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The developmental origin of heart size and shape differences in *Astyanax mexicanus* populations

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ABSTRACT

Regulation of heart size and shape is one of the least understood processes in developmental biology. We have for the first time analysed the hearts of *Astyanax mexicanus* and identified several differences in heart morphology between the surface (epigean morph) and cave-dwelling (troglomorph) morphs. Examination of the adult revealed that the troglomorph possesses a smaller heart with a rounder ventricle in comparison to the epigean morph. The size differences identified appear to arise early in development, as early as 24 h post-fertilisation (hpf), while shape differences begin to appear at 2 days post-fertilisation. The heart of the first-generation cross between the cave-dwelling and river-dwelling morph shows uncoupling of different phenotypes observed in the parental populations and indicates that the cardiac differences have become embedded in the genome during evolution. The differences in heart morphology are accompanied by functional changes between the two morphs, with the cave-dwelling morph exhibiting a slower heart rate than the river-dwelling morph. The identification of morphological and functional differences in the *A. mexicanus* heart could allow us to gain more insight into how such parameters are regulated during cardiac development, with potential relevance to cardiac pathologies in humans.

1. Introduction

Tight control of heart morphology during development is crucial for normal cardiac function, as abnormalities can affect tolerance to varying physiological activities, ultimately affecting the overall viability of an organism. Many human congenital defects affecting heart size (hypertrophic cardiomyopathy), shape (single-ventricle defects) and tissue structure (non-compaction cardiomyopathy) are known to exist (Kloesel et al., 2016; Towbin et al., 2015), with genetic testing aiding in the identification of causative genes (Arndta and MacRae, 2014). Although the molecular and cellular biology of cardiovascular development has been intensely studied in a variety of vertebrates, the mechanisms behind the regulation of heart morphology is not clearly understood. Furthermore, it is not entirely known whether molecular mechanisms exist to confer cardiovascular differences to organisms with active or sedentary lifestyles. Organ size can be dictated by a wide variety of cellular activities, such as cell proliferation, apoptosis and fates of stem/progenitor cells and cellular hypertrophy. Several pathways have been implicated in heart size regulation, most notably the Hippo (Zhou et al., 2016) and FGF pathways (S. R. Marques et al., 2008).

Here, we introduce *Astyanax mexicanus* (Mexican cavefish) as a new model for understanding the regulation of heart size and shape. *A. mexicanus* is a single fish species comprising troglomorph (cave-dwelling) and epigean (surface) river populations. This species possesses unique traits that make it a useful model organism to study various aspects of adaptation to a food-scarce environment in perpetual darkness. Several cavefish populations are known to have derived from a surface fish ancestor, suggested from a couple of thousand to several million years ago (Fumey et al., 2016; Gross, 2012). Surface fish and cavefish are still inter-fertile and can produce fertile progeny, making genotyping studies such as a Quantitative Trait Locus (QTL) analysis feasible. Furthermore, due to its close ancestry to zebrafish, many tools are transferrable, including transgenesis and live imaging of embryos. By combining the unique traits of *A. mexicanus* and established tools in zebrafish, we can study how different selection pressures and genetic factors influence heart development.

Currently, the majority of teleost cardiovascular knowledge stems from zebrafish research. The adult fish heart is anatomically different from most vertebrates, with only a single atrium and ventricle. Blood

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from the body enters the atrium through the sinus venosus, is pumped to the ventricle, from where it is directed to the gills via the bulbus arteriosus. The blood is oxygenised in the gills, before directly being pumped towards the body (Simões et al., 2002). Cardiovascular development begins with the specification and differentiation of cardiac cell precursors in two bilateral heart fields, which migrate and fuse in the midline to form the cardiac disc, which gives rise to the initial linear heart tube. The heart tube then elongates by the addition of cells to its anterior and venous poles, and begins to beat by 24 h post fertilisation (hpf). By 48 hpf, the heart tube starts to loop and form chambers, acquiring an adult-like morphology by 5 dpf (Bakkers, 2011).

In this study, we provide the first characterisation of heart development, morphology and function in *A. mexicanus*. We show that while the hearts of both cavefish and surface fish have a general teleost fish morphology, there are several differences between the two. We find that shape and size differences of the heart arise early in development, suggesting that such traits are genetically determined. Moreover, heart rate measurements also reveal differences between the surface and cavefish. While functional differences appear to originate during developmental stages, coinciding with morphological differences, analysis of F1 hybrids indicates uncoupling of phenotypes.

2. Materials and methods

2.1. Ethical approval and fish husbandry

The experiments were performed on laboratory stock of teleost *Astyanax mexicanus*, surface fish and Pachón, Tinaja and Chica cavefish. All the experimental procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and institutional guidelines. Laboratory strain of surface fish were originally collected at Nacimiento Del Rio Choi, San Luis Potosí, Mexico. The three laboratory strains of cavefish population are originally from Cueva de El Pachón in Tamaulipas, Mexico (Pachón cavefish), El Sótano de la Tinaja (Tinaja cavefish) in San Luis Potosi, Mexico, and La Cueva Chica (Chica cavefish) originally provided by the Steinhart Aquarium (San Francisco, CA USA). The fish used in this study have been bred for multiple generations at the Yamamoto laboratory after having been obtained from University of Maryland (Jeffery laboratory). All the fish were maintained and fed once a day at 20 °C in pH 7.2 tank water with a 14-h light and 10-h-dark light cycle. Embryos were obtained by temperature induced spawning after increasing the tank water temperature to 25 °C.

2.2. Embryo collection and fixation

Embryos were collected and incubated in petri dishes at 25 °C in zebrafish embryo medium 2 (15 mM NaCl, 0.5 mM KCl, 0.05 mM Na_2HPO_4, 0.15 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4{\cdot}7H_2O$, 0.7 mM $NaHCO_3$). At specific time stages, embryos were fixed in 4% paraformaldehyde in PBS overnight at 4 °C. Embryos were then dehydrated gradually by increasing gradients of methanol/PBS and stored at − 20 °C.

2.3. Adult heart isolation

Fish hearts of surface and Pachón cavefish were isolated at 50-days, 6-months, 1 year and 2.5-years old and hearts of Tinaja and Chica cavefish were isolated at 6-months old. The fish were anaesthetised and killed using MS222 (Tricaine methanesulfonate, Sigma, A5040, 0.16 mg/ml), after which the standard body length from snout to tail base and the body weight were measured. Then the fish were placed on a cut sponge in a ventral-side-up orientation. Spring scissors were used to make an incision to penetrate the thorax and open the pericardial sac. The ventricle was exposed and hearts were isolated by cutting the artery superior to the bulbus arteriosus and the veins inferior to the atrium. The isolated hearts were directly fixed in 4% paraformaldehyde in PBS at 4 °C overnight, followed by washing with PBS and stored in PBS containing 0.05% sodium azide at 4 °C or processed for paraffin embedding.

2.4. Paraffin embedding and sectioning

To visualise the transparent embryos in paraffin, the embryos were transferred from 100% methanol to 0.1% eosin staining in 100% ethanol to stain the embryos for 30 s, followed by 3 washes with 100% ethanol. The ethanol solution was replaced by 1-butanol, the samples transferred to liquid paraffin wax and kept at 65 °C for 30 min. After washing twice with liquid paraffin wax, embryos were embedded. For embedding of adult hearts, after 4% paraformaldehyde fixation, the hearts were dehydrated by increasing ethanol gradients (70%, 80%, 90%, 95%, 100% twice, 1 h per step), kept in 1-butanol overnight, after which the hearts were transferred to liquid paraffin wax at 65 °C. After washing twice for 1 h with liquid paraffin wax, the hearts were embedded. 7–12 µm sections were cut using a microtome (AO Spencer 820) and mounted onto Superfrost slides (Thermo Scientific), then dried overnight at 37 °C.

2.5. Acid Fuchsin Orange-G (AFOG) staining and fluorescent immunohistochemistry

Sections were deparaffinised by xylene and rehydrated by decreasing ethanol gradients (100% three times, 95%, 90%, 80%, 70%, 50%) to PBS, or distilled water in case of AFOG staining. For AFOG staining, the sections were placed in Bouin's fixative at 60 °C for at least 2 h and washed in distilled water until sections were clear. The sections were then placed in aqueous 1% phosphomolybdic acid for 5 min, again rinsed in distilled water, before stained with AFOG solution for 5 min (5 g/l Water Blue, 10 g/l Orange G, 15 g/l Acid Fuchsin (Sigma), pH1.09). The sections were quickly rinsed in distilled water before dehydration and mounting using DPX (Sigma).

For fluorescence immunohistochemistry, 30 min of 3% (w/w) hydrogen peroxide was followed by high-pressure antigen retrieval with antigen unmasking solution (H-3300, Vector Laboratories Inc.). Sections were then blocked for 30 min with TNB (0.1 M Tris-HCl, pH = 7.5: 0.15 M NaCl; 0.5% blocking powder). The primary antibody against monoclonal mouse anti-myosin heavy chain 2 (MF20) (1:50 in TNB, Developmental Studies Hybridoma Bank) was incubated with samples overnight at room temperature. AlexaFluor 488 goat anti-mouse IgG secondary antibody (1:200 in TNB) and DAPI (2.5 µg/ml; Sigma) were incubated for 2 h at room temperature. Immunohistological stained sections were mounted with hydromounting solution (Applied Genetic Technologies Corporation) or Mowiol 4-88 (Applichem).

2.6. Whole mount immunohistochemistry and light sheet imaging

2 dpf embryos were rehydrated from 100% methanol to PBS followed by 10 min of proteinase K treatment at 65 °C (10 µg/ml in PBS). After washing in PBS-T, the embryos were fixed in 4% paraformaldehyde for 10 min and blocked for 2 h in TNB followed by primary antibody (MF20) overnight at room temperature. After washing in PBS-T, the embryos were incubated with secondary antibody and DAPI (2.5 µg/ml; Sigma) for 2 h (AlexaFluor 488 goat anti-mouse IgG secondary antibody, 1:200 in TNB). After washing in PBS-T, the embryos were embedded in 0.5% agarose/PBS in glass capillaries for light sheet microscopy (Zeiss Z1).

2.7. 3D reconstructions and volume measurements

Pictures of stained sections were taken by fluorescent microscope (Leica DM4500 LED). Three-dimensional (3D) reconstructions of