLC, nkx, Tbox and grl (Bakkers 2011; Reiter et al. 1999; Staudt and Stainier 2012). Currently, the zebrafish is one of the most widely used animal models in biomedical science, including cardiology (Kimmel 1989; Stainier 2001). The zebrafish model has multiple advantages over other experimental models. Zebrafish have a high reproductive rate and rapid development. The zebrafish genome is similar to that of humans (approximately 70 % genes are orthologous), and zebrafish also have functionally similar cardiovascular systems to that of humans (Howe et al. 2013). Early-stage zebrafish do not depend on the circulatory system to survive, as their tissues are sufficiently oxygenated via diffusion in their aquatic medium; therefore, early-stage zebrafish can be used for embryological, physiological and genetic studies. Additionally, zebrafish populations are easily maintained at relatively low cost in the laboratory (Kimmel 1989; Stainier 2001).

The development of the cardiovascular system (i.e., the heart and the blood vessels) in zebrafish originates from the primitive mesoderm where early differentiation occurs. Initially, a group of cells gives rise to an early cardiac and hemangioblasts disk. The disk gradually develops into a cardiac cone, from which a tubular structure is formed and the heart tube begins to contract in a coordinated and rhythmic fashion at 22 h post-fertilization (hpf). At 36 hpf, the segmentation process and the specific rotation of the cardiac tube give rise to different well-defined cardiac chambers: a venous sinus, an atrium, a ventricle and an arterial bulb. These four compartments are arranged in a series and are separated by constrictions and heart valves that ensure unidirectional blood flow (Bakkers 2011; Dahme et al. 2009; Holden et al. 2011; Hu et al. 2000; Liu and Stainier 2012; Staudt and Stainier 2012). At the same time, the mesoderm produces hemangioblasts that produce angioblasts and endothelial cells. At 24 hpf, a simple vascular circuit is formed from the dorsal aorta and the axial vein (Serbedzija et al. 1999). Intersegmental vessels that give rise to different vascular circuits are derived from the aorta (Childs et al. 2002). Although there is extensive research regarding cardiovascular development in zebrafish from early embryonic to late larval stages, literature on the cardiovascular system of adult zebrafish is scarce. Because many chronic heart diseases in humans primarily affect the adult population, more information is needed on the structure and function of the cardiovascular system in adult zebrafish. By doing so, normal points of reference may be established that can be compared with abnormal processes; this information can be extrapolated to other animal species, including humans. The aim of this study is to provide a method and some information on the morphological, functional, macroscopic and microscopic aspects of the adult zebrafish heart.

Methods

The present study was conducted using wild-type zebrafish specimens. Zebrafish were obtained from local suppliers. Fish were kept in aquaria with permanent aeration and light–dark cycles of 14:10 p.m. and were fed a commercial diet once daily. A constant temperature and pH (25 °C and 7.4, respectively) were maintained. The protocol for maintaining the specimens was approved by the AICUC of the Pontificia Universidad Javeriana. We used 10 specimens; this sample size is consistent with the principle of the 3 R's, which advises minimizing the number of replicates and optimizing their use.

Anesthesia

Fish were anesthetized using a solution of distilled water and 0.6 mM MS-222 (tricaine methanesulfonate ethyl-3 aminobenzoate; Sigma-Aldrich) for handling and conducting the various procedures. Under general anesthesia, we measured weights and lengths. Afterwards, euthanasia was performed using a 6 mM of MS-222 solution. Then, the specimens were fixed in a solution of 4 % paraformaldehyde for 24 h to facilitate dissection and organ removal.

Dissection

For dissection, we used plastic foam with a central recess where the specimen was placed in a supine position. To extract the organs, we made a ventral midline incision from the gills to the anal fin (Fig. 1b). We then fixed the walls to the sponge with pins to expose the internal organs. First, we extracted the gonads, which are located most superficially, and then, we extracted the digestive tract, kidney, pancreas and heart. The organs of each specimen were stored in vials containing 4 % paraformaldehyde for subsequent histological processing.

Histology

The zebrafish hearts were fixed with 4 % paraformaldehyde for 24 h. Whole hearts were embedded, sectioned into 20–50 μm sections and mounted on slides. Sections were deparaffinized, rehydrated and stained with hematoxylin/eosin. Sample processing was performed in the Department of Pathology, Faculty of Medicine, Pontificia Universidad Javeriana. Digital images were taken from slides using a conventional microscope (Leica DM500) and a video camera (S3CMOS, Touptek Photonics). Measurements of lengths and angles of the ventricular chamber and thickness of ventricular walls were made using the free software, ImageJ (Fig. 2a).

Measurements

In this study, we obtained both direct and indirect measurements. Direct basic measurements included the size and weight of each specimen and the weight and length of the heart of each animal. When the animal was under anesthesia, the weights of the animal and heart were measured using a Sartorius analytical balance. Digital images were taken of the adult zebrafish and the isolated heart using a stereomicroscope (2X–4X Scientific) and a video camera (S3CMOS, Touptek Photonics). Then, measurements (length, cardiac diameters, angles) were obtained from these images using ImageJ. In addition, measurements of ventricle wall were taken in 4 random zones in each histological section in order to determine compact and spongy myocardium thickness. Indirect measurements were obtained from direct measurements using validated formulas of body mass index (BMI), cardiosomatic index (CSI) and Fulton's condition factor (Factor K). For all variables, basic descriptive statistics (mean, standard deviation and error) were obtained.

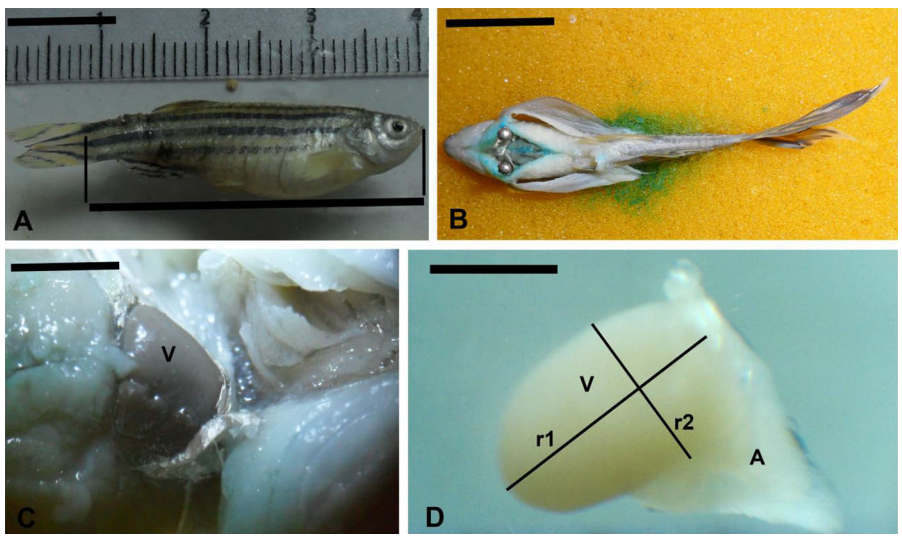

Fig. 1 Procedures implemented for zebrafish. **a** Method of measuring adult zebrafish size. **b** Adult zebrafish in supine position with median incision for evisceration. **c** In situ zebrafish heart, as viewed through a scientific stereo microscope at ×20 magnification. **d** Isolated zebrafish heart, showing the major (*r1*) and minor (*r2*) axes at ×20 magnification. In **a** and **b**, the *top left line* represents 1 cm. In **c** and **d**, the *upper left line* represents 1 mm. *V* ventricle, *A* atrium

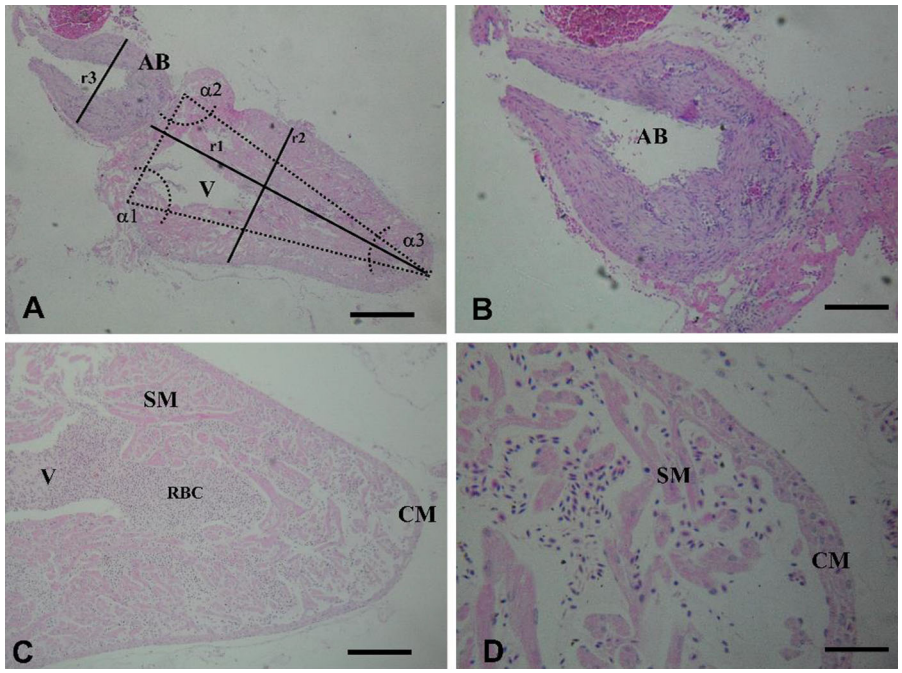

Fig. 2 Histology of the zebrafish heart. **a** Zebrafish heart with two visible chambers: the ventricle (*V*) and arterial bulb (*AB*). **b** Arterial bulb of the zebrafish heart. **c** Ventricular apex of the zebrafish heart. The compact myocardium (*CM*) and the spongy myocardium (*SM*) are shown. **d** Ventricular wall. The compact myocardium and spongy myocardium are well defined. *Lines* represent the major axis (*r1*), the minor axis (*r2*) of the ventricle, and the diameter of the arterial bulb (*r3*). The angles shown are *α1*, *α2* and ventricular *α3*. RBC: red blood cells. Image magnification is ×4 or ×10, and images were taken with an upright microscope (Leica DM500). The *scale bar* on the *bottom right* in **a** is 0.2 mm. The *scale bars* in **b** and **c** are 0.1 mm, and the *scale bar* in **d** is 0.05 mm

Results

Direct or primary parameters

Basic measurements were performed for both the specimens and the isolated heart or histological preparations. These measurements include weight and body size, weight and heart ventricular lengths, angles and wall thickness of the ventricle and diameter of the arterial bulb (Figs. 1, 2; Table 1).

Weight and body size

The average weight and size of all specimens ($n = 10$) were 0.743 ± 0.181 g and 4.19 ± 0.32 cm, respectively. The average weight and size of males ($n = 6$) were 0.689 ± 0.10 and 4.3 ± 0.3 g cm, respectively. For females ($n = 4$), average weight and size were 0.824 ± 0.28 g and 4.1 ± 0.4 cm, respectively.

Heart weight

Hearts were measured separately from specimens. Average heart weight was 1.37 ± 0.59 mg. The heart weight represents no more than 2 % of the total body weight of adult zebrafish, and a positive correlation was observed between heart and body weight (Fig. 3a–b).

Heart morphometry

Measurements of the main cardiac chamber, the ventricle, were obtained from both the isolated organ and from histological preparations. We measured two axes: the major axis (r1), defined as the distance from the apex to the midpoint of the base of ventricle, and the minor axis (r2), defined as widest segment perpendicular to the ventricular major axis (Pombo et al. 2012). The average length of the major axis was

Table 1 Cardiosomatic measurements in zebrafish

Primary parameters	
Weight (g)	0.743 ± 0.181
Height (cm)	4.19 ± 0.32
Heart (mg)	1.37 ± 0.59
Cardiac parameters	
Wall thickness	
Atrium	0.18 ± 0.053
Ventricle	0.084 ± 0.013
Ventricle	
Length (isolated organ)	
Major axis (mm)	1.714 ± 0.296
Minor axis (mm)	1.192 ± 0.144
Width–length ratio	0.704 ± 0.059
Length (histology)	
Major axis (mm)	1.543 ± 0.132
Minor axis (mm)	1.142 ± 0.093
Width–length ratio	0.741 ± 0.047
Ventricular angles	
α1	84.82 ± 9.51
α2	68.66 ± 9.35
α3	30.36 ± 2.96
Arterial bulb	
Diameter (mm)	0.478 ± 0.019
Secondary or derived parameters	
Body mass index (g/cm^2)	0.0425 ± 0.0093
Cardiosomatic index (%)	0.1851 ± 0.0697
Factor K (g/cm^3)	1.0567 ± 0.2501

Primary data were obtained from direct measurements of the specimen or sample. Secondary parameters were calculated from the primary parameters by applying the formulas for BMI, cardiosomatic index and Factor K. Data are shown as the mean ± SD

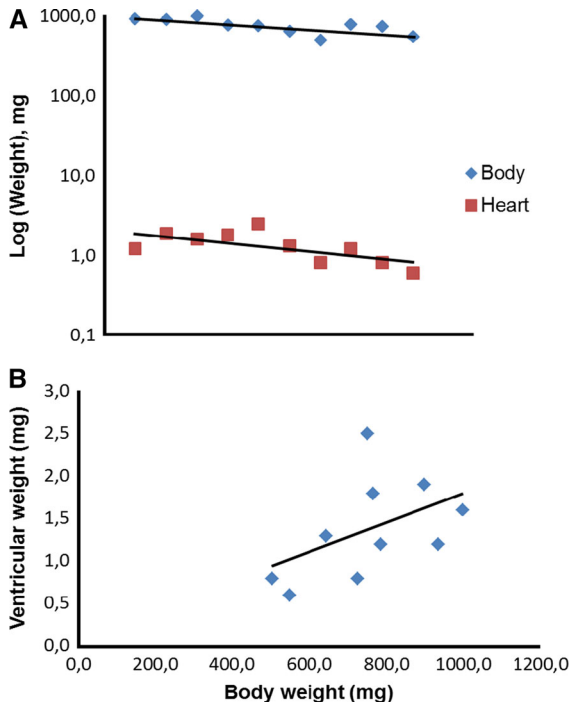

Fig. 3 Body and heart weight. **a** Semilog plot of body and ventricular weight of each adult zebrafish. Heart weight represents <2 % of the total body weight. **b** Ventricular weight versus body weight plot. *Graph* shows a little bit linearity ($y = 0.0018x - 0.0071$)

1.714 ± 0.296 mm and that of the minor axis was 1.192 ± 0.144 mm. The ratio of the minor axis to the major axis (i.e., r2/r1, sphericity index; Claireaux et al. 2005) was 0.704 ± 0.059; that is, the minor axis was approximately 70 % the length of the major axis (Fig. 1d).

In histological sections, the ventricular length of major axis was 1.543 ± 0.132 mm and the minor axis was 1.142 ± 0.093 mm. The ratio of the minor axis to the major axis was 0.741 ± 0.047; that is, the minor axis was 74 % the length of the major axis (Fig. 2a). Although there are differences between the isolated organ and histological measurements, the sphericity index was similar, in both cases, to previous reports (Claireaux et al. 2005). We also measured ventricular angles: α1 and α2, which were the angles at the base of the ventricle, and α3, which was the vertex angle (Fig. 2a). The value of α1 was 84.82 ± 9.51, and the value of α2 was 68.66 ± 9.35. The α3 value was 30.36 ± 2.96. Other authors have described these angles and ventricle shapes in several wild and farmed species of fish; it has been suggested that these measurements can be used to determine the impact of environmental factors on cardiac structure and function (Pombo et al. 2012).

The ventricular wall was thicker than the atrial wall (0.18 ± 0.053 vs. 0.084 ± 0.013 mm, respectively). Two clearly defined layers were evident in the ventricular wall: the compact myocardium and the spongy myocardium. The spongy myocardium represents approximately 70–80 % of the thickness of the ventricle wall (Fig. 2c, d). These data are consistent with data reported for other fish species, where the

thickness of the compact layer fluctuates between 0.05 and 0.25 mm (Pombo et al. 2012).

The diameter of the arterial bulb was also measured (Fig. 2a). Because of its globular shape, the arterial bulb diameter was measured at its widest segment perpendicular to the longitudinal axis of the arterial bulb. The average diameter of the arterial bulb was 0.478 ± 0.019 mm. According to some authors, this parameter is important because it reveals the function of the arterial bulb; in some species, the arterial bulb has limited utility, and in other species, it protects branchial circulation due to the presence of smooth muscle and elastic tissue (Pombo et al. 2012).

Secondary or derivatives parameters

From our initial measurements, we calculated the additional parameters of BMI, CSI and Fulton's condition factor. BMI links body weight and size and is obtained by the following formula:

$$\text{BMI} = \frac{\text{Body weight (g)}}{\text{Length}^2\,(\text{cm}^2)}$$

This index has been traditionally used to identify overweight and obese individuals (Oka et al. 2010).

The CSI relates heart weight and total body weight. This index is expressed as a percentage and is obtained from the following formula:

$$\text{CSI} = \frac{\text{Heart weight (g)}}{\text{Total body weight (g)}} \times 100$$

Deviations from an average CSI implies increased or decreased heart mass, which may be an indicator of specific cardiac conditions such as ventricular hypertrophy (Hu et al. 2000).

Fulton's condition factor (Factor K) is a parameter that quantifies general condition of an animal. It is obtained from the following formula:

$$\text{Factor K} = \frac{\text{Body weight (g)}}{\text{Length}^3\,(\text{cm}^3)} \times 100$$

This factor can identify disorders in an animal's nutritional condition (Siccardi et al. 2009).

In this study, we documented an average cardiosomatic index of 0.18 %, body mass index of 0.04 g/cm^2 and Fulton's condition factor of 1.05 g/cm^3. These data are similar to those reported in the literature (Oka et al. 2010; Siccardi et al. 2009).

Discussion

In the present study, a method to evaluate morphometric parameters was described and basic somatic and cardiac morphometric parameters were obtained from healthy adult zebrafish specimens. Morphometric parameters included somatic body size data and cardiac characteristics, which were obtained from the entire animal, individual organ or histological preparations. Cardiac parameters included measurements of the ventricle size, inner ventricular angles, ventricular wall thickness and arterial bulb diameter. The other parameters that were calculated from these basic measurements were BMI, cardiosomatic index and Fulton's condition factor. The data that we obtained coincide with some other partial datasets for adult zebrafish and other commercially farmed species.

Various somatic and cardiac morphometric parameters are widely used to assess the developmental conditions of various commercial and wild fish species; they provide indirect information on growth, maturation, nutrition, reproduction and overall health status for individuals and populations.

Some authors have suggested that changes in the ratios of the cardiac axes and ventricle angles cause alterations in cardiac blood flow and adversely affect cardiac output and, therefore, development and survival. Additionally, they suggest that globular hearts in some fish species represent low cardiac output and low survival, whereas a triangular shape may reflect improved cardiac output (Poppe et al. 2002). Ventricular structure is a key parameter because it can reveal stress-related disorders, which may be part of the natural life cycle for some species, such as salmon. In salmon, the effort involved in countercurrent swimming increases the thickness of the compact myocardium to ensure higher tension in the ventricular walls, which maintains cardiac output and supports the muscular demand for additional oxygen. This situation also may occur in response to improper handling in aquaculture environments, deficiency in oxygen supply to aquatic tanks or environmental stressors, such as noise (Poppe et al. 2003).

Cardiosomatic parameters are tools that allow comparisons between individuals, populations or species. They facilitate the evaluation of the impacts of food, disease and/or environment on growth, health and fertility and allow predictive models to be developed for communities or populations (McCallum

2008). We found that, surprisingly, these parameters have not previously been fully examined in zebrafish, and it is essential to establish reference values for healthy adult zebrafish such as those that already exist for other commercial species (e.g., trout, cod and salmon). Because zebrafish are currently an important model for studying cardiovascular disease, the parameters reported here can be used as reference values for cardiac disease studies, including heart disease, arrhythmias and ischemic heart disease. These parameters may also be used to assess the influence of cardiac function on body development of captive specimens for which is important to know body condition and to ensure their proper care.

Heart disease studies using zebrafish as a study organism have focused on establishing models of dilated and hypertrophic cardiomyopathy and examining the relationships between pathology, gene alterations (e.g., HAND, NkX2,5, T-box, EYA4 and GATA; Dahme et al. 2009; Schönberger et al. 2005; Tu and Chi 2012) and/or specific proteins, such as troponin T, troponin C and sarcomeric protein titin (Becker et al. 2011; Gerull et al. 2002; Ho et al. 2009; Huang et al. 2013; Xu et al. 2002). In addition, other authors have shown that there are changes in the compact and spongy myocardium relationship as a consequence of physical activity and oxygenation levels in the aquatic environment (Gamperl and Farrell 2004). Studies of these diseases and their impacts on the physical development of zebrafish may benefit from including parameters such as BMI, ICS, Fulton's condition factor and sphericity index. Similarly, knowledge of ventricular size, myocardium structure and the relationship of compact and spongy myocardium may also be a key in these types of studies.

Cardiac arrhythmias may affect the development of an individual; zebrafish have also served as a model for studies of the heart conduction system and related disorders, as there are fundamental structural and functional similarities in the heart conduction system among higher vertebrates (Sedmera et al. 2003). Some types of arrhythmias have been developed via mutagenic studies of ion channels (e.g., sodium channels) and optogenetic zebrafish techniques to understand the underlying pathophysiological mechanisms of arrhythmias (Arrenberg et al. 2010; Chi et al. 2008; Huttner et al. 2013). In these cases, the cardiosomatic parameters can be used to identify the impact of altered electrical cardiac function on heart chamber function and structure and its impact on development. ICS, BMI, Fulton's condition factor, heart diameters, angles of the ventricular chamber and the sphericity index are useful parameters with which to evaluate these pathologies, as they complement electrophysiological and molecular studies (Chaudhari et al. 2013; Milan et al. 2006).

Finally, zebrafish are also used to model and study cardiac regeneration and ischemic heart disease. Experimental hypoxia and reperfusion protocols were developed to simulate cardiac ischemia and reperfusion processes (which are common in humans) by surgical recession or cryoinjury-induced infarction in the zebrafish ventricle (Chablais et al. 2011; González-Rosa et al. 2011; Parente et al. 2013; Poss et al. 2002; Schnabel et al. 2011). A reduction in myocardial mass can alter cardiac performance and adult zebrafish development. The impact of this injury can be measured using cardiosomatic parameters. Similarly, due to the regeneration process in the myocardium after damage, it is important to evaluate the role of new myocardial tissue during recovery of cardiac function and the impact of the spongy and compact myocardium ratio on fish growth and development after the injury.

Conclusion

Cardiovascular diseases are a global health problem, and studies using animal models can contribute to our understanding of the pathophysiological mechanisms that trigger these problems. The zebrafish is one of the most interesting emerging animal models that are used to study these types of diseases. However, there is insufficient information about cardiosomatic parameters in healthy adult zebrafish. These parameters may be useful because they serve as a reference values for cardiovascular pathophysiology research. Ample cardiac pathologies affect the adult population, and the elderly population is proportionally increasing in our society; it is therefore necessary to use animal models to represent these disorders at advanced stages of the human life cycle. The adult zebrafish is a good potential model for the biomedical investigation of the various mechanisms associated with chronic heart disease development and the evaluation of new therapeutic alternatives. However, it is necessary to have a proper understanding of this species' adult

physiology, including reference parameters. In the present study, we provide a method and some cardiosomatic parameters that can be used for subsequent studies that evaluate the pathophysiology of the zebrafish cardiovascular system.

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Compliance with ethical standards

Conflict of interest The authors confirm that they have no conflicts of interest in relation to this article.

Ethical statement No ethical issues are involved.

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