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Make no mistake, I only achieve simplicity with enormous effort.

- **Clarice Lispector**

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1. Abstract

This dissertation advances a new theoretical framework for explaining vertebrate heart evolution, grounded in philosophical considerations and evolutionary developmental biology (evo-devo). Integrating Woodward's (2003) interventionist account of causation with an expanded mechanistic perspective, the work develops the Environmentally Dependent Developmental Induction framework, conceptualizing environmental conditions, especially oxygen availability, as constitutive parts of developmental mechanisms, not just as external influences. By combining phylogenetical comparative analysis with theoretical approaches and experimental interventions, the dissertation demonstrates how environmental factors historically became embedded within the causal architecture of the heart, shaping both the generation and stabilization of phenotypic variation. This approach bridges the gap between causal (mechanistic) and constitutive (organizational) explanations and reframes disease as an outcome of developmental systems evolved to be responsive to environmental difference-makers. It thus reframes congenital heart defects as manifestations of the same developmental mechanisms that underpin evolutionary innovation and constraint. The resulting theory offers a unified mechanistic and phylogenetic explanation of heart evolution and developmental vulnerability, connecting the historical emergence of novel cardiac forms to present-day susceptibilities in vertebrate development.

2. Zusammenfassung

Diese Dissertation entwickelt einen neuen theoretischen Ansatz zur Erklärung der Evolution des Wirbeltierherzens, der in philosophischen Überlegungen und der evolutionären Entwicklungsbiologie (Evo-Devo) verankert ist. Durch die Integration von Woodward's (2003) interventionistischem Kausalitätsverständnis mit einer erweiterten mechanistischen Perspektive entwickelt die Arbeit den Ansatz der Environmentally Dependent Developmental Induction (umweltabhängige entwicklungsbiologische Induktion). Dieses Modell konzeptualisiert Umweltbedingungen, insbesondere die Verfügbarkeit von Sauerstoff, als konstitutive Bestandteile entwicklungsbiologischer Mechanismen, nicht nur als äußere Einflüsse.

Durch die Verbindung phylogenetisch-vergleichender Analysen mit theoretischen Ansätzen und experimentellen Interventionen zeigt die Dissertation, wie Umweltfaktoren historisch in die kausale Architektur des Herzens eingebettet wurden und so sowohl die Entstehung als auch die Stabilisierung phänotypischer Variation geprägt haben. Dieser Ansatz überbrückt die Kluft zwischen kausalen (mechanistischen) und konstitutiven (organisationalen) Erklärungen und beschreibt angeborene Herzfehler als Ergebnis von Entwicklungssystemen, die evolviert sind, um auf umweltbedingte „Unterschiedsmacher“ zu reagieren. Demnach beschreibe ich angeborene Herzfehler hier als Manifestationen derselben entwicklungsbiologischen Mechanismen, die auch evolutionäre Innovation und Einschränkung ermöglichen. Die daraus resultierende Theorie bietet eine einheitliche mechanistische und phylogenetische Erklärung der Herzevolution und deren entwicklungsbiologischen Vulnerabilität, und verbindet damit das historische Entstehen neuartiger Herzformen mit deren gegenwärtigen Anfälligkeit in der Wirbeltierentwicklung.

3. Introduction

Heart development (cardiogenesis) in vertebrates has been studied extensively, largely because congenital heart defects (CHDs) represent the most common category of birth defects (Kelly et al. 2014; Gruber and Epstein 2004; Pierpont et al. 2018). As a result, a significant body of descriptive and experimental data has accumulated across a range of model organisms. However, much of this research approaches phenotypic variation from a medical perspective, focusing more on deleterious outcomes than on the mechanisms underlying the formation of phenotypes.

This dissertation takes a complementary perspective by reinterpreting these data within an evolutionary framework. By comparing cardiogenesis across vertebrates, it seeks to elucidate how different cardiac phenotypes evolved and how the same developmental mechanisms that ensure normal morphogenesis also influence susceptibility to disease. This approach builds on a central assumption underlying the use of animal models in biomedical research: phenotypically similar traits across species often reflect conserved developmental pathways (Pavlicev and Wagner 2022).

More broadly, the work is grounded in the idea that medicine and evolutionary biology share a fundamental interest in phenotypic variation, though they focus on different aspects of it. Medicine is primarily concerned with deleterious variation (what we call disease), whereas evolutionary biology seeks to understand how variation arises, is constrained, and becomes available to selection (Pavlicev and Wagner 2022)—so with how development of a phenotype *evolved*.

Before turning to the specific case of the heart, I outline the theoretical considerations that inform this approach and the framework through which developmental and evolutionary mechanisms are connected in this work.

4. Evolvability and Robustness

The variational properties of a phenotype (expressed as the amount, direction, and distribution of phenotypic variation) describe its potential and constraints for change, shaping what kinds of variation can arise and thus how it can evolve under natural selection (Illari and Williamson 2012; Machamer et al. 2000; Kaiser and Krickel 2017; Austin and Nuño De La Rosa 2018; Nuño De La Rosa and Villegas 2022). This idea is captured in the concept of *evolvability*, defined as the capacity of a biological system to generate heritable phenotypic variation that can be acted upon by selection. Evolvability is high when small perturbations, whether genetic, developmental, or environmental, enable developmental systems to reach a wide range of

phenotypic outcomes. In contrast, *robustness* refers to the ability of a system to maintain its core functionality despite such perturbations (Conrad 1990; Aldana et al. 2007; Gerhart and Kirschner 2007; Mayer and Hansen 2017; Aldana et al. 2007).

At first glance, the relationship between evolvability and robustness appears dichotomous. Excessive robustness limits the capacity for adaptation, while excessive evolvability threatens organismal stability by allowing too frequent or disruptive change. This tension between maintaining function and generating variation has long framed a central question in evolutionary theory (Wagner and Altenberg 1996; Pigliucci 2008; Kirschner and Gerhart 1998; Wagner 2007; Mayer and Hansen 2017; Watson 2021; Masei and Trotter 2010).

From an evolutionary developmental (evo-devo) perspective, however, this tension is not a true dichotomy. The evo-devo framework treats evolvability and robustness not as traits to be optimized by natural selection but as emergent properties of developmental systems (Sniegowski and Murphy 2006; Lynch 2007). Importantly, a large body of work has argued that robustness and evolvability are not mutually exclusive but can be mutually enabling, depending on how developmental processes structure variation. Robustness can arise through buffering, redundancy, and modular organization, which stabilize phenotypes against many perturbations while simultaneously allowing latent or conditionally expressed variation to accumulate (Wagner 2007; Wagner and Altenberg 1996; Masei and Trotter 2010; Masei and Siegal 2009). Such architectures generate biased or structured variation, in which some phenotypic changes are far more likely than others, thereby shaping evolutionary trajectories without requiring direct selection for evolvability itself.

This view shifts attention from abstract correlations between genetic variation and phenotypic change toward the mechanisms that produce and structure variation itself. Evo-devo explains how interactions among genes, cells, and tissues constrain and facilitate phenotypic evolution. In other words, it seeks to uncover the architectural features of development that underlie an organism's variational properties: the genotype–phenotype map. These properties determine not only how much phenotypic diversity can arise but also which kinds of variants are more or less likely to be produced, and therefore what natural selection can act upon (Hendrikse et al. 2007; Wagner and Altenberg 1996; Brun-Usan et al. 2022; Watson 2021; Austin and Nuño De La Rosa 2018; Nuño De La Rosa and Villegas 2022). For example, while tetrapod limbs vary greatly in digit length, the number of digits shows little variation, illustrating how developmental systems bias the production of certain phenotypes while excluding others (Saxena et al. 2017).

By beginning with evolvability, I highlight a central theme of this dissertation: understanding how development generates, stabilizes, and biases phenotypic variation. This choice is not

meant to foreground the contrast with robustness, which will not play a central role in what follows, but rather to emphasize a particular way of posing explanatory questions. Framing the problem in terms of evolvability directs attention toward the developmental mechanisms that structure variational properties: how certain phenotypic changes are made more or less likely to arise. My aim is therefore to identify the developmental mechanisms that underlie the evolution of vertebrate heart development. To do so, I first clarify what counts as a mechanistic explanation in evolutionary developmental biology.

5. Explanations in Evo-Devo

Explanations in evolutionary developmental biology are mechanistic: they describe how developmental processes produce specific traits, how these mechanisms change over evolutionary time, and how such changes bias the production of phenotypic variation (Calcott 2009; Villegas 2024).

Unlike classical evolutionary biology, which typically examines phenotypic change at the population level by correlating trait variation with allele frequencies and processes such as selection, drift, and mutation, evo-devo emphasizes individuals and lineages. It focuses on how changes in developmental mechanisms give rise to both quantitative and qualitative variation in the phenotype. In this context, the explananda of evo-devo include the origin and transformation of morphological structures, the emergence of developmental constraints and biases, and the conditions under which novel phenotypes become possible. Mechanisms are therefore individuated relative to these explananda: they consist of organized components and interactions that produce and stabilize particular developmental outcomes (Kaiser 2021; Bich and Bechtel 2021).

A key question in evolutionary developmental biology is how new traits emerge from preexisting developmental systems (Wagner et al. 2000). One way to address this is through lineage explanations, which trace a continuous sequence of trait modifications across generations. While mechanistic explanations describe how a system works in individual organisms, lineage explanations add a historical perspective, showing how developmental mechanisms evolve. Evo-devo explanations therefore span both the functioning of developmental systems in individual organisms and the historical transformations of these systems over evolutionary time. They do not rely on population-level processes or invoke natural selection directly; instead, they show how the components of a mechanism and their organization could transform to produce novel traits (Calcott 2009; Kaiser 2021; Laubichler 2009).

In this framework, developmental mechanisms are analyzed within comparative and phylogenetic contexts, with each step in the sequence corresponding to a mechanism that produces the observed phenotype. The aim is to uncover the mechanistic bases of both phenotypic commonalities and diversity. This approach brings a mechanistic dimension to the study of phenotypic variation, which is a necessary condition for evolution to occur, yet one that classical evolutionary theory leaves unaddressed. Lineage explanations therefore capture both how developmental mechanisms generate particular traits and how these mechanisms themselves can gradually change over evolutionary time. This helps us understand how development evolved to give rise to different phenotypes (Bechtel and Abrahamsen 2013; Laland et al. 2011).

5.1. Challenges

Developmental mechanisms include components such as gene products, signaling molecules, environmental inputs, nutrient availability, and physical factors like mechanical stress. Their organization, meaning their spatial arrangement, temporal sequence, and hierarchical relationships, determines how the mechanism functions. Because development is dynamic and activities change over time, mechanistic explanations must account for nonlinear, temporally structured processes. This dynamic nature of developmental systems makes it difficult to individuate mechanisms, especially in a comparative or phylogenetic context (Kaiser pp. 124-126 and Love p. 343 in Bueno et al. 2018) . Nevertheless, these difficulties do not preclude mechanistic explanation. In practice, evo-devo individuates mechanisms pragmatically, by stabilizing explanatory targets around specific phenomena, developmental stages, or levels of organization, and by tracing recurring patterns of components and interactions across contexts. Comparative strategies, idealized models, and partial mechanistic sketches allow us to identify conserved organizational features even when the full mechanism varies across taxa or developmental time.

Lineage explanations face another layer of complexity because mechanisms evolve as their components and organization are modified (Illari and Williamson 2012). Determining when a mechanism has changed sufficiently to be distinct from its precursor requires careful consideration of both ontogenetic and phylogenetic context (Machamer et al. 2000; Craver 2007; Craver and Darden 2013). Despite these challenges, evo-devo demonstrates that mechanistic explanations are achievable, precisely by treating mechanisms as historically and developmentally situated rather than as fixed entities (Gilbert 2012; Wagner et al. 2000).

6. Environment and Development

The previous section described developmental mechanisms as organized systems composed of interacting components whose coordinated activities give rise to the phenotype. These components include gene regulatory networks, environmental factors, and epigenetic influences (epigenetics sensu Newman and Müller, 2000: conditional responses of tissues to each other and to external forces). The latter two provide the physico-chemical substrate necessary for development (Brun-Usan et al. 2022; Newman et al. 2006; Newman and Müller 2000; Draghi and Wagner 2008). This means that the phenotype can be altered not only by changes in the genotype but also by modifying the physico-chemical substrate in which development takes place. In other words, the internal organization of a developmental system underlies its variational tendencies in evolution (Brun-Usan et al. 2022; Crispo 2007; Emlen and Nijhout 2000; Levis and Pfennig 2016; Newman et al. 2006; Waddington 1959; 1942; 1940; West-Eberhard 2005).

The precise composition of a developmental mechanism, however, is not fixed (Hansen 2006; Watson 2021; Wagner and Altenberg 1996). Considerable work over the past decades has gone into identifying what kinds of entities and processes constitute such mechanisms. Gene products within cells, such as proteins and transcription factors, are key molecular components, driving signaling and regulatory cascades that translate genetic and environmental inputs into phenotypic outcomes like cell differentiation. Yet many extracellular and environmental components also participate in these processes, along with physical factors such as mechanical stress. All of these contribute to the behavior and expression patterns of cells at specific points in development. Which components are relevant depends on the phenomenon under investigation: developmental mechanisms are both numerous and context-dependent, and the entities that matter most can differ from one system or stage to another (Kaiser 2021; Kaiser and Krickel 2017; Love 2008; Jäger and Sharpe in Minelli and Pradeu 2014).

As embryos develop, they encounter a characteristic sequence of environmental conditions, involving factors such as temperature, oxygen availability, mechanical load, and nutrient supply. These factors act as components that modulate or enable the activities of other entities, such as genes, signaling molecules, or cellular structures. They also vary markedly across taxa, depending on the mode and timing of development, and often shift dynamically as development proceeds (Brun-Usan et al. 2022). Comparing these environmental components across vertebrates provides insight into how developmental mechanisms have diverged and how this divergence contributes to phenotypic variation.

This framework allows us to pose a causal question that is both developmental and evolutionary: which environmental factors truly make a difference to the formation of the heart, and how have these differences shaped the evolution of cardiogenesis? Woodward's (2004) interventionist account of causation provides a useful way to address this question. According to Woodward, a causal explanation identifies factors that, if changed through intervention, would systematically alter the phenomenon of interest. Waters (2007) extends this logic to biology, arguing that causal explanations must identify *actual difference-makers*, so those variables that, when varied, produce reproducible and explanatorily relevant changes. From this perspective, my aim has been to identify which environmental factors act as genuine difference-makers in vertebrate heart development and how they have influenced the evolution of cardiac morphology.

6.1. Environment and Development in Cardiogenesis

From this comparative, mechanistic perspective, I investigate how variation in physical and environmental conditions during cardiogenesis contributes to differences in heart morphology across vertebrates. I believe the vertebrate heart to be particularly well suited for this inquiry: It has to start functioning while it is developing. The heart thus allows us to study how environmental conditions, such as oxygen tension, pressure load, and shear stress, feed back into its own ongoing morphogenesis. Additionally, as it is the first organ to form and function, its development is less interdependent on that of other organ systems, making it comparatively easy to individuate developmental processes. Thus, the heart serves as an ideal model to unravel some of the intricate interactions governing development rather than organ systems that develop at later stages, when developmental interactions are more convoluted (Poelmann et al. 2008; Poelmann and Gittenberger-de Groot 2019; Brand 2003; Giussani 2021; Fisher and Burggren 2007).

Cardiac development also appears particularly sensitive to environmental perturbation. Although the underlying mechanisms remain poorly understood, alterations in oxygenation, temperature, or flow are consistently correlated with congenital heart defects (Fahed et al. 2013; Giussani 2021; Gruber and Epstein 2004; Burggren 2004; Burton and Jauniaux 2018). This sensitivity makes cardiogenesis an ideal system for examining how environmental factors become integrated into developmental mechanisms. Comparative analysis of vertebrate embryos thus provides an opportunity to identify which environmental factors are present in each developmental mode, how they influence the organization of the developmental system, and how these differences have contributed to the diversification of cardiac phenotypes across vertebrates (Illari and Williamson 2012; Glennan 2017; Craver 2007; Craver and Darden 2013; Machamer et al. 2000; Brun-Usan et al. 2022; Fisher and Burggren 2007).

6.1.1. Mechanical Stress

I initially set out to focus on the mechanical forces that vertebrate embryos experience during cardiogenesis, as they constitute the first environmental input the developing heart encounters. In particular, wall shear stress (WSS), the frictional force exerted by blood flowing parallel to the vessel wall, has long been considered a key regulator of cardiac morphogenesis (Liu et al. 2009).

With the looping of the initially almost straight heart tube, WSS starts to vary as the heart gets remodeled and rapidly changes its geometry, a process that is intricately related to shear stress patterns (Poelmann and Gittenberger-de Groot 2019; Courchaine et al. 2018). Endothelial cells are responsive to such changes in flow patterns via their cytoskeleton. But while the cytoskeleton notices shear stresses in general, endothelial cells equipped with primary cilia are particularly sensitive to changes in WSS and function as the main shear stress sensor. However, cilia are only present in areas of low shear stress, as they disassemble under high shear stress (Van der Heiden et al. 2006). This phenomenon is of particular interest in the context of force-dependent protein activation and gene expression patterns of shear-responsive genes: non-ciliated endothelial cells that were subject to fluid shear stress showed significantly less induction of shear marker genes (Hierck et al. 2008; Egorova et al. 2011; 2012). The distribution of these cilia is both regional- and developmental-stage-dependent, meaning that different amounts of cilia are found in different areas of the heart in different developmental stages depending on the current flow profile, as shown for the chick model by Van der Heiden et al. (2006). Such force-dependent protein activation and gene expression are important regulators of vascular function: upon shear stress activation, endothelial cells react by regulating the expression of shear-stress responsive genes (Hierck et al. 2008).

In short, the changing heart morphology influences shear stress patterns by altering vessel diameters and introducing bends and curvatures into the heart's geometry. These, in turn, affect the expression of shear-responsive genes, which orchestrate further changes to heart morphology, again leading to shifts in WSS distribution and gene expression. This feedback between mechanical forces and gene expression forms a central part of vertebrate heart morphogenesis (Goddard et al. 2017; Menon et al. 2015; Steed et al. 2016).

Paper I: OCT Meets micro-CT: A Subject-Specific Correlative Multimodal Imaging Workflow for Early Chick Heart Development Modeling

Setting out to explore how shear stress guides heart development in detail, I developed a correlative multimodal imaging workflow combining optical coherence tomography (OCT) and lab-based X-ray microtomography (micro-CT). Chick embryos were used as a model system

due to their optical accessibility and developmental similarity to mammalian hearts. By integrating in ovo structural and Doppler velocity data from OCT with high-resolution ex vivo imaging from micro-CT, this approach enabled specimen-specific modeling of early heart development. The workflow provides both precise shear-stress quantification and detailed assessment of cardiac morphology using only standard reagents and freely available software.

Together, these complementary modalities offer a comprehensive view of early cardiac morphogenesis and provide the technical foundation for investigating mechanical influences on development. This paper is published in Journal of Cardiovascular Development and Disease (accepted 31.10.2022).

This paper itself is methodological in scope, but by engaging with the literature and by extending my work on shear stress comparatively, it became clear that mechanical forces alone could not account for the diversity of cardiac forms across vertebrates: shear stress proved indispensable for proper morphogenesis by guiding chamber formation, valve development, and myocardial maturation, but its influence appeared modulatory rather than determinative. In other words, shear stress provides necessary conditions for correct development, yet it does not explain the origin of distinct cardiac architectures. Fittingly, manipulations of flow consistently induce heart defects but do so in a non-systematic way. In Woodward's (2003) terms, shear stress thus acts as an *enabling condition*, but not a genuine *difference-maker* in the diversification of cardiogenesis.

Author Contributions

I conceptualized the study and designed the overall research approach. I performed the micro-CT imaging together with Brian Metscher and conducted all associated image analyses. I wrote the manuscript, with the exception of the sections pertaining to optical coherence tomography (OCT). The OCT data were collected by Fabian Placzek and me; Fabian Placzek carried out the OCT analysis and authored all OCT-related sections of the manuscript. Brian Metscher contributed to the conceptual development of the project and supported both the micro-CT work and the preparation of the manuscript. All authors provided critical feedback and approved the final version of the manuscript.

Article

OCT Meets micro-CT: A Subject-Specific Correlative Multimodal Imaging Workflow for Early Chick Heart Development Modeling

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Abstract: Structural and Doppler velocity data collected from optical coherence tomography have already provided crucial insights into cardiac morphogenesis. X-ray microtomography and other ex vivo methods have elucidated structural details of developing hearts. However, by itself, no single imaging modality can provide comprehensive information allowing to fully decipher the inner workings of an entire developing organ. Hence, we introduce a specimen-specific correlative multimodal imaging workflow combining OCT and micro-CT imaging which is applicable for modeling of early chick heart development—a valuable model organism in cardiovascular development research. The image acquisition and processing employ common reagents, lab-based micro-CT imaging, and software that is free for academic use. Our goal is to provide a step-by-step guide on how to implement this workflow and to demonstrate why those two modalities together have the potential to provide new insight into normal cardiac development and heart malformations leading to congenital heart disease.

Keywords: heart development; optical coherence tomography; micro-CT; correlative imaging; multimodal imaging; chick embryo; heart morphogenesis

1. Introduction

As developmental processes are highly conserved among amniotes, the chick heart represents an exemplary model for heart morphogenesis in human heart development, particularly because the development of avian embryos inside an egg allows for easy access for cell and tissue manipulations, longitudinal follow up of manipulations, and in vivo imaging [1]. Additionally, just as in humans, the embryonic avian heart undergoes extensive morphological changes during early cardiogenesis: it starts out as a single-chambered tube; through extensive growth and remodeling, it develops into a four-chambered pump with valve leaflets, ventricular trabeculae and double parallel blood flow which, despite some differences, resembles the mammalian and thus, human heart [2]. Most importantly, cardiac defects in human babies can be replicated in avian embryos, allowing a unique developmental window into human congenital heart disease (CHD; [3–6]. All of this makes the chick model attractive for researchers aiming to understand the mechanistic connection between altered blood flow patterns and congenital heart defects—the most prevalent and often lethal type of birth defect [7]. Attempts at elucidating the role of blood flow in heart development and CHD formation often make use of computational modeling to quantify blood flow velocities and flow stresses.

Subject-specific computational fluid dynamic (CFD) models based on structural and Doppler velocity data collected from optical coherence tomography (OCT) are currently the gold standard in modeling of early cardiac development [8]. This is because OCT allows for non-invasive, label-free, and cross-sectional (B-scan) imaging of tissues in vivo with micrometer-scale spatial resolution [9,10]. So far, OCT is routinely used as a diagnostic tool

in ophthalmology and as a fast volumetric guidance during ophthalmic surgery [11], but it is also gaining interest in endoscopy [12], multimodal approaches combining complementary imaging methods to provide deeper understanding of diseases [13], and of signal origin and imaging outcome in diagnosis [14]. Furthermore, functional extensions to OCT can provide additional layers of information to the morphological data: e.g., polarization sensitive OCT [15], Doppler-OCT, or OCT-angiography [16]. Thus, OCT is capable of simultaneous sequence acquisition of structural cardiac images and of blood flow data [8,17]. True subject-specific CFD models are of particular importance when studying treatments or interventions that alter blood flow, especially when they can lead to different blood flow conditions from embryo to embryo [8].

No single imaging modality can give comprehensive information and single-handedly decipher the inner workings of an entire developing organ [18]. Although OCT gives highly resolved *in vivo* insight into cross-sectional micro structures, the actual dimensions, especially in the axial direction, remain difficult to determine: OCT gives the optical path length in the axial direction. To access the geometrical length in depth, the refractive indices are essential. Usually, an average for the present biological tissue is assumed [19], since refractive index measurements of even homogenous tissue is complex [20]. Therefore, there is a lack of geometrical information, especially for CFD model validation. Furthermore, the OCT imaging depth depends on the optical scattering properties of the investigated tissue and relies on natural tissue contrast for image construction, which limits imaging to the endogenous contrast [21]. Normally, penetration depths range between 1–2 mm in depth, hence it is limited to chick embryonic stages before 3½–4 days of gestation (around Hamburger-Hamilton stage (HH)24; [22]) (out of 21 days of incubation): after 4 days, the embryo starts sinking into the yolk. However, combining different imaging techniques in a multimodal workflow allows overcoming potential shortcomings by integrating the best features of the combined techniques. This provides complementary information about structure, function, dynamics, and molecular composition of the sample [18]. OCT is ideal for correlative multimodal imaging with other modalities as it preserves the tissue, and is non-invasive and label-free. Therefore, sample preparation for OCT imaging does not interfere with any treatments for subsequent imaging.

An especially suitable complementary imaging modality for OCT imaging in early cardiac modeling is *ex vivo* X-ray microtomography (XRM or micro-CT), as it allows for subject-specific acquisition of genuine and isotropic 3D image volumes at micron resolution [23]. Isotropic refers to equilateral voxels (volumetric pixel) used in a volume data set, where the spatial resolution in the transaxial plane (X-Y plane) and that in the longitudinal direction (Z direction) are equivalent, and spatial resolution is identical in all three spatial directions. This by itself is a great advantage over all anisotropic imaging methods (such as OCT, or reconstruction from serial sections), as this allows one to directly assess the 3D spatial distribution of the cardiac tissue (sub-)structures, such as myocardial fiber orientation, or localization of “tethers” (the ultra-fine connections observed between the endocardium and myocardium), and other morphological features associated with cardiomyopathy [24,25]. Such details in the cardiac microstructures can affect action potential propagation depending on the precise orientation of the cardiomyocytes [26], as well as the mechanical tissue properties and concomitant blood-tissue interactions. Here, the combination of OCT and micro-CT (together with soft tissue mechanics and custom experimental tools [27]) can provide important subject-specific information on how blood flow interacts with the developing cardiac tissue. This is essential for an integrated biomechanical understanding of embryonic heart development [28].

As micro-CT is inherently volumetric, automatically aligned, and size-calibrated, it can also provide a basis for different kinds of quantitative analysis of heart morphogenesis, such as volumetry, morphometry, geometric morphometrics, and densitometry [24,29]. Several studies qualitatively analyzed developmental malformations based on visual inspection and comparison to normal development [29–33]. This makes the addition of micro-CT imaging particularly valuable for studies aiming to understand and phenotype

how altered hemodynamic loads caused by certain treatments or interventions can lead to congenital heart defects: OCT imaging can inform about subject-specific changes to hemodynamics and overall heart geometry, while micro-CT can provide subsequent subject-specific information needed to quantitatively describe (micro-)structural alterations to the heart [21,29].

Micro-CT was initially used mainly for mineralized animal tissues [34,35], but the introduction of X-ray dense contrast agents enabled micro-CT imaging of soft (non-mineralized) tissues with high contrast as well [36,37]. This allows the visualization and quantitative description of the structure and microstructure of healthy and diseased cardiovascular tissues during development and in adults using micro-CT. General contrast stains have been in use for over a decade now, and many of them already allowed for valuable insights into heart tissue substructures, such as myocardial fiber direction, or localization of collagen and elastin [38–42]; see [24] for an extensive review on different contrast agents already used in heart imaging and their characteristics. For further microscopic analyses, the sample preparation methods and the non-destructive nature of micro-CT allow for subsequent investigation by histology, scanning electron microscopy, immunohistochemistry, and immunofluorescence [43,44]. However, micro-CT imaging itself is moving in the direction of 3D histology with the development of new tissue-specific X-ray contrast enhancement protocols [45–49] and protocols showing that micro-CT can even be utilized for imaging of molecular signals [50,51]. These new methods will increase the potential of X-ray microscopy for 3D histological investigation of tissues and organisms even further.

The present study introduces a detailed workflow for specimen-specific correlation of dynamic *in vivo* OCT imaging of the developing chick heart with high-detail 3D micro-CT data. Our workflow allows for overview and dynamic imaging of the whole heart over the cardiac cycle and can provide 3D virtual histology information in intact fixed specimens (Figure 1). This multimodal approach has the potential to provide new and valuable insights into cardiac morphogenesis by not only overcoming the limitations of isolated standard imaging approaches, but also by providing unique and complementary information only achievable through the correlation of these data.

As proof of concept, we acquired OCT B-scans of live chicken embryos at HH12–14 *in ovo* before subsequent fixation in end-diastole, staining in PbOAc, a nuclei specific X-ray dense contrast enhancement [49], micro-CT data acquisition with a commercial laboratory system, and co-registration of the data sets using software that is free to use for academic research. Our goal is to provide an extensive guide facilitating the implementation of this workflow for other research groups and to discuss potential use and the advantages this multimodal approach provides over using a single imaging modality.

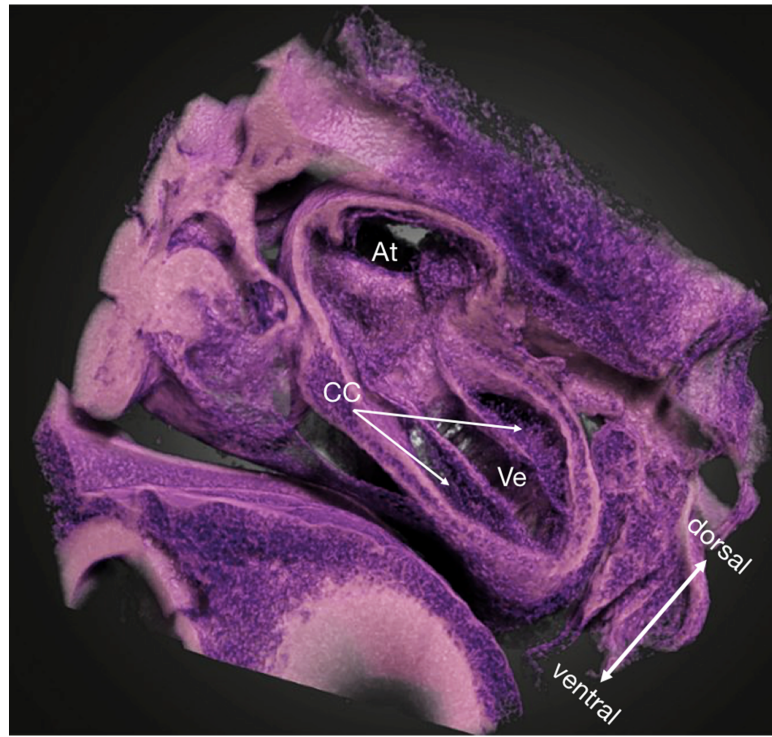


Figure 1. Micro-CT volume rendering of a PbOAc stained HH18 chicken embryo showing a near-histological 3D view into the ventricle and the atrium (arbitrary cutaway). The cardiac cushions are in the process of being populated by cells. Owing to the high specificity of the PbOAc staining, the lookup table and the transfer function could be manipulated as to resemble the classic Hematoxylin/Eosin-staining used in histology to locate nuclei more easily. At atria, CC cardiac cushions, Ve ventricle.

2. Materials and Methods

2.1. Embryo Preparation

Fertilized chicken eggs were incubated blunt-end up at 39.5 °C and 80% humidity for 45–53 h (HH12–14; mid-looping of the heart tube), as is typically done when incubating only up to early developmental stages [22]. Embryos were then removed from the incubator, staged according to Hamburger and Hamilton [22], and prepared for subsequent OCT imaging. The eggs were opened up at the blunt-end by creating a small hole in the shell using a scalpel; tweezers were then used to remove a small area of the shell and the inner shell membrane to access the embryo. Embryos that bled upon membrane removal or that had obvious structural defects were not used for subsequent imaging. All imaging steps were carried out at ambient room temperature and humidity as only a few B-scan positions were imaged per embryo, keeping data acquisition time short. However, for longer imaging time and for accurate assessment of blood flow data, temperature and humidity control during the imaging procedure is highly recommended.

2.2. Optical Coherence Tomography

A custom made, fiber-based OCT system in a laser scanning microscope setup was used in this study. It incorporated an akinetic swept source laser (Insight Photonic Solutions)

centered at 1304 nm, providing a bandwidth of 90 nm (rectangular spectrum), leading to an axial resolution of 12 μm in air. The sweep frequency was approximately 173 kHz. The scan lens ($f = 40$ mm; Figure 2) provided a measured lateral resolution of 17.2 μm . The field of view (FOV) can be set to cover 5.5×5.5 mm or 2.7×2.7 mm with 1000×1000 sampling points, exceeding the Nyquist requirement. It is possible to acquire a full three dimensional image stack, or to acquire a cross-section at a specific position (B-scan only), so that cross-sections over time could be acquired from the same position. The pixel size in depth after post-processing was 7.6 μm in air. Assuming a refractive index in tissue of 1.33, the pixel size inside tissue was reduced to 5.7 μm . With an output power of approximately 18 mW on the sample, a sensitivity of 110 dB was measured.

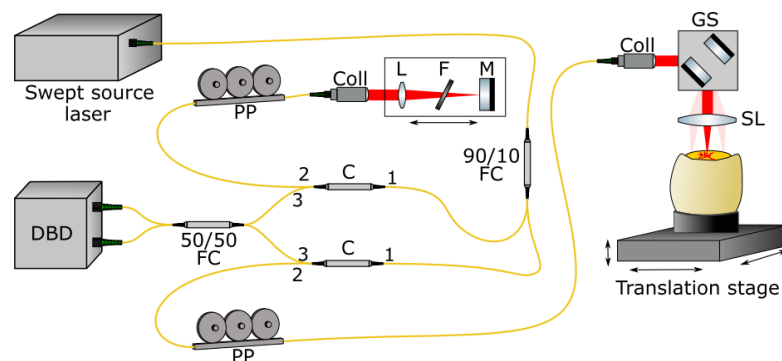


Figure 2. In-house-developed optical setup for performing SS-OCT in a fiber based, Mach-Zehnder configuration. The swept source laser is connected to the interferometer via a 90/10 fiber coupler (FC) transmitting 10% of the optical power to the reference arm via a circulator (C). The collimator (Coll) collimates the laser light, and a lens (L), filter wheel (F), and mirror (M) are mounted on a linear translation stage to match the arm lengths. 90% of the optical power is transmitted to the sample arm in which scanning is performed with galvanometric scanners (GS). The scan lens (SL) focuses the laser light onto the embryo, which is mounted on a three-axis translation stage. Reference and sample arm signals are combined in a 50/50 fiber coupler and detected in a dual balanced detector (DBD). The DBD is connected to the computer for data acquisition as well as driving the swept source laser and the GS. The polarization paddles (PP) provide adjustment capabilities for the polarization state of the laser light.

The egg was placed in a tea light holder (IKEA) which can accommodate the pointy end of the egg. The chosen size of the holder was important to not reduce the flexibility of the OCT system in translating the egg in all three directions due to space restrictions. Three small balls of UHU Patafix adhesive putty were put on the walls of the tea light holder to adjust the holder to different sized eggs and to prevent the eggs from moving or tilting. The adhesive putty also allowed us to mount the egg with a slight angle for an improved OCT signal acquisition: this way, strong yolk/egg white surface reflection could be reduced or even removed from the region of interest (ROI). The mounted embryo was placed on a three-axis translation stage to position the embryo precisely to the desired ROI. The live preview of cross-sectional B-scans was used to select an imaging position. After the acquisition of the OCT data (acquisition time < 8 s), raw data is stored on the computer. An in-house developed post-processing pipeline (LabView) was used to perform the Fourier transformation, numerical dispersion compensation and signal optimization of the raw OCT data. Using Fiji, the brightness and contrast was adjusted and a golden lookup table was applied.

2.3. Subsequent micro-CT Imaging

Embryos used for OCT data collection were immediately prepared and fixed in end-diastole (fully relaxed state) for micro-CT imaging. For this, the eggs were carefully cracked into an embryo dish at the pointy end, so that the yolk does not break and the embryo—initially situated on the blunt-end of the egg—now lies on top of the yolk in the dish (Figure 3). Then, the embryo was carefully lifted out of the yolk using a homemade perforated embryo spoon [52] leaving behind as much yolk as possible (as to not dilute the subsequent treatment), while at the same time keeping the blood vessels intact. Once on the spoon, a small hole was made into the embryonic membranes over the ventricle using forceps-scissors, so the treatment reaches the heart more readily, all while making sure the embryo's heart is still beating.

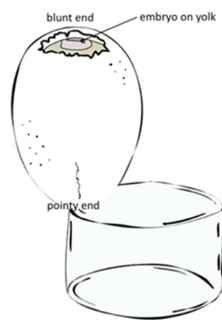


Figure 3. Schematic drawing of embryo preparation and egg orientation required for micro-CT imaging subsequent to OCT image acquisition.

For fixing the heart in end-diastole, a few drops of 5 mM verapamil/EGTA (0.6 M KCl, 10 mM verapamil, 50 mM EGTA in chick Ringer's solution) were put directly on top of the ventricle using a sterile transfer pipette (see [5]). For fixing the heart in end-systole (fully contracted), a few drops of 2M NaCl (see [53]) were applied to the ventricle in the same fashion. Once the heart stopped beating, the embryo was cut out of the surrounding blood vessels and embryonic membranes, transferred into an embryo dish containing chick Ringer's solution to clean it from yolk, and dissected out of residual embryonic membranes with the help of a stereomicroscope. The embryos were then fixed in 4F1G (10% formalin, 1% glutaraldehyde in PBS) to reduce shrinkage.

After 3 h to overnight of fixation in 4F1G, embryos were given a quick wash in ddH₂O (>5 min) followed by two 15 min long washes in ddH₂O. Then, the embryos were contrast-enhanced for Micro-CT imaging using lead(II) acetate trihydrate (PbOAc; CAS 6080-56-4; Sigma-Aldrich 467863) dissolved in distilled water at 2% *w/v* [49]. Whole embryos were stained overnight; for hearts dissected out of the embryo at stages up to HH18, 4 h of staining sufficed. Subsequent to staining, specimens were washed again with a quick wash in ddH₂O (>5 min), followed by two 15-min washes in ddH₂O.

After staining, specimens were mounted in polypropylene pipette tips or small polyethylene tubes in 1% low-melting temperature agarose in water [51] for scanning. All specimens used in this study were scanned using a commercial laboratory system, the Xradia MicroXCT microtomography imaging system (Zeiss X-ray Microscopy) with a tungsten X-ray source operated at 40–60 kV and 4 W. Individual projection images were taken every 0.20° over a half-rotation (182–184°) scan, with 40 s exposure times. The tomographic sections were reconstructed with voxel sizes of 0.89–3.99 µm using the XMReconstructor software supplied with the system. The tomograms were reconstructed with the XMReconstructor software supplied with the scanner, and reconstructed tomography volumes were saved in *.txm format.

Obtained images of specimens were examined and described using the Xradia XM3DViewer software, and the free software Dragonfly 3.6 (<http://www.theobjects.com/dragonfly>, accessed on 31 October 2022) and Fiji (ImageJ; <http://www.fiji.sc>, accessed on 31 October 2022).

2.4. Additional micro-CT Imaging

To further demonstrate near-histological image quality of micro-CT data over more developmental stages and to demonstrate a more commonly used staining protocol, scanning data of additional specimens was collected. For this, specimens between the stages of HH13–HH21 were used.

For micro-CT imaging only, eggs were incubated as described above, but lying on their side instead of blunt-end up. This way, the eggs could be cracked into an embryo dish on the side, reducing the risk of breaking the yolk. Subsequent embryo preparation and fixation in end-systole and end-diastole was done as described above. This data was also used to see whether fixation at certain points in the heart cycle actually represent reported cardiac-cycle related changes in the cross-sectional shape of the primary ventricular tube. The opened endocardial tube during diastole has an elliptic cross-section, while the collapsed endocardial tube during systole is slit-shaped as a consequence of an uneven distribution of cardiac jelly, which facilitates complete occlusion of the endocardial lumen at end-systole [54,55].

The fixed specimens were stained using either the lead(II) acetate trihydrate protocol described above or with 0.3% phosphotungstic acid (PTA) in 70% EtOH [56]. For PTA staining, specimens were washed three times in 70% EtOH for about 15 min each and then transferred to the staining solution for 24–48 h, depending on size. Subsequently, specimens were washed again in 70% EtOH for at least 1 h with one change to fresh 70% EtOH during this period. After staining, specimens were mounted and scanned as described above.

3. Results

3.1. Embryo Preparation

Optimizing embryo preparation for a multimodal workflow is no trivial matter, as compromises need to be made at each step so that the embryo can be used for both modalities. Incubating eggs blunt side up is the easiest and most reliable way to have the embryo develop at the desired spot and be able to open the egg without causing damage. However, this makes opening up the egg for embryo retrieval more difficult, as the egg now cannot be cracked from the side (easiest breaking point). Opening up at the egg at the blunt-end for OCT imaging makes it necessary to crack it from the pointy-end for subsequent treatment for OCT imaging. However, as this is the most robust part of the egg, utmost care and practice are needed as to not break the yolk; this is crucial with very young embryos (at least up to HH16 in our experience), as otherwise, the tiny, transparent embryo gets lost in the yolk. The methods described above (Figure 3) proved to be the most reliable to facilitate embryo collection for both imaging modalities.

3.2. Image Registration

We explored how well micro-CT stacks and OCT B-scans can be correlated and tried to improve the workflow for consistent results other groups can implement in their research.

Fixing hearts in end-systole for micro-CT was found to facilitate reliable co-registration. This allowed us to manually choose a B-scan at end-diastole rather than co-registering data for a different, unknown point in the heart-cycle. As the characteristic cardiac-cycle related changes in the cross-sectional shape of the primary ventricular tube reported [54] are also found in specimens fixed at end-diastole (or end-systole), we judged our fixation methods as sufficiently realistic (Figure 4).

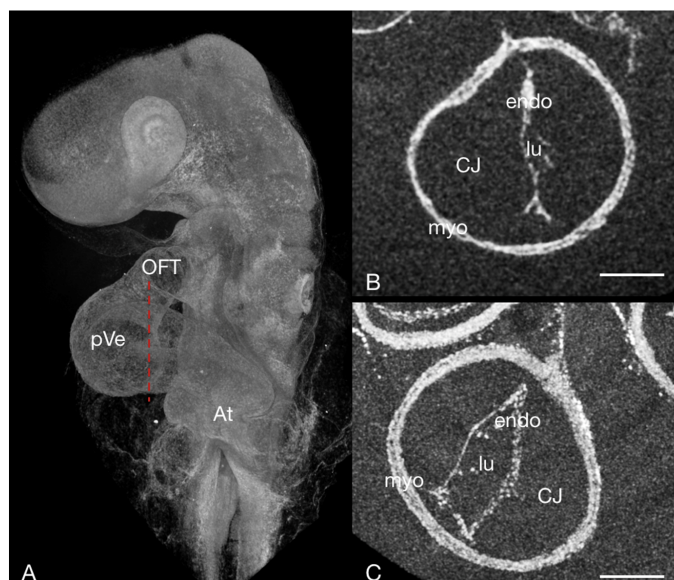


Figure 4. The embryonic heart tube of a HH13 chick stained with PbOAc. (A) Overview, micro-CT volume rendering: the red dotted line corresponds to the sectioning plane of (B,C) Virtual cross-section through the embryonic heart tube in the area of the primitive ventricle fixed at different phases in the heart cycle. (B) systole—the lumen is slit-shaped; (C) diastole—the lumen appears more oval in shape. At atrium, CJ cardiac jelly, endo endocardium, lu lumen, myo myocardium OFT outflow tract, pVe primitive ventricle; scale bar = 100 μ m.

It proved to be important that only hearts themselves were co-registered as other positional information (such as the neural tube) could come to lie in different positions in relation to the heart in ovo versus mounted in agarose. Hence, anything but the heart itself was cropped out of the OCT B-scans used. For further processing, *.txm files were converted into *.tiff stacks using a Fiji (ImageJ) plugin [57].

For image registration we first opened the required datasets in Dragonfly's Fusion Mode, adjusting opacity and changing the Lookup Table (LUT) to improve visualization of fused images. To bring the two data sets in close proximity, their centroids were aligned (select one of the datasets in the Data Properties and Settings panel > Align > Centroid with ...). Then, data sets were registered manually, which proved to be necessary for adequate results. For this, we first split the view into four equal views (x- y- & z-axis and 3D). After that, the micro-CT data was selected in the Data Properties and Settings panel. Then, by clicking the Move tool in the Move panel (Dynamic refresh on), the Translate and Rotate tools appear in the selected view. Dragging the Translate tool allows to move the selected dataset within the view, the Rotate tool rotates the selected dataset from the pivot position (pivot position can be moved if desired). Those tools were used until adjustments lead to the best co-registration possible. In order to save manual adjustments, the modified datasets have to be exported (e.g., as *.ORSObject or *.tiff).

All registration procedures in this demonstration used only affine transformations. Thus, elastic transformations between the two data sets (such as minor differences in the heart cycle, small differences in size due to shrinkage caused by the contrasting agent for micro-CT, or uncertainty in actual dimensions in OCT scans) were not compensated. However, this did not affect interpretation as data acquisition at end-diastole allowed for detection and correlation of the same structures with affine transformation (Figures 5 and 6).

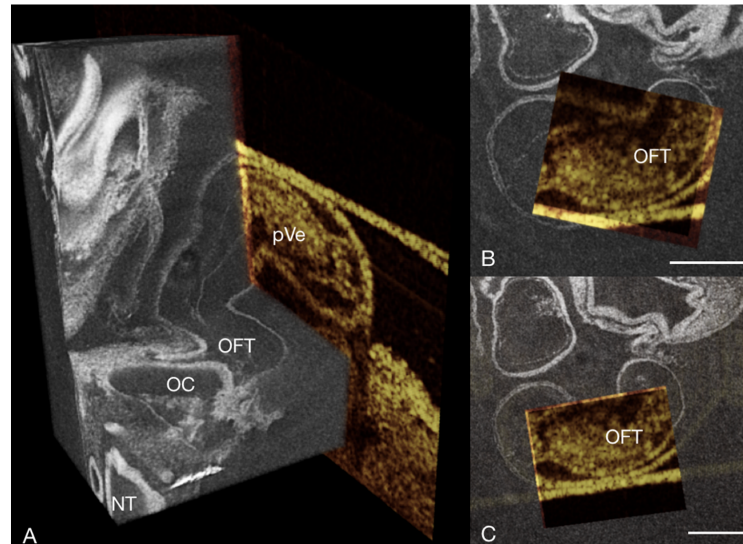


Figure 5. 3D micro-CT volume (grayscale LUT) of the heart of a chick embryo stained with PbOAc at HH13 in end-diastole correlated with an OCT B-scan (golden LUT) of the same specimen at the same stage of the heart cycle. (A) Virtual cutaway (dorsal) of a micro-CT volume rendering showing the early heart tube correlated with a specimen specific OCT B-scan of the same position of the heart. (B,C) Rostral view of virtual thick sections in the transverse plane of a micro-CT volume rendering with a window cut out to show the co-registered OCT B-scan of the same specimen. NT neural tube, OC oral cavity, OFT outflow tract, pVe primary ventricle; scale bar = 100 μ m.

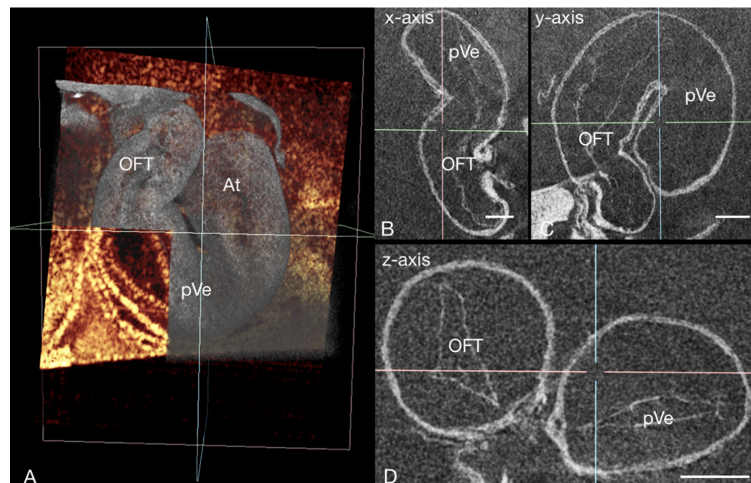


Figure 6. 3D micro-CT data (grayscale LUT) of the heart of a chick embryo stained with PbOAc at HH13 in end-diastole, correlated with an OCT B-scan (golden LUT) of the same specimen demonstrating how specimen-specific micro-CT image acquisition subsequent to OCT imaging allows a better understanding of the spatial distribution of cardiac (micro-)structures, as the capability of

micro-CT to provide 3D virtual slicing of the whole heart allows for an extensive cardiac assessment. (A) Virtual cutaway of a micro-CT volume rendering of the early heart tube co-registered with a OCT B-scan of the same specimen providing an overview of the developing chick heart. The axis position (x: blue, y: pink, z: green) in (A) corresponds with the axis position in (B–D), which shows virtual sections through the heart at different cutting planes. At atrium, OC oral cavity, OFT outflow tract, pVe primary ventricle; scale bar = 100 μ m.

3.3. Other Findings and Considerations

3.3.1. Staining Used

We decided to focus on PbOAc staining [49], as it is more specific to nuclei, meaning that more of the staining agent binds to nuclei than to other components of the imaged tissue; blood cells stain especially heavily with PTA (Figure 7). This is valuable in understanding cardiac development as alteration in the number of cells migrating into cardiac cushions during endothelial to mesenchymal transition (EndoMT) have been associated with CHD. The process of EndoMT is responsible for populating the cushions with cells crucial for normal development into functional heart valves [58–61]. In normal heart development, the number of cells found in the cardiac cushions has been found to increase linearly over development, but when disrupted by hemodynamic interventions, the rate of EndoMT is altered [5,60,62]. The resolution of both OCT [63] and micro-CT (Figure 7) allows for assessing EndoMT via the cell-counting method. While OCT is ideal for longitudinal progression of EndoMT, micro-CT allows more robust positioning of the imaging plane and assessment of the spatial distribution of invading cells. The inherent optical tissue contrast of OCT suffices for cell imaging in the developing cardiac jelly. However, when evaluating the number of invading cells, we recommend PbOAc staining [49]. Other stainings, such as PTA, also allow the visualization of nuclei (Figure 7), but when stained with PbOAc, nuclei absorb more X-rays and therefore have higher grayscale values than other tissues components (see Figure 1). Based on this, we were able to use Fiji's (ImageJ) "Find Maxima" tool on a virtual thick section of a selected area of the OFT cushions to evaluate the number of invading cells (Figure 8). Cells thus marked can then be enumerated using the cell counting tools in Fiji or other software.

However, co-registration proved to be independent of whether PbOAc or PTA was used for X-ray contrast enhancement. We are confident that all general contrast agents will provide sufficient contrast for co-registration of the heart tissues. However, some stainings (such as PTA) can cause significant tissue shrinkage, which would lead to less precise co-registration. Whenever possible, we recommend stains that are already less prone to cause shrinkage, such as PbOAc. When using a very tissue-specific contrasting agent, dual energy micro-CT (microDECT; [64]) might be necessary to simultaneously image the histology and the general morphology.

3.3.2. Using micro-CT for Doppler OCT Based CFD Model Validation

Micro-CT data has frequently been used by itself for creating the flow domain of a CFD model (e.g., [60]), as it can be combined with ultrasound methods which do not provide geometric information. Micro-CT also provides a more direct way to create the geometry, as the anisotropic nature of OCT and the motion of the beating heart poses a challenge. However, this of course only provides static information. Hence, methods facilitating the acquisition of the 3D vessel geometry from OCT data have been developed [65,66], allowing for more realistic, dynamic CFD models. Nonetheless, OCT still relies on refractive indices averaged over all tissues to provide geometric information, leading to inaccurate heart lumen diameters that could affect the accuracy of CFD models. Many efforts are made to precisely measure and correct for different refractive indices [67–69], but whole organs, like the developing chick heart, are made up of many different tissues, complicating any attempts at acquiring realistic geometries from OCT data.

As micro-CT data is calibrated in geometry, it could, in theory, serve as an excellent tool for validating the geometry obtained from OCT imaging. However, tissue preparation

necessary for micro-CT imaging can lead to significant tissue shrinkage, caused by the fixation and staining method used [29], among other things. However, tissue shrinkage can be minimized either by polymer perfusion [70] or by the combination of formaldehyde-fixation, hydrogel-embedding [71], and neutral-buffered iodine infiltration [72]. Hence, careful embryo preparation for micro-CT imaging in combination with our proposed workflow could circumvent the technical challenge of correcting for different refractive indices in the multi-layered heart tube, while still allowing for specimen-specific geometry validation: cross-sectional OCT B-scans at different positions of the heart tube in end-diastole would need to be co-registered with the specimen-specific end-diastole fixed micro-CT data. Then, the lumen surface needs to be assessed for all cross-section images using OCT. In Dragonfly ORS, this is done by segmenting lumen in the virtual slices and B-scans of interest; basic measurements like surface size are then calculated automatically if the image data was loaded with the correct voxel size. Then, the quotient of the lumen surfaces of the same position in an OCT B-scan and a micro-CT scan can be used to validate the dynamic geometry retrieved from OCT imaging to model changes in blood flow over the cardiac cycle even more accurately.

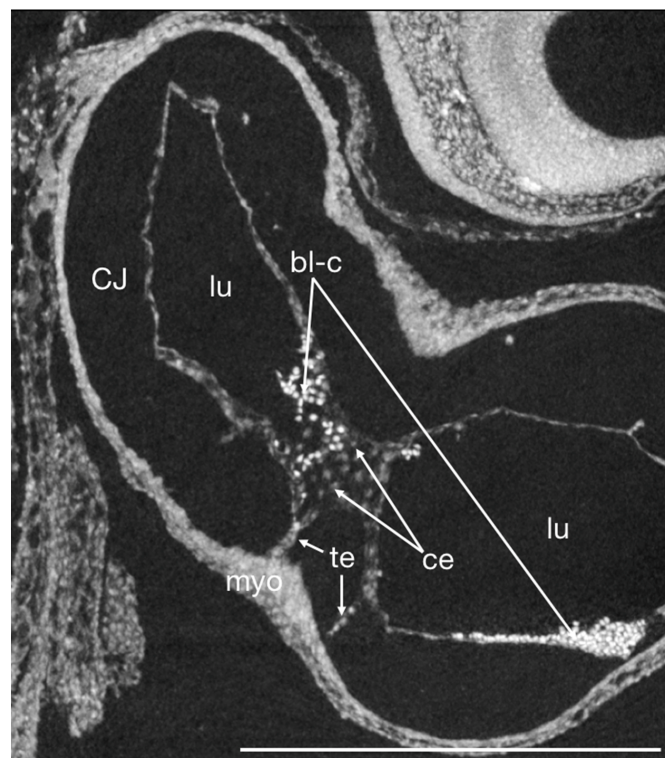


Figure 7. Virtual thick section of a micro-CT scan of a PTA stained HH16 chick embryo heart in sagittal plane showing the OFT and ventricle at early onset of cells migrating into the endocardial cushions (EndMT) and the two-layered myocardium in near histological resolution. The cardiac jelly (CJ) appears empty as the collagens, glycoproteins, and mucopolysaccharides that it consists of do not stain sufficiently with PTA to be detectable at this resolution. Note that blood cells stain heavily with PTA. bl-c blood cells, ce cell nuclei, CJ cardiac jelly, endo endocardium, lu lumen, myo myocardium, te tether. scale bar = 500 μ m.

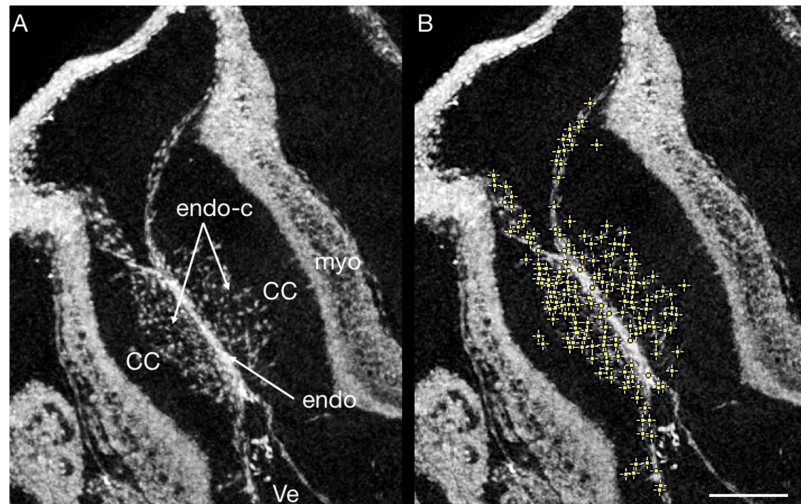


Figure 8. (A) Virtual thick sections of a PbOAc stained HH17 chick OFT. The cardiac cushions are invaded by endothelial cells undergoing EndoMT. (B) Maxima in grayscale values found in a selected area (cardiac cushions) using Fiji's (ImageJ) "Find Maxima" tool (yellow dots; here, maxima correspond to nuclei, as PbOAc is nuclei-specific, which means they absorb more X-rays and therefore have higher grayscale value). CC cardiac cushion, endo endothelium; endo-c endothelial cells, myo myocardium, Ve ventricle; scale bar = 100 μ m.

3.3.3. Reproducibility

During OCT imaging of the embryo, the strong signal of the interface between air and egg needs careful handling. Depending on where exactly the embryo lies within the egg, this strong reflection can potentially saturate the detector and create image artifacts overlaying the beating heart. This is particularly problematic if the embryo lies close to the surface or in the middle of the opening, where the surface is more flat than curved, which causes stronger reflections. Besides refractive index matching by adding a few drops of chick Ringer's, we found that tilting the egg to orient the interface between embryo and yolk/egg white oblique to the incident laser beam helped to reduce strong back reflection artifacts. Thus, the area of strong detected signal from the surface can be properly managed.

Regarding reproducible B-scan orientation, variations in egg size and embryo positioning require individual changes to the settings of the OCT system with respect to the reference arm length and the z-position of the egg itself. The live preview of the OCT data is helpful for the desired orientation of the embryo. Experience with the mentioned parameters led to a reproducible OCT data acquisition from the chicken embryos.

With micro-CT imaging, the orientation of the specimen during image acquisition does not matter, as the isotropic 3D image volumes can be arbitrarily resliced. However, the times for staining we specified might need to be adjusted depending on sample size.

4. Discussion

We have demonstrated a correlative multimodal imaging workflow using OCT and micro-CT imaging for early development heart modeling in the chick. As discussed, both OCT and micro-CT imaging individually have already greatly enhanced our understanding of cardiac morphogenesis and the interaction of hemodynamics with the developing heart, but the integration of both into a multi-modal workflow will allow for new insights impossible to achieve using either method alone.

OCT delivers dynamic in vivo anatomical and morphological information at acquisition speeds of up to multiple volumes per second [73], and also functional information if Doppler OCT is applied to detect axial blood flow velocity at the same spatiotemporal resolution as structural OCT [21]. Micro-CT data provides high-detail isotropic 3D data that is calibrated for both geometry and intensity and that can be acquired over a large number of developmental stages [29]. The combination of these modalities, however, not only overcome the limitations of isolated standard imaging approaches, but also provide unique and complementary information only achievable through the correlation of these data. Integrating those two modalities is particularly valuable when studying treatments or interventions that alter blood flow in order to replicate CHD, especially when those interventions can lead to varying blood flow conditions from embryo to embryo [8].

Even without subsequent analysis, the proposed workflow will allow for a deeper understanding of the downstream effects hemodynamic interventions can have on cardiac morphogenesis, as many visible developmental changes are already known to be associated with CHD. We hope to provide a useful guide on how to implement this workflow and to have demonstrated that a vast amount of open questions about cardiac development can be addressed using this multimodal approach. The potential of this multimodal approach has not yet been fully explored, and the integration of OCT and micro-CT imaging will allow for even further insights into heart development in the future. With the continued advancement of technology, the potential for studying heart morphogenesis will expand even further. While micro-CT imaging is already a well-established technique, efforts for moving toward virtual 3D histology are only just starting to take off.

With the development of new, tissue-specific contrast agents, even more specific questions aiming to better our understanding of normal cardiac development and the formation of CHD can be addressed with the presented workflow. However, despite expected advancements in contrast agent development, the low molecular sensitivity of micro-CT imposes a limit on its use for analyzing molecular signals. While potential ways of amplifying weak signals are feasible, another way of circumventing this limitation could be the use of micro-CT as a bridge technique. As both OCT and micro-CT are non-destructive, they lend themselves to integrating even further imaging modalities [21,29,74,75]. In such a workflow, micro-CT imaging will provide complete, isotropic 3D data sets enabling easy co-registration and co-localizations of ROIs in OCT data with either histology or immunohistochemistry, allowing for subsequent downstream analysis [29,43].

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6.1.2. Oxygen

Developmental pathways that are modulated by shear forces intersect with signaling cascades modulated by oxygen availability. Thus, by reading more about oxygen's role during cardiogenesis, I started to suspect that oxygen availability during cardiogenesis plays an instrumental role in shaping the developmental system of the heart. Unlike mechanical load, oxygen levels differ predictably between developmental modes and across vertebrate taxa. Yet medical literature often remains agnostic to these differences, treating embryonic environments as uniform despite substantial variation in oxygenation among model organisms such as zebrafish, *Xenopus laevis*, mouse, or chicken – and despite oxygen's known influence on cellular metabolism, developmental rate, and the timing of morphogenetic events.

Paper II: Environmentally Dependent Developmental Induction as a Potential Driver of Heart Evolution

For Paper II, I apply the comparative, mechanistic argument advanced in this thesis, focusing primarily on oxygen. Using published physiological and gene expression data from different vertebrates, I propose how variation in developmental oxygen environments corresponds to distinct underlying mechanisms. The paper introduces the Environmentally Dependent Developmental Induction (EDDI) framework, which conceptualizes environmental factors as genuine difference-makers that shape both the generation and stabilization of phenotypic variation. EDDI offers a mechanistic account of how repeated environmental influences, such as oxygen levels or mechanical load, can become constitutively embedded within developmental mechanisms over evolutionary time, without requiring classical genetic assimilation.

In this framework, environmental factors serve as *difference-makers* that shape both the generation and stabilization of phenotypic variation. EDDI thus provides a mechanistic explanation for how the same environmental conditions that once facilitated evolutionary innovation can now underlie developmental vulnerability. This paper has been accepted in Journal of Experimental Biology (05.12.2025).

Author Contributions

I am the single author of the manuscript and as such was solely responsible for the conceptualization, literature review, interpretation, and writing of the manuscript. Although I am the single author of the paper, my supervisor, Brian Metscher, provided valuable feedback that helped refine the clarity of the writing and he served as a discussion partner during the development of certain ideas. All revisions incorporated this feedback, but the final work is my own. Brian Metscher approved the final manuscript prior to submission.

COMMENTARY

Environmentally dependent developmental induction as a potential driver of heart evolution

Nina Kraus^{1,2,*}

ABSTRACT

Developmental biology and evolutionary theory have traditionally emphasized gene mutations as the primary drivers of new traits, with natural selection shaping the resulting variation. However, recent insights highlight the role of environmental factors during development in shaping trait evolution. In this Commentary, I introduce the 'environmentally dependent developmental induction' (EDDI) model, which proposes that phenotypic evolution is driven not only by genetic changes but also by environmentally induced modifications to the core developmental program. Using cardiogenesis as an example, I argue that environmental triggers such as oxygen levels and mechanical forces expand the genotypic toolkit available to heart development, activating new pathways that lead to the emergence of novel cardiac structures. These lineage-specific environmental changes might thus influence the differentiation of cardiac progenitor cells, resulting in modifications to the cardiac building plan. The EDDI model provides a novel explanation for how the basic cardiac plan was expanded during evolution while simultaneously explaining why cardiogenesis is vulnerable to malformations, even in the absence of genetic defects.

KEY WORDS: Evo-devo, Cardiogenesis, Environmentally induced modifications, Devo-Evo, Theory

Introduction

Gene mutations are often considered the primary source of new phenotypic traits. However, the development of a phenotype arises from a dynamic interplay between gene-regulatory networks, environmental factors and epigenetic influences (epigenetics *sensu* Newman and Müller, 2000: conditional responses of tissues to each other and to external forces). Epigenetics and the environment provide the physico-chemical substrate necessary for development (Brun-Usan et al., 2022; Newman et al., 2006; Newman and Müller, 2000). As embryos develop, they encounter a characteristic sequence of environmental conditions. Thus, development produces the phenotype, and the variation upon which natural selection acts depends not only on the genotype but also on how the genotype interacts with its environment during development. This suggests that phenotypes evolve not only through genetic changes but also in response to environmental factors (Brun-Usan et al., 2022; Crispo, 2007; Emlen and Nijhout, 2000; Levis and Pfennig, 2016; Newman et al., 2006; Waddington, 1940, 1942, 1959; West-Eberhard, 2005).

The classical Baldwin (1896) effect (see Glossary), Levis and Pfennig's (2016) plasticity-first hypothesis (see Glossary) and West-Eberhard's (2005) genetic accommodation model (see Glossary) put the plastic phenotype at the center of their evolutionary theory. They all suggest that a phenotype's initial plastic response to a novel environment involves the overproduction of random variation, some of which may confer adaptive benefits. If the environment consistently evokes this plastic response over evolutionary time, genetic variation accommodating this adaptive phenotype may emerge, leading to the phenotype's heritable stabilization.

The stress-induced evolutionary innovation model of Wagner et al. (2019) describes the initial response to a new environmental stressor not as random but as a protective stress response within an organism's normal reaction range. If this stressor becomes a persistent feature of the species' life cycle, the stress response may become internalized, producing a compensatory phenotype that is independent of the environment. Over time, this is said to lead to a genetic shift in the reaction norm (see Glossary), facilitating the consistent production of the compensatory phenotype (Love and Wagner, 2022; Wagner et al., 2019).

The aforementioned models have different ideas about how a plastic phenotype allows for evolutionary change, but they all assume that genetic assimilation (see Glossary) of the new developmental program is eventually required to consistently evoke similar adaptive phenotypes. Fittingly, findings from: (1) epigenetics (in the now more common use of the word: 'the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence'; Wu and Morris, 2001) and (2) the field of developmental origins of health and disease have shown that environmental conditions during development can produce long-lasting, sometimes transgenerational phenotypic changes without altering the DNA sequence (Lacagnina, 2020).

In contrast to models emphasizing the eventual genetic control of adaptive phenotypes, here, I argue that genetic assimilation might not always occur, particularly in cases where environmental perturbations are known to cause developmental defects. I use vertebrate cardiogenesis to demonstrate that key aspects of the phenotype could remain persistently dependent on environmental triggers. This provides an instructive example of sustained phenotypic plasticity without genetic assimilation while also explaining how development can reliably produce consistent phenotypes in the absence of genetic assimilation.

Environmentally dependent developmental induction (EDDI)

In this Commentary, I propose that the cardiac developmental program was expanded in response to changes in the developmental environments of different vertebrate classes. I posit that changes to the core developmental program do not become genetically assimilated, but still rely on environmental cues to become induced during cardiogenesis. This model challenges the conventional gene-centric view of heart development and explains how the

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Glossary

Ambiogenic induction

A process in which highly localized micro-environmental conditions within a specific tissue or cellular niche act as direct triggers for developmental responses.

Atavism

The reappearance of a lost character typical of remote ancestors but not seen in the parents or recent ancestors. Features of the atavistic character are its persistence into adult life, its presence in only one or a few individuals within a population and its close resemblance to/shared identity with the same character possessed by all members of an ancestral population (Hall, 1984).

Baldwin effect

The idea that behavioral or developmental plasticity can guide evolution by allowing organisms to survive and reproduce until genetic changes stabilize the adaptive trait.

Carnegie stages

A standardized series of 23 stages in the embryonic period of normal human development (Fierman et al., 2023).

Conotruncal heart defects

A group of congenital heart malformations involving improper development of the heart's outflow tract, affecting the connection between the ventricles and the great arteries (e.g. truncus arteriosus, Tetralogy of Fallot).

Endothelial-to-mesenchymal transition (EndoMT)

Endothelial cells are organized in two dimensions and sit on a basal membrane. Mesenchymal cells have no basal layer and can thus be organized three dimensionally. EndoMT refers to the process where endothelial cells lose their endothelial characteristics and transdifferentiate into a mesenchymal phenotype that has a different genetic expression profile and the ability to migrate.

Environmentally dependent developmental induction (EDDI)

A model proposed here that encompasses the expansion of an ancestral developmental network through plastic responses to environmental cues. Within this model, newly recruited developmental circuits are not under the genetic control of the initial network but are triggered by ambiogenic induction.

Genetic assimilation

'The process by which a phenotype that is initially induced by an environmental stimulus becomes stably inherited in the absence of the stimulus after a few generations of selection' (Raju et al., 2023).

Haecklian explanation

Ernst Haeckel's recapitulation theory (also termed 'biogenetic law') states that ontogeny (development of an individual organism) recapitulates phylogeny (the evolutionary history of the group) – a view that tends to be seen as overly simplistic by most evolutionary biologists.

Hamburger–Hamilton stage

A standardized series of 46 stages in normal chick development from egg deposition to hatching (Hamburger and Hamilton, 1951).

Levis and Pfennig's plasticity-first hypothesis

This hypothesis proposes that environmentally induced phenotypes can precede and facilitate genetic evolution by exposing cryptic variation to selection.

Reaction norm

'The spectrum of phenotypic variation produced when individuals of the same genotype are exposed to varying environmental conditions' (Manuck, 2010).

West-Eberhard's genetic accommodation model

A model describing how phenotypic variation, whether environmentally or developmentally induced, becomes stabilized or refined through selection acting on its underlying regulatory mechanisms.

List of symbols and abbreviations

CAM	chorioallantoic membrane
CC	cardiac cushion
CHD	congenital heart disease
cNCC	cardiac neural crest cell
EDDI	environmentally dependent developmental induction
EndoMT	endothelial-to-mesenchymal transition
HIF	hypoxia-inducible factor
OFT	outflow tract
SHF	second heart field
VEGF	vascular endothelial growth factor

the induction of which does not fall under genetic control. In this view, the regulation of the developmental program remains conditional on certain environmental triggers, and thus depends on properties and mechanisms beyond the strictly genetic. I call this model 'environmentally dependent developmental induction' (EDDI; see Glossary). I term the process by which environmental changes activate additional circuits 'ambiogenic induction' (see Glossary). Rather than being permanently incorporated into a genetic network, these sub-circuits rely on specific, local, extrinsic factors to be activated during development. Thus, EDDI posits that certain environmental changes during development – such as the significant shift in oxygen levels at the onset of placentation or egg deposition and chorioallantoic membrane (CAM) formation (Carroll et al., 2021; Nowak-Sliwinska et al., 2014) – could lead to ambiogenic induction, which potentially serves as a protective stress response within an organism's normal reaction range (Love and Wagner, 2022; Wagner et al., 2019). The resulting stress response could then recruit new regulatory pathways into the core developmental program, leading to new developmental interactions, altered cell-fate decisions and the subsequent emergence of new phenotypes. Evolutionary shifts in developmental timing – such as changes in egg deposition or placentation – would thus have the potential to influence heart phenotypes in developing embryos.

Within the EDDI model, phenotypic changes could become evolutionarily persistent without requiring genetic assimilation by the core developmental program, as long as the developmental environmental sequence remains stable over evolutionary time (Brun-Usan et al., 2022). If the developmental sequence of environmental cues is consistent across generations, it should reliably evoke the same adaptive phenotype without fixing it through genetic mutations (Kuijper and Hoyle, 2015; Minelli, 2016). This does not suggest that genetic mutations are unimportant but rather that they alone cannot fully explain morphological changes. EDDI posits that genetic changes, although necessary, may not be sufficient to produce novel phenotypes without environmental triggers that also change how genes are expressed. For instance, a gradual genetic shift in a cell's response to oxygen deprivation may only reveal a new phenotype when combined with sudden changes in developmental conditions, making the genetic variation subject to natural selection (Lofft et al., 2023; Waddington, 1940).

Within this Commentary, I discuss how the theoretical framework of EDDI might explain: (1) how developmental programs were expanded during the evolution of different heart phenotypes in vertebrates and (2) why the evolutionary history of the heart leaves its phylogenetically youngest structures especially vulnerable to malformations during development, even in the absence of genetic defects. For this, I review ongoing research in cardiogenesis and present testable predictions made using the EDDI framework.

evolutionary history of the heart leaves its newer accessory structures particularly vulnerable to pathogenesis.

To place this view in a theoretical framework, I introduce a model for the expansion of developmental programs (i.e. networks of gene interactions) through plastic responses to environmental changes,

A new perspective on developmental gene expression: integrating oxygenation, apoptosis and evolution in cardiogenic development

Vertebrate heart evolution

At its core, the vertebrate heart is a muscular pump comprising two functional segments: an inflow tract that receives blood from the venous vascular network and an outflow tract (OFT) that ejects it into the arterial vascular network (Fisher and Burggren, 2007; Simões-Costa et al., 2005). This basic architecture is present during early cardiogenesis in all vertebrates, but the developmental trajectory from this common starting point diverges in phylum- and lineage-specific ways. In jawed fishes, the heart comprises a two-chambered pump with one atrium, one ventricle and an undivided OFT. Amphibians typically have two atria, one ventricle and an undivided OFT containing a septum that might help maintain partial separation between oxygenated and deoxygenated blood (Kraus and Metscher, 2022). Most reptiles also have three chambers, with the ventricle and OFT variably and incompletely divided. In crocodilians, birds and mammals, the heart consists of four chambers forming a functional double circulation (i.e. two completely separated blood circulatory systems) that directs oxygenated blood to the body and deoxygenated blood to the lungs (Fig. 1; Stephenson et al., 2017).

In this Commentary, I focus on the different OFT morphologies. Among vertebrates, modifications to the OFT are phylogenetically the youngest cardiac structures and exhibit considerable adaptability (Fig. 1). This adaptability is essential for regulating blood flow

to different gas exchange organs (e.g. lungs, gills, skin; Gittenberger-de Groot et al., 2005; Restivo et al., 2006; Rothenberg et al., 2003). Interestingly, altered cardiac phenotypes in the human OFT often resemble normal phenotypes found in other vertebrates. This raises the possibility that the mechanisms driving macroevolutionary innovations in the OFT may be clinically relevant, potentially contributing to cardiac malformations (Fisher and Burggren, 2007).

Cardiogenesis and congenital heart malformations

Cardiac morphogenesis is driven by the specification, proliferation, differentiation and migration of cardiac progenitor cells (Yuan et al., 2017). Specifically, two major populations of cardiac progenitor cells drive early cardiac morphogenesis. The first heart field, composed of early-differentiating cardiac cells, gives rise to part of the left ventricle. Later-differentiating progenitor cells, known as the second heart field (SHF), originate from the pharyngeal mesoderm and contribute to the progressive extension of the heart tube, forming the myocardium of the atria, the ventricle and the inflow and outflow tracts (Fig. 2; Buckingham et al., 2005; Kelly et al., 2014; Kelly and Buckingham, 2002).

Disruptions in the pathways regulating these processes can lead to congenital heart disease (CHD; Figs 3 and 4; Kelly et al., 2014). Although genetic mutations have been identified in some familial and spontaneous cases of CHD, the majority of cases cannot be attributed to a single genetic cause (Fahed et al., 2013). However,

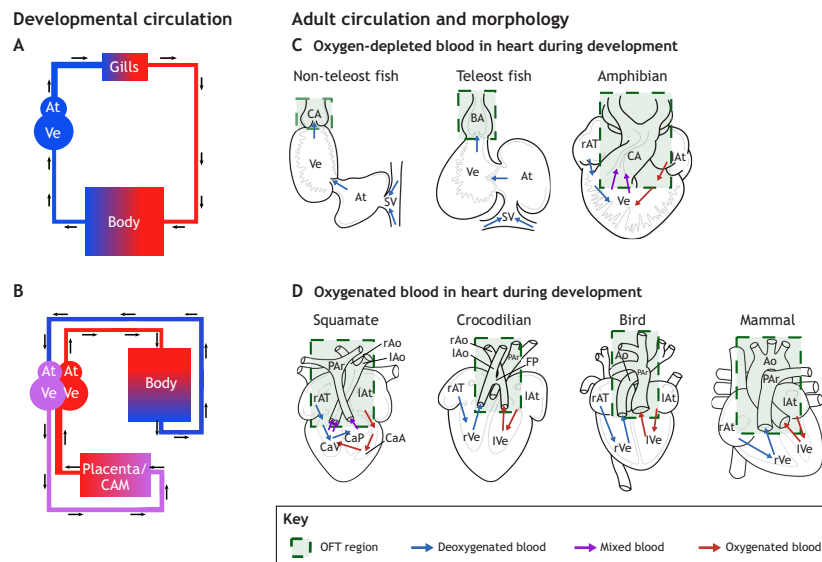


Fig. 1. Modifications to the outflow tract (OFT) of the heart show greatest variability and complexity among different vertebrates. (A) Schematic depiction of blood flow patterns during development for animals with (B) oxygen-depleted blood in the heart during development. (C) Schematic depiction of blood flow patterns during development for animals with (D) oxygenated blood in the heart during development. Arrows indicate the direction of blood flow; colors correspond to the oxygenation status of blood. Note that all hearts of all classes receiving oxygen-depleted blood during development do not show shortening of the OFT (green boxes) by apoptosis during cardiogenesis. In vertebrate hearts receiving oxygenated blood during development, the OFT shortens and becomes partially incorporated into the ventricle. This completes ventricular separation and leaves each ventricle with its own outflow vessel, leading to a complete, functional double-circulation. (r/l)Ao, (right/left) aorta; (r/l)At, (right/left) atrium; BA, bulbus arteriosus; CA, conus arteriosus; CaA, cavum arteriosum; CAM, chorioallantoic membrane; CaP, cavum pulmonale; CaV, cavum venosum; FP, foramen panizza; OFT, outflow tract; PA, pulmonary artery; (r/l)Ve, (right/left) ventricle; SV, sinus venosus.

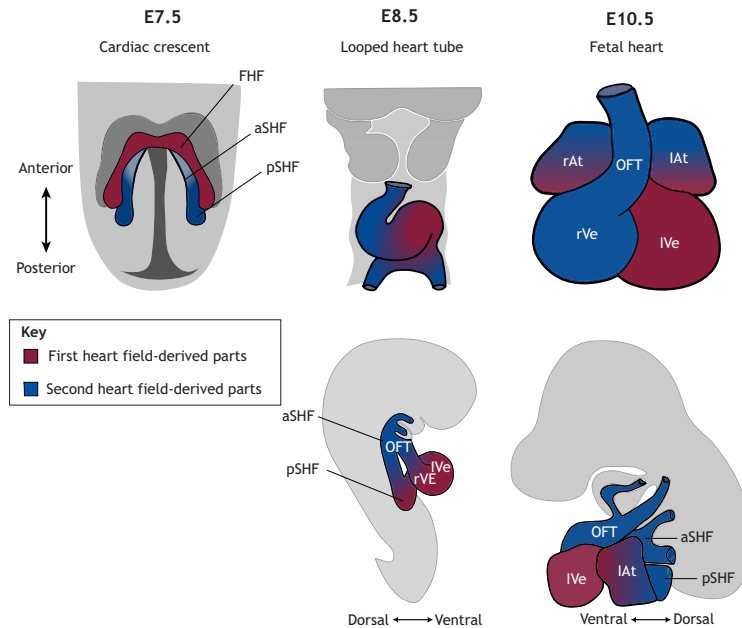


Fig. 2. Schematic illustration of mouse heart development from the cardiac crescent (E7.5) to the fetal heart (E10.5). The figure highlights the contributions of the first heart field (FHF, red) and second heart field (SHF, blue) to different heart structures. Between E7.5 and E10.5, the SHF progressively provides progenitor cells that differentiate and go on to contribute to various cardiac structures during this developmental window. Drawings based on Hempel and Kühl (2016) (<http://creativecommons.org/licenses/by/4.0/>) and Kelly (2012). (pr/p)At, primitive (right/left) atrium; a, anterior; p, posterior; other abbreviations as in Fig. 1.

non-genetic factors, such as placental abnormalities (Burton and Jauniaux, 2018; Kalisch-Smith et al., 2024; Nijman et al., 2024), high-altitude living (Hasan, 2016; Miao et al., 1988; Song et al., 2021), smoking (Lee and Lupo, 2013; Wang et al., 2022; Zhang et al., 2017) and prenatal diabetes (Basu and Garg, 2018; Choudhury et al., 2021; He et al., 2024; Ibrahim et al., 2023; Morishima et al., 1996; Zhao et al., 2020; Zhao, 2010), have been associated with an increased risk of CHD, though the exact mechanisms remain unclear (Dunwoodie, 2009; Giussani, 2021; Patterson and Zhang, 2010; Kenchegowda et al., 2014; Patel and Burns, 2013; Simeone et al., 2016; Webster and Abela, 2007).

Notably, most CHDs in humans involve disruptions in the terminal stages of the SHF contribution to the heart, particularly affecting OFT formation. This process, which transforms the heart's single OFT into a separated pulmonary artery and aorta, is frequently disrupted, leading to malformations of the double-circulatory system (Fisher and Burggren, 2007; Gittenberger-de Groot et al., 2005; Pierpont et al., 2018; Zaidi and Brueckner, 2017).

I propose that the variability of the OFT and its susceptibility to malformation both stem from temporal differences in progenitor cell differentiation. Early cardiogenesis, and thus early progenitor differentiation, occurs under hypoxic conditions for all vertebrate species. But SHF differentiation, which is critical for OFT septation, coincides with species-specific changes in oxygen availability linked to developmental mode (e.g. placentation, timing of egg deposition and CAM formation). Oxygen levels influence proliferation and differentiation of multipotent progenitor cells (Fisher and Burggren, 2007; Gittenberger-de Groot et al., 2005), and shifts in oxygen regime could therefore shape OFT development, leading to lineage-specific phenotypes (Figs 3 and 4). Additionally, altered oxygen levels are frequently associated with cardiac malformations (Dunwoodie, 2009; Giussani, 2021; Patterson and Zhang, 2010;

Kenchegowda et al., 2014; Patel and Burns, 2013; Simeone et al., 2016; Webster and Abela, 2007).

This view contrasts with the idea that the basic cardiac blueprint was expanded during evolution by genetically assimilating pre-existing developmental pathways into the core program of cardiogenesis. In the 'genetic assimilation' model, mutations in the expanded cardiac program are thought to underlie CHD (Olson, 2006; Taussig, 1988). However, the high number of CHD cases with unknown causes and the numerous external factors that can disrupt normal cardiogenesis suggest that formation of these additional cardiac structures may not be driven by genetic mutations in the core heart development program. Below, I discuss major events in cardiogenesis and outline how certain developmental abnormalities correspond with the cardiac phenotypes we see in phylogeny.

Oxygen delivery to the embryo and embryonic heart

The hearts of developing anamniotes (fishes and amphibians) pump oxygen-poor blood to the gills, where it is oxygenated and dispersed to supply all organs. Thus, the embryo receives enough oxygen for overall development but the cardiac tissue contacts oxygen-depleted blood during critical developmental stages (Fig. 1; Kardong, 2019; Kraus and Metscher, 2022). In contrast, the hearts of amniotes (squamates, crocodilians, birds and mammals) receive oxygenated blood during development, owing to different strategies of embryonic gas exchange (Fig. 1). In reptiles, crocodilians and birds, oxygen diffuses into the blood through the eggshell. As the CAM forms and expands, the surface area for gas exchange increases further to keep up with an increasing metabolic demand of the growing embryo (Schlatter et al., 1997).

In mammals, placental respiration begins once the embryonic circulation is fully closed. This is achieved when the developing placenta invades the maternal uterine spiral arteries. Initially, blood

flow from these arteries to the embryo is blocked by endovascular plugs. In humans, small channels within these plugs begin to form around 6–7 weeks of gestation (Carnegie stages 10–17; see Glossary; note that Carnegie stages are counted from fertilization, whereas pregnancy is usually dated from the last menstrual period, creating a ~2-week offset). Over time, the plugs gradually disintegrate, allowing continuous blood flow by 8–10 weeks of gestation (Carnegie stages 18–23; Pijnenborg et al., 2006; Roberts et al., 2017; Sato, 2020; Faber et al., 2021). This transition coincides with the heart's shift from a simple, unidirectional tube to a structurally developed organ at the end of embryonic development. A similar time line occurs in mice, despite the species being at otherwise different stages of development: as fetal–maternal blood flow starts at around embryonic day (E)9.5, the heart undergoes abrupt developmental changes (Figs 3 and 4; Jauniaux et al., 2000; Graham et al., 2015; Buijtenlijk et al., 2020; Hu and Zhang, 2021). Although oxygen saturation in mammals remains low compared with that experienced during postnatal life, disintegration of the spiral artery plugs increases oxygen availability from about 2.5% to 8% during the first trimester (Morton and Brodsky, 2016; Saghian et al., 2019; Burton et al., 2021).

Apoptosis and OFT remodeling

In mammals, birds and crocodilians, OFT remodeling involves the incorporation of the conus arteriosus into the ventricle, proper alignment of the OFT with the ventricles and the shortening of the truncus arteriosus through programmed cell death (i.e. apoptosis; see Fig. 3 for a depiction of the conus arteriosus and truncus arteriosus; Gittenberger-de Groot et al., 2005; Barbosky et al., 2006). Previous studies suggest that tissue hypoxia induces this apoptosis (Anderson et al., 2003; Barbosky et al., 2006; Rothenberg et al., 2003; Sugishita et al., 2004a) through hypoxia-inducible factor (HIF), which upregulates the main biochemical pathway for gene-based defenses against oxygen deprivation (Fisher and Burggren, 2007; Semenza, 1998, 1999). Forced expression of *HIF-1a* has been shown to protect cardiomyocytes from apoptosis during OFT remodeling, actually causing OFT defects (Sallee et al., 2004; Sugishita et al., 2004a,b,c). This suggests that the presence of oxygen-rich blood and consequent HIF degradation potentially triggers OFT remodeling (Figs 3 and 4).

In fishes and amphibians, the conus arteriosus does not integrate into the ventricle, and the truncus arteriosus does not shorten by apoptosis during OFT development (Fig. 1; Wang et al., 2018; Park et al., 2023). As their developing hearts contact oxygen-poor blood, *HIF* expression is likely to be upregulated, as seen in *Xenopus laevis* (Nagao et al., 2008). Taken together, this suggests that when the OFT contacts oxygen-poor blood, HIF maintains its downstream cell-protective effects, hindering apoptosis-driven OFT shortening. Once oxygenated blood flows through the heart, *HIF* downregulation triggers apoptosis and OFT remodeling, as seen in amniotes (Fig. 3; Sugishita et al., 2004a,b,c; Barbosky et al., 2006; Liu and Fisher, 2008).

If the above predictions are correct, then restricting oxygen during the apoptotic window in development of mammals, birds or crocodilians should inhibit correct OFT development (Watanabe et al., 1998). Indeed, our lab was able to experimentally confirm this prediction in chick embryos (Kraus et al., 2025 preprint).

Cardiac cushions

Vascular endothelial growth factor (VEGF), a family of growth factors that stimulate angiogenesis, is downstream of HIF. As *HIF* expression is downregulated in the presence of oxygen, so is *VEGF* expression. Although the downregulation of *VEGF* is necessary to

initiate apoptosis and OFT remodeling, complete inhibition of *VEGF* can lead to heart defects in itself (Sugishita et al., 2004a,b,c). This can potentially be explained by variations in local oxygen availability within the developing heart. In early mammalian and avian development, the endocardium and myocardium are separated by an extracellular matrix that is thin enough to allow oxygen diffusion to the myocardium (Gittenberger-de Groot et al., 2005; Sugishita et al., 2004b,c). As development progresses, endocardial cells lining the inside of the heart undergo an endothelial-to-mesenchymal transition (EndoMT; see Glossary) and migrate into the extracellular matrix. The resulting mesenchymal cells proliferate and form thick cardiac cushions, or heart valve progenitors, between the myocardium and the endocardium. As a result, oxygen no longer diffuses all the way to the myocardium, causing local hypoxia in the myocardium beneath the cushions, with consequential local upregulation of *HIF* and subsequent *VEGF* expression (Fig. 3; Camenisch et al., 2010, 2000; Djenoune et al., 2022; Garside et al., 2013; Runyan et al., 1990). *VEGF* expression is needed locally for proper cardiac cushion remodeling, as EndoMT is regulated by *VEGF* in a dose-dependent manner (Lambrechts and Carmeliet, 2004). Alterations in both global and local OFT tissue oxygenation can thus lead to conotruncal heart defects (see Glossary), providing a good example of ambiogenic induction (Fig. 3).

Neural crest cells

Neural crest cells are a vertebrate-specific innovation. They originate from the developing central nervous system, and migrate extensively via the aortic arches to produce a plethora of cell lineages (Bronner and LeDouarin, 2012). A subpopulation of neural crest cells, the cardiac neural crest cells (cNCCs), migrates towards the heart. In amniotes, cNCCs migrate deep into the distal OFT, where they differentiate into smooth muscle cells and connect the arterial arches with the ventricular septum, thereby forming a complete aorticopulmonary septum. In anamniotes, cNCCs migrate towards the OFT but remain in the pharyngeal arches. Thus, amniotes either completely lack an aorticopulmonary septum or have an incomplete one, as is often seen in squamates (Figs 3 and 4; Hutson and Kirby, 2003; Abu-Issa et al., 2004; Kulesa et al., 2010).

In humans, failure of cNCC migration can result in an absent aorticopulmonary septum, causing congenital heart defects (Poelmann et al., 2017). This can be seen in DiGeorge syndrome, a chromosomal disorder where one copy of the *Tbx1* gene is absent (Burn et al., 1993). Affected individuals show cardiovascular abnormalities characterized by an incomplete aorticopulmonary septum (Théveniau-Ruissy et al., 2008). *Tbx1* has been found to upregulate *Fgf8*, which inhibits *Sema3C*, a chemoattractant that guides cNCCs into the OFT (Barriga et al., 2013; Kodo et al., 2017; Neeb et al., 2013; Toyofuku et al., 2008; Xu et al., 2004). *Fgf8* also fails to be upregulated under hypoxic conditions, phenocopying defects caused by *Tbx1* deletion (Fig. 3; Gandhi et al., 2020).

Taken together, the above discussion suggests that the oxygenated OFT of amniotes is likely to inhibit cNCC migration. However, as described above, hypoxia occurs in the myocardium underlying the OFT cushions (Liu and Fisher, 2008). It is possible that this local hypoxia downregulates the *Tbx1* target *Fgf8* in the cushion area and thus locally upregulates *Sema3C*. *Sema3C* expression might then prompt a subset of cNCCs to migrate from the pharyngeal arches into the distal OFT (Fig. 3). This idea is supported by *Sema3C* expression observed in the developing mouse OFT at E10.5, coinciding with endocardial cells populating the cushions (Keyte and Hutson, 2012; Keyte et al., 2014). In avian species, cNCCs reach the pharyngeal arches around Hamburger–Hamilton stage (HH)11 (see Glossary),

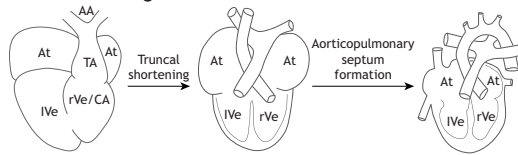
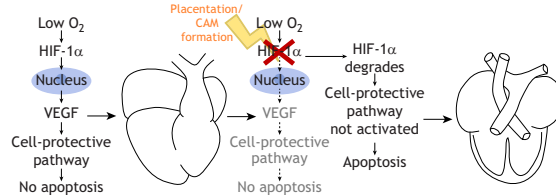
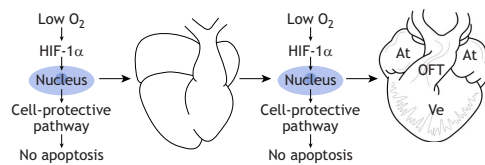
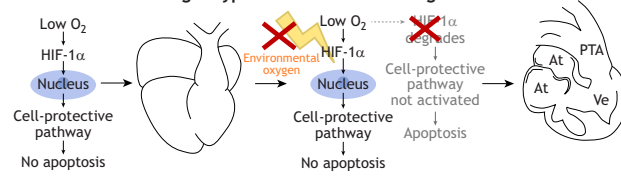
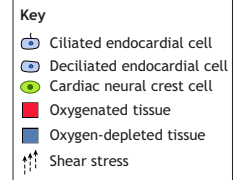
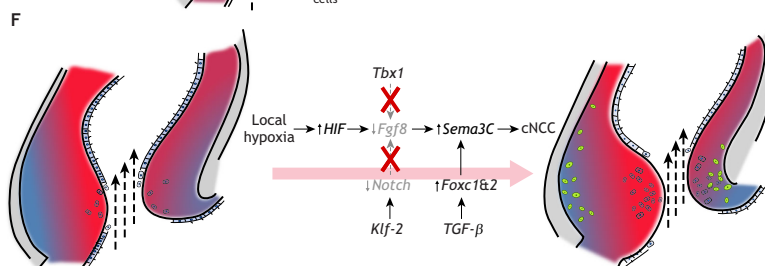
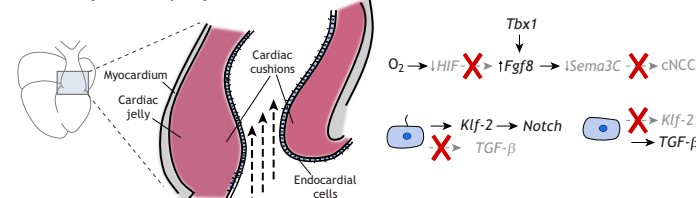
A Mammalian cardiogenesis**B Truncal shortening – normal amniote cardiogenesis****C Truncal shortening – normal anamniote cardiogenesis****D Truncal shortening – hypoxic anamniote cardiogenesis****E Aorticopulmonary septum formation**

Fig. 3. See next page for legend.

Fig. 3. Mechanistic depiction of crucial steps in OFT development.

(A) Normal mammalian cardiogenesis. As the conus arteriosus portion of the OFT is incorporated into the ventricle, the truncus arteriosus (TA) shortens by apoptosis, coincident with the ingress of oxygenated blood. (B) TA shortening during normal amniote cardiogenesis, where sufficient oxygen availability leads to HIF-1 α degradation. This prevents the activation of downstream cell-protective pathways, triggering apoptosis. Apoptosis facilitates OFT remodeling, ultimately reducing the common TA shared by the aorta and pulmonary artery. (C) The normal process in anamniote cardiogenesis, where oxygen-depleted blood flows over the heart. This prevents apoptosis and results in a long TA. (D) Illustration of how the mechanism in amniotes is affected if oxygenation is insufficient to cause HIF degradation. This prevents apoptosis, with a consequent persistent truncus arteriosus (PTA). Completion of the aorticopulmonary (AP) septum requires the migration of cardiac neural crest cells (cNCCs) into the distal OFT. (E) How shear stress regulates gene expression in endocardial cells (ECs). As long as shear stress remains moderate, ECs remain ciliated, but as cardiac cushions continue to swell, some ECs are exposed to higher levels of shear stress, causing their cilium to break. This inhibits *Klf-2* expression, but causes upregulation of *TGF- β* , which is associated with the endothelial-to-mesenchymal transition (EndoMT), where ECs migrate into the cardiac jelly. Together, ciliated and de-ciliated ECs coordinate EndoMT and cNCC migration. In E, OFT tissue is sufficiently oxygenated, so the chemoattractant *Sema3C* remains downregulated. As depicted in F, when EndoMT progresses sufficiently and the cardiac cushions become thicker and populated with ECs, oxygen diffusion no longer suffices to supply the myocardium, causing local hypoxia and upregulation of *HIF* and its downstream target *Sema3C*, which attracts cNCCs into the developing cardiac cushion, where they contribute to AP septum formation. Abbreviations as in Fig. 1.

before EndoMT initiation at HH16. However, these cells do not migrate into the OFT until about HH21, despite the OFT being relatively normoxic earlier than in mammals. This suggests a threshold of hypoxia must be crossed in the cushions to downregulate *Fgf8* and upregulate *Sema3C* (Hamburger and Hamilton, 1951; Hutson and Kirby, 2003; Kirby et al., 1983; Kodo et al., 2017; Martinsen, 2005). This mechanism fits with ambiogenic induction and EDDI, as all necessary factors must converge to induce certain changes to the core developmental program, even if the sequence of events differs between birds, crocodilians and mammals (Figs 3, 4 and 5).

Mechanical forces

In addition to hypoxia, mechanical forces such as shear stress further regulate cNCC behavior and OFT development through *Klf-2* and *TGF- β* signaling pathways. Shear forces, combined with oxygen gradients, create a multifactorial environment that ensures precise OFT remodeling in amniotes (Fig. 3; Hu and Clark, 1989; Liu et al., 2009; Vermot et al., 2009; Yalcin et al., 2011; ten Dijke et al., 2012).

The endocardium lining the inside of the heart consists of a single layer of endothelial cells, each of which is equipped with a mechanosensitive cilium. In normal cardiogenesis, shear stress bends this cilium, which causes *Klf-2* expression. When *Klf-2* is expressed, endothelial cells maintain a quiescent, anti-inflammatory phenotype. *Klf-2* is also needed to regulate the deposition of matrix proteins in the cardiac cushions and has important downstream effects on *Wnt* and *BMP4*, both of which are needed for correct OFT development (Combs and Yutzey, 2009; Garside et al., 2013; Luxán et al., 2016; Potts and Runyan, 1989). As the cardiac cushions expand during EndoMT, shear forces continually increase in the cushion area, eventually causing the cilia to break away from endothelial cells exposed to higher levels of shear stress. De-ciliated endothelial cells are no longer able to express *Klf-2*, which triggers *TGF- β* signaling, leading to a proinflammatory phenotype (Boon et al., 2007; Egorova et al., 2012, 2011a,b). *Klf-2* expression

in still-ciliated cells is needed to control essential downstream targets of *TGF- β* while simultaneously upregulating *Notch* signaling. Downregulation of *Notch* has been shown to downregulate *Fgf8*; thus, shear stress is predicted to modulate cNCC behavior through *Klf-2* and *TGF- β* . Additionally, *TGF- β* controls the expression of *Foxc1* and 2, which have been shown to positively regulate *Sema3c* expression (Duchemin et al., 2019; Goddard et al., 2017; Inman et al., 2018; Seo and Kume, 2006). Indeed, the expression of *TGF- β* and that of its downstream targets *Foxc1* and 2 spatiotemporally coincides with *Sema3c* expression and cNCC migration in amniotes. Thus, shear stress is another potential trigger for ambiogenic induction (Fig. 3; High and Epstein, 2008; Keyte et al., 2014; Neeb et al., 2013).

A note on squamates and aortic arches

The predictions outlined above suggest that, in squamates, developmental oxygenation is sufficient to downregulate global HIF expression in the OFT, leading to apoptosis-driven shortening of the truncus arteriosus. Uniquely, squamates are the only order in which this stage of OFT remodeling occurs without subsequent migration of cNCCs into the distal OFT to complete the aorticopulmonary septum.

In birds, the left visceral aorta, derived from pharyngeal aortic arch 4 (PAA4), regresses during development, whereas the right visceral aorta persists and merges with cNCC-derived elements to form the aorticopulmonary septum. In mammals, by contrast, both PAA4-derived structures persist, but the right PAA6 regresses, whereas the left PAA6 contributes to the septum. Thus, the aorticopulmonary septum in birds incorporates cNCC- and SHF-derived cells associated with PAA4, whereas in mammals it incorporates cNCC- and SHF-derived cells from PAA6 (see Poelmann et al., 2017, for a detailed discussion).

Squamates differ in that the left visceral aorta is positioned slightly differently than in birds and crocodilians. Rather than regressing during development, it persists and appears to act as a barrier that separates cNCC and SHF populations (Poelmann et al., 2017). This arrangement is likely to produce shear stress patterns that are distinct from those of birds and crocodilians. As discussed above, such differences in hemodynamics can influence EndoMT directly through the *Klf-2/TGF- β* pathway, and indirectly by modifying the extracellular matrix composition of the cardiac cushions. Consistent with this, changes in the expression of *Fibulin-1* (a matrix protein) have been shown to alter cNCC migration and apoptosis (Cooley et al., 2008), while altered *TGF- β* expression is associated with changes in apoptosis and with arch artery abnormalities in mammals (Molin et al., 2002). Although definitive data are currently lacking, this again emphasizes how different environmental cues might influence and coordinate development.

Mechanisms and evolutionary consequences of EDDI in heart development

According to the EDDI model proposed here, developmental changes might be regulated by environmental conditions such as hypoxia, which modulate gene expression to guide morphogenesis. This would allow for species-specific variation in cardiogenesis, influenced by local factors such as oxygen availability. Under this model, in placental mammals, the onset of placental oxygenation triggers the degradation of HIF, altering the interaction between interdependent parts of the cardiac developmental program, including cushion formation, EndoMT and shear response. These changes affect localized conditions, such as hypoxia in the cushion myocardium, which attracts cNCCs and incorporates additional developmental networks into cardiogenesis. Ultimately, these factors shape cell-fate decisions, altering how cardiac progenitors differentiate.

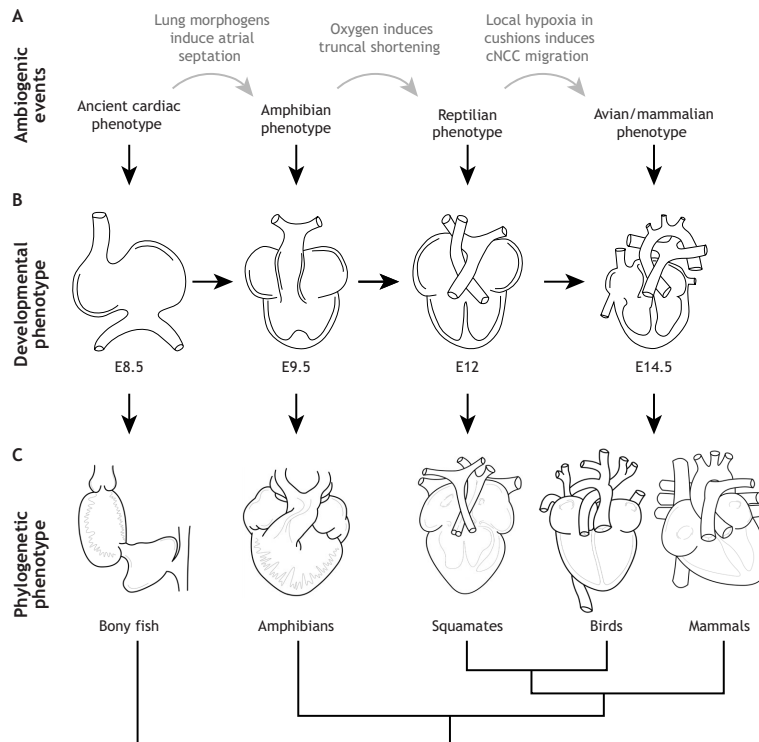


Fig. 4. Normal mammalian cardiogenesis and ambiogenic events hypothesized to be required for proper outflow tract development during placentalization, along with associated developmental and phylogenetic phenotypes. (A) Mammalian heart morphology at different times in development, along with the ambiogenic event potentially required to bring about the next morphological change. (B) Associated mouse cardiac phenotypes at different developmental stages. (C) According to the EDDI model, the genetically fixed developmental program underlying cardiogenesis produces the coiled heart tube as seen in bony fish. If lungs are present, atrial septation is mediated by molecular signals secreted from the developing lungs, which leads to the development of an 'amphibian' phenotype. Oxygenation of cardiac tissue via the onset of placental respiration induces shortening of the TA so that the aortic arches directly contact the ventricle, as is seen in the 'reptilian' phenotype. Intricate interactions between mechanical stressors and local oxygen environments then coordinate the migration of cNCCs into the cardiac cushions, where they complete the aorticopulmonary septum. This leads to the formation of a functional double circulation. Abbreviations as in Figs 1 and 3.

EDDI provides a potential explanation for why certain congenital heart defects in humans resemble normal phenotypes in other species. As the embryo develops and encounters environmental cues, ambiogenic induction expands the genetic toolkit available for the cardiac developmental program, allowing new regulatory patterns to emerge and new cardiac structures to form. For example, persistent truncus arteriosus in humans, which presents as a single arterial trunk (unshortened truncus arteriosus) with malalignment-type ventricular septal defects, resembles the normal OFT development in amphibians and lungfish, where low oxygen and the absence of apoptosis are part of standard development. The EDDI model suggests that one potential reason for this condition in humans is insufficient oxygenation at the onset of placental respiration, which limits apoptosis and restricts further developmental expansion in the heart (Figs 3, 4 and 5).

Oxygen deprivation at other points in development might not block apoptosis but could still bias development, affecting processes such as cushion formation and cNCC migration. In these cases, the EDDI model predicts milder cardiac malformations resembling a 'reptilian' phenotype, where the OFT partially divides but lacks a complete aorticopulmonary septum. Therefore, EDDI offers an alternative to Haeckelian explanations (see Glossary) for why some cardiac defects mimic ancestral vertebrate phenotypes. When key environmental triggers – such as sufficient oxygen during OFT apoptosis – are absent, cardiogenesis might revert to its core developmental processes, a process known as atavism (see Glossary;

Figs 3 and 4). This view aligns with the observation that tissues evolve similar morphologies under comparable environmental pressures (Loffet et al., 2023), supporting Garstang's (1922) famous conclusion: 'Ontogeny does not recapitulate phylogeny, it creates it'.

Conclusions and future directions

The concept of using ontogenetic rules to understand trait evolution is not novel, but applying this framework to cardiogenesis and congenital heart disease can offer new insights. My exploration of this idea led to the hypothesis that the cardiac developmental program has not fully genetically assimilated the co-opted sub-circuits needed to modify cell-fate decisions and create accessory structures in response to increased metabolic demands. Instead, I propose that the expansion of the core developmental program is driven by ambiogenic induction, where environmental triggers initiate physiological responses that expand the genetic toolkit available to cardiac progenitor cells. These newly activated elements interact with the core developmental program, leading to the formation of new cardiac structures through coordinated environmental and genetic interactions (Figs 3, 4 and 5).

This model builds on prior frameworks that emphasize phenotypic plasticity, but it diverges in a key way: although earlier models require genetic assimilation to stabilize new phenotypes across generations (Baldwin, 1896; Levis and Pfennig, 2016; Wagner et al., 2019; West-Eberhard, 2005), EDDI does not depend on such assimilation. By offering a mechanism in which environmental cues play a

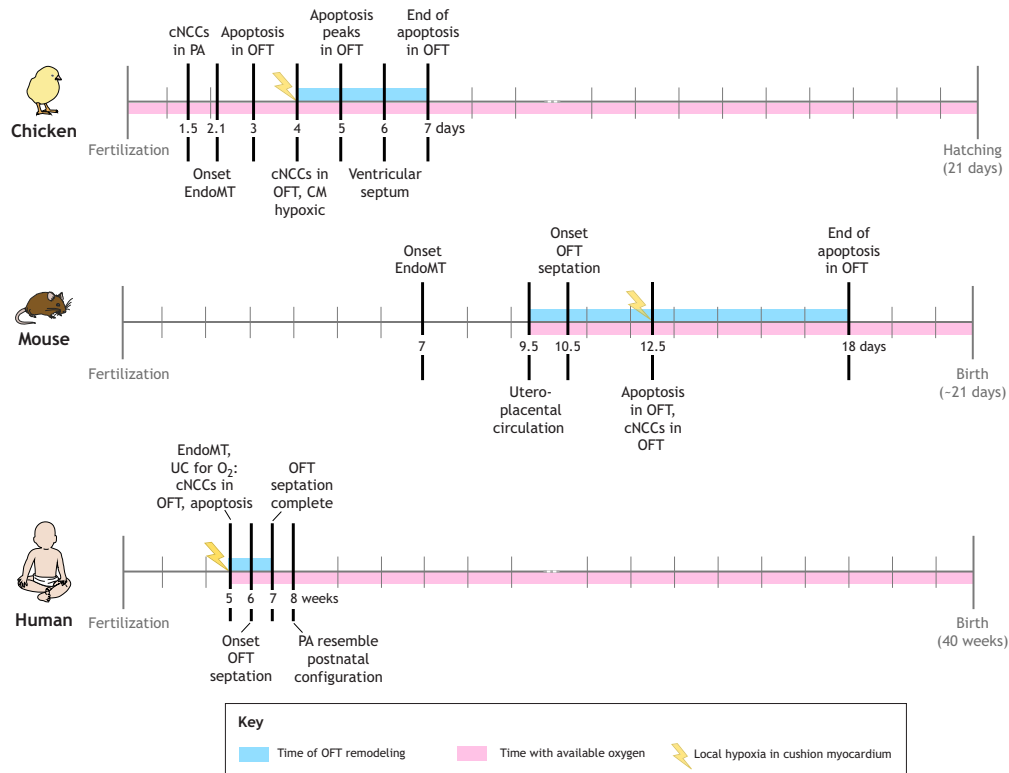


Fig. 5. Developmental time line of outflow tract remodeling during ontogeny of chick, mouse and human, with corresponding environmental conditions. Independent of the order of individual events and external triggers, the EDDI model predicts that all precursors needed for the transition to a double circulation need to be present to initiate OFT remodeling. Once the heart receives oxygenated blood, apoptosis sets in. However, it is only if the EndoMT has proceeded far enough to produce hypoxia in the cushion myocardium that cNCCs start migrating into the OFT, culminating in drastic OFT remodeling. Note the differences in time scale between different species. (Information taken from Al Naieb et al., 2013; Buijtenijk et al., 2020; Graham et al., 2015; Hamburger and Hamilton, 1951; Hikspsors et al., 2022; O’Rahilly and Müller, 2010.) CM, cushion myocardium; PA, pharyngeal arches; UC, umbilical cord; other abbreviations as in Figs 1 and 3.

primary role, EDDI provides a fresh perspective on why the heart remains vulnerable to environmental perturbations throughout its evolutionary history. Moreover, EDDI might broaden our understanding of congenital heart disease in cases without clear genetic defects, while also helping to clarify how development might be disrupted in genetically based heart defects, such as those associated with DiGeorge syndrome.

As the heart is the first organ to form and function, its development is not dependent on that of other organ systems, making it comparatively easy to tease apart different (micro-)environmental factors. Thus, the heart serves as an ideal model to unravel some of the intricate interactions governing development. As cardiogenesis is vulnerable to environmental perturbation, it exemplifies how developmental processes are sensitive to non-genetic signals, such as oxygen levels and shear stress.

Although this work focuses on how specifically oxygen and shear stress affect vertebrate heart development, the principles of EDDI

could be extended to other organ systems and environmental factors (for examples, see Gilbert, 2012; Miyakawa et al., 2010). Ambigenic induction might also be crucial in guiding species-specific limb development in vertebrates (Newman and Müller, 2005).

The EDDI model proposed here potentially offers a new framework for generating testable hypotheses for various fields of study, particularly in the light of new methods such as single-cell -omics and metabolomics, and gene–environment interaction studies. Considering development through the lens of the EDDI model might thus reveal broader patterns of environmentally driven development, providing a deeper understanding of evolution across biological systems.

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Competing interests

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Summary: This Commentary introduces the 'environmentally dependent developmental induction' model, a potential means through which phenotypic evolution is influenced by environmental effects on development.

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Paper III: A Developmental Mechanism Linking Heart Evolution and Congenital Defects

Paper III tests a concrete prediction of that framework by experimentally asking whether oxygen functions as a causal variable whose manipulation systematically alters a key morphogenetic event – in other words, whether oxygen fulfills the criteria of a developmental difference-maker. Using chick embryos, I manipulated oxygen levels during the critical period of outflow tract (OFT) septation, a developmental process that requires spatially controlled apoptosis. As predicted, under hypoxic conditions, apoptosis was inhibited, producing an atavistic phenotype resembling a more ancestral vertebrate heart phenotype.

These results establish oxygen as a true causal regulator: if oxygen levels are altered, developmental outcomes change in a consistent and mechanistically interpretable way. Oxygen therefore satisfies the condition of causation outlined by Woodward (2003), acting as an active organizing parameter that determines whether and how major structural transformations occur. Viewed comparatively, they also suggest that oxygen's role reflects an evolutionary process of constitutive embedding: developmental modes such as placentation or chorioallantoic respiration have integrated oxygen supply into the organization of the mechanism itself. Oxygen thus operates both as a manipulatable cause and as an evolutionarily stabilized component of the system's causal architecture. Consequently, its phylogenetic distribution can mechanistically explain normal vertebrate heart phenotypes, while developmental perturbations provide an ontogenetic explanation for the morphological patterns observed in cardiac defects. This article has been submitted to Journal of Experimental Biology (13.01.2026)

Author Contributions

I conceptualized the study and developed the hypotheses tested in the experiments. Fabian Placzek, Rostyslav Samonov, and Qhuang Nguyen designed and built the custom-made incubator, wrote the associated control software, performed the oxygen analyses, and authored all methodological sections related to the incubator. I conducted all experiments, including fluorescence imaging, micro-CT imaging, and all data analyses not involving the incubator system, and I wrote all parts of the manuscript not pertaining to the incubator and its set-up. Brian Metscher assisted in establishing the experimental workflow and contributed to refining the manuscript. All authors contributed through discussions related to imaging and experimental considerations, provided constructive comments and approved the final version of the manuscript.

1 **Oxygen as both cause and constituent of vertebrate heart**
2 **development**

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25 **1. Abstract**

26 Mechanistic studies of heart development have primarily emphasized causal explanations by
27 identifying factors whose manipulation alters morphogenesis. Here, we extend this perspective
28 by asking how environmental conditions that once acted as external triggers have become
29 embedded in the organization of vertebrate cardiogenesis. Given that changes in
30 developmental mode from aquatic to placental forms or amniote eggs alter oxygen availability,
31 we examined how such evolutionary shifts might have become incorporated in the mechanism
32 of heart development. By reducing oxygen during outflow tract remodeling in chick embryos,
33 we suppressed apoptosis in the truncal myocardium, producing single-outlet hearts
34 reminiscent of ancestral vertebrate morphologies. These findings show that oxygen availability
35 acts as a developmental difference-maker in cardiogenesis. From a comparative perspective,
36 they suggest that evolutionary shifts in developmental mode, such as placentation and
37 chorioallantoic respiration, embedded oxygen as a constitutive condition of heart development,
38 making it an integral part of the developmental mechanism. This continuity between cause and

1 constitution provides a framework for understanding how environmental factors shape both the
2 emergence of evolutionary novelties and the persistence of developmental vulnerabilities.

3 **2. Introduction**

4 One of the central questions in evolutionary developmental biology is how developmental
5 systems generate new phenotypes while maintaining functional coherence (Gregory 2008;
6 Rorick and Wagner 2011; Mayer and Hansen 2017). Explaining this process requires more
7 than identifying correlations between genes and traits: it necessitates uncovering the causal
8 structure of development itself (Wagner et al. 2000; Hall 2003; Müller 2007; Brun-Usan et al.
9 2022).

10 Woodward's (2004) interventionist account of causation offers a powerful framework for such
11 inquiry, proposing that explanations are causal when they answer *what-if-things-had-been-*
12 *different* questions: if an intervention on a variable systematically changes an outcome, that
13 variable counts as a cause of the outcome. Waters (2007) extends this logic to biology, arguing
14 that robust explanations must identify actual *difference-makers*: factors that, when varied,
15 produce reproducible and explanatorily relevant changes within biological systems.

16 Heart development (cardiogenesis) in vertebrates has been studied extensively, largely
17 because congenital heart defects (CHDs) represent the most common category of birth defects
18 (Gruber and Epstein 2004; Kelly et al. 2014; Zaidi and Brueckner 2017; Pierpont et al. 2018).
19 As a result, a significant body of causal explanations has accumulated across a range of model
20 organisms, mapping how genetic pathways, cellular behaviors, and biomechanical forces
21 interact to produce cardiac form. Yet, as Love (2015) and Fagan (2012) note, causal
22 mechanistic explanations alone do not exhaust the explanatory space of developmental
23 biology: such results show which manipulations change outcomes, but only constitutive
24 mechanistic explanations help us understand how those variables become part of the
25 organized mechanism itself. To explain not only how the heart develops, but how its
26 development has evolved, we need to connect causal difference-making to the evolving
27 architecture that sustains it (Craver 2007; Illari and Williamson 2012; Craver and Darden 2013;
28 Glennan 2017).

29 In developmental biology, this task is particularly complex because causal interactions unfold
30 across multiple levels – molecular, cellular, tissue, and environmental – each contributing to
31 the organization of the developmental system (Newman and Müller 2000; Brigandt 2015;
32 Moczek et al. 2015). Evo-devo adds a further, historical dimension. As Calcott (2009)
33 observes, “genuine explanations of evolutionary change” must describe not only how
34 mechanisms produce traits, but also how those mechanisms themselves transform over

1 evolutionary time. Developmental mechanisms are thus *dynamic causal architectures* that
2 evolve by incorporating new components or reorganizing existing ones (Calcott 2009; Nuño
3 de la Rosa and Villegas 2022).

4 Comparative embryology and theoretical reasoning indicate that early vertebrate
5 cardiogenesis is characterized by deeply conserved morphogenetic processes, followed by
6 lineage-specific divergence in the final organization of the heart (Simões-Costa et al. 2005;
7 Fisher and Burggren 2007). Vertebrate cardiogenesis therefore provides an ideal case for
8 exploring how systematic changes to a developmental system alter the resulting phenotype.
9 Oxygen availability is known to influence gene regulation, tissue differentiation, and
10 programmed cell death (Cordeiro und Tanaka, 2020; Zhu *u. a.*, *preprint*) and varies predictably
11 across vertebrate classes and developmental modes (Kardong 2019). It may therefore serve
12 as a repeatable, evolutionarily significant difference-maker in cardiogenesis, one that helps
13 explain both developmental divergence and evolutionary transitions in cardiac form.

14 In this paper, we use oxygen availability as a case study to link mechanistic-extended causal
15 and constitutive explanations. This means we aim to understand a particular phenomenon
16 during cardiogenesis not only by describing the internal components and causal interactions
17 that produce it, but also by considering the historically and environmentally structured
18 conditions that have become constitutive of the mechanism itself.

19

20 Experimentally, we test whether manipulating oxygen during outflow tract remodeling
21 produces predictable developmental changes; a causal analysis. Conceptually, we interpret
22 these results within an evolving mechanistic framework, asking how oxygen's developmental
23 role might have become constitutively embedded through evolutionary changes in
24 developmental mode, such as placentation or chorioallantoic respiration. In doing so, we aim
25 to connect the extensive causal knowledge of heart development to a broader account of how
26 environmental conditions become integrated into the mechanisms that generate and evolve
27 form, thus providing a constitutive mechanistic explanation of heart development.

28

29 **2.1. Background: Vertebrate Heart Evolution and Outflow Tract Remodeling**

30 Across vertebrates, the heart develops from a simple linear tube comprising an inflow tract that
31 receives venous blood and an outflow tract (OFT) that ejects it into the arterial network. This
32 basic organization is conserved across all major vertebrate clades. From this shared starting
33 point, the heart differentiates into discrete chambers through looping, regionalization, and

1 septation, following developmental trajectories that diverge in lineage-specific ways (Simões-
2 Costa et al. 2005; Fisher and Burggren 2007; Stephenson et al. 2017).

3 In fishes, the heart typically consists of two chambers, a single atrium and ventricle, with an
4 undivided OFT that pumps oxygen-poor blood toward the gills, maintaining a single circulation.
5 Amphibian larvae begin with a similar configuration but later develop a second atrium and a
6 partial internal septum in the OFT, which enables partial separation of oxygen-depleted and
7 oxygen-enriched blood as they transition from gill to lung respiration toward the end of
8 metamorphosis. During gill respiration however, the developing heart continues to transport
9 only deoxygenated blood (Kraus and Metscher 2022). Squamate hearts consist of two atria
10 and two ventricles, but the degree of septation in both the ventricle and OFT varies among
11 species. In crocodilians, birds, and mammals, the OFT undergoes complete remodeling such
12 that the pulmonary artery and aorta are each positioned over one of two fully separated
13 ventricles. This transformation establishes a double circulation system that directs oxygen-
14 poor blood to the lungs and oxygen-rich blood to the body.

15 Developmentally, this major morphological transition depends on precise remodeling of the
16 OFT (Kelly and Buckingham 2002; Gittenberger-de Groot et al. 2005; Barbosky et al. 2006;
17 Kelly et al. 2014). The OFT initially comprises two segments: the conus arteriosus, which
18 contacts the ventricles, and the truncus arteriosus, which connects the conus to the aortic
19 arches (Gittenberger-de Groot *et al.*, 2005; Barbosky *et al.*, 2006). During early development
20 in mammals, birds, and crocodilians, the conal portion becomes incorporated into the
21 ventricles, while the truncal portion shortens through spatially restricted apoptosis (Anderson
22 et al. 2003; Barbosky et al. 2006; Rothenberg et al. 2003; Sugishita, Leifer, et al. 2004). This
23 process reduces the common truncus so that the pulmonary and systemic outflows become
24 separated and aligned over their respective ventricles, allowing full division of pulmonary and
25 systemic circulation (Gittenberger-de Groot et al. 2005).

26 Previous studies have shown that oxygen availability changes markedly during this stage of
27 OFT remodeling in amniotes. The transition from a hypoxic to a more oxygen-rich environment
28 happens through the onset of placentation in mammals (Pijnenborg, Vercruysse and
29 Hanssens, 2006; Roberts *et al.*, 2017; Sato, 2020; Faber *et al.*, 2021) or the formation of the
30 chorioallantoic membrane in birds and crocodilians (Schlatter et al. 1997) and coincides with
31 a wave of apoptosis in the OFT that shortens the truncus and ensures separation of
32 oxygenated and deoxygenated blood (Pijnenborg, Vercruysse and Hanssens, 2006; Roberts
33 *et al.*, 2017; Sato, 2020; Faber *et al.*, 2021).

34 Experimental results from literature suggest that the causal mechanisms at the molecular level
35 connecting hypoxia and OFT transformation involves the degradation of hypoxia-inducible

1 factor HIF-1 α (Anderson et al. 2003; Barbosky et al. 2006; Rothenberg et al. 2003; Sugishita,
2 Leifer, et al. 2004). Under hypoxic conditions, HIF-1 α is stabilized and prevents cell death by
3 maintaining the expression of cell-protective genes such as VEGF. As oxygen levels rise, HIF-
4 1 α degradation potentially removes this protective effect, likely permitting apoptosis to proceed
5 and thus driving the structural remodeling of the OFT (Sugishita, Watanabe, et al. 2004;
6 Sugishita, Leifer, et al. 2004).

7 This relationship suggests a developmental and evolutionary logic: environmental oxygen acts
8 as a trigger that reorganizes the causal architecture of cardiogenesis. In lineages where the
9 developing heart carries only oxygen-depleted blood, such as fishes, and amphibians before
10 pulmonary respiration begins, stable HIF expression prevents apoptotic remodeling,
11 maintaining a single circulation with a pronounced truncus arteriosus. In birds, crocodilians,
12 and mammals, increased developmental oxygen levels potentially destabilize HIF, enabling
13 apoptosis and facilitating the evolution of double circulation. If this assumption is correct,
14 reducing available oxygen should thus hinder shortening of the truncus arteriosus via
15 apoptosis, resulting in an atavistic phenotype reminiscent of the adult amphibian heart. Then,
16 this variation in environmental oxygen would provide a mechanistic bridge between
17 evolutionary transitions in vertebrate cardiogenesis and the developmental processes that
18 continue to shape heart formation today.

19

20 **3. Methods and Predictions**

21 Building on this comparative framework, we sought to test whether oxygen availability
22 functions as a causal difference-maker in vertebrate cardiogenesis; specifically, whether
23 reducing oxygen during development predictably alters the morphology of the outflow tract
24 (OFT) and its associated pattern of apoptosis.

25 Our experiments focused on the early stage of OFT remodeling (Hamburger–Hamilton stages
26 19–27; Hamburger and Hamilton 1951; Martinsen 2005), when the truncus arteriosus
27 shortens to fully separate aorta and pulmonary artery. Based on comparative and
28 developmental evidence, we hypothesized that oxygen availability acts as a key determinant
29 of this process by regulating the onset of apoptosis required for OFT remodeling. Specifically,
30 we predicted that reducing oxygen levels during this critical window would suppress apoptosis
31 in the truncal myocardium, leading to incomplete OFT separation and resulting in single-
32 outflow morphologies reminiscent of ancestral vertebrate hearts.

33 **3.1. Animals and Incubation**

1 Fertilized chicken eggs were obtained from a commercial breeder (Schropper GmbH). To
2 control for batch effects, treatment groups were repeated with eggs from a private breeder.
3 Eggs were incubated at 39.5°C and 80% humidity under three conditions: (1) normal
4 atmospheric oxygen levels (~20.9%), (2) partial reduction of gas exchange by covering 50%
5 of the eggshell surface with modeling clay and aluminum foil, and (3) precise hypoxia using a
6 custom-designed incubator set to controlled oxygen concentrations (including 10.5% oxygen).

7 After 48 hours (2 days) of incubation, the apoptotic window in chick heart development begins.
8 This time point was used as the starting point for all subsequent treatments. At this stage, the
9 oxygen concentration in our custom-made incubator (described in Supplementary Material)
10 was adjusted to the desired level.

11 Clay and aluminum foil were also added at the 2-day mark. For instructions on applying clay
12 and aluminum foil to eggs, see <https://youtube.com/shorts/gu7s5xdexVI> and
13 <https://youtube.com/shorts/U8zjm3INhB0>.

14 For each treatment category, 30 eggs were initially used. At harvesting, embryos showing
15 developmental abnormalities unrelated to the heart were discarded. Unfertilized eggs were
16 also excluded from the sample size. However, dead embryos and embryos with severe
17 hematoma (Supplementary Fig. 1) were included in the sample size.

18 **3.2. Fluorescent Staining and Imaging**

19 Embryos were harvested at HH27 and rinsed in phosphate-buffered saline (PBS). They were
20 then stained with 2 µM LysoTracker Red DND-99 (ThermoFisher Scientific) in PBS (50 µL in
21 25 mL for approximately 25 embryos) for 15 minutes in the dark.

22 This protocol was adapted from Schaefer et al. (2004), who compared LysoTracker to TUNEL
23 staining and found that LysoTracker provided superior detection of early apoptotic cells while
24 distinguishing apoptosis more clearly from necrosis. This distinction was crucial for our
25 experiments, as low oxygen concentrations resulted in poor embryonic conditions, likely
26 increasing necrotic cell death.

27 After staining, embryos were:

- 28 1. Rinsed briefly in PBS.
- 29 2. Washed three times for 10 minutes each in PBS.
- 30 3. Fixed in 4% paraformaldehyde (PFA) in PBS for 1–2 hours.
- 31 4. Rinsed again in PBS.
- 32 5. Washed three additional times for 10 minutes each in PBS.

1 Next, hearts were dissected and gradually transitioned to 80% glycerol for imaging:

- 2 • 25% glycerol → 50% glycerol → 80% glycerol, incubating for at least 30 minutes at
3 each step.

4 Specimens were stored in the dark at 4°C for up to 36 hours before fluorescence microscopy
5 imaging using an EVOS microscope.

6

7 **3.3. MicroCT Imaging**

8 Following fluorescence microscopy, specimens were re-fixed in 4% PFA/PBS until further
9 processing.

10 For micro-CT imaging, specimens were contrast stained (Metscher 2021):

- 11 1. Rinsed briefly in deionized distilled water (ddH₂O).
12 2. Washed three times for 10 minutes each in ddH₂O.
13 3. Stained in 2% lead acetate (PbOAc) in ddH₂O overnight (Metscher 2021)
14 4. Rinsed briefly in ddH₂O, followed by three additional 10-minute washes in ddH₂O.
15 5. Mounted for scanning in 200 µL pipette tubes using 1% low-melting agarose (Metscher
16 2009, 20)

17 Micro-CT images in Fig. 1 were acquired from a Bruker Skyscan 1272 with a tungsten X-ray
18 source, set at 50-70kVp and 3-5µm reconstructed voxel size. The micro-CT images in Fig. 3
19 were obtained from a Xradia (Zeiss) MicroXCT using a tungsten source at 40kVp and 5W, with
20 2µm or 4µm voxel size. These scans were used for gross assessment of morphology and so
21 the results were not sensitive to differences in scanning parameters.

22 **3.4. Data Analysis**

23 Both apoptosis and morphology exhibited distinct differences between affected and non-
24 affected embryos; therefore, data analysis was conducted qualitatively (Supplementary Table
25 1).

- 26 • Unedited fluorescent and micro-CT data were analyzed
27 • Apoptosis patterns were compared to those described by Schaefer et al. (2004) and to
28 untreated embryos in our own experiment.
29 • 3D morphology was compared to normal chick heart development at equivalent stage

1 **3.5. Data Visualization**

3 **3.5.1. Fluorescence Data Processing**

4 For optimal visualization, 4x fluorescent images were processed differently based on the
5 presence or absence of apoptosis:

- 6 • **Apoptosis present:**

- 7 ○ Fiji (ImageJ) (Schindelin et al. 2012) was used for background subtraction:
 - 8 ▪ *Process > Subtract Background > Rolling ball radius: 50 pixels*
- 9 ○ Manual focus stacking was applied:
 - 10 ▪ *Stacks > Z Project > Max Intensity*
- 11 ○ Gamma and window level adjustments were applied for optimal visualization.

- 12 • **Apoptosis absent:**

- 13 ○ Background subtraction was not performed, as this would eliminate weak
14 signals.
- 15 ○ Images were stacked directly as described above.
- 16 ○ Gamma and window level adjustments were applied to enhance contrast.

17 To ensure comparability, Supplementary Figure 2 presents apoptotic and non-apoptotic hearts
18 adjusted in an identical manner.

19 **3.5.2. Micro-CT Data Processing**

21 Micro-CT data were processed using Dragonfly ORS.

- 22 • **Heart segmentation/Surface renderings:**

- 23 ○ Cardiac tissue was segmented from virtual stacks.
- 24 ○ The segmented area was displayed using its original lookup table (LUT).
- 25 ○ The LUT was adjusted for optimal contrast.
- 26 ○ Brightness and contrast of exported screenshots were adjusted using Fiji
27 (ImageJ).

- 28 • **Internal morphology visualization:**

- 29 ○ The Dragonfly ORS box tool was used to create cut-out views.
- 30 ○ Window functions of the screenshots were adjusted as described above.

31 **4. Results**

32 **4.1. Defining the Apoptotic Window for OFT Remodeling**

1 Apoptosis and remodeling of the outflow tract (OFT) in chick embryos initiate around HH19
2 (3 days incubation) with peak apoptotic activity by HH27 (5 days). This period was designated
3 as the "apoptotic window" and targeted for oxygen manipulation experiments.

4 **4.2. Initial Evidence: Gas Exchange Reduction and Atavistic Heart Phenotypes**

5 Reducing available gas exchange by covering half the eggshell with modeling clay and foil
6 generated embryos exhibiting predicted atavistic cardiac phenotypes: a persistent, undivided
7 truncus arteriosus, and single-outflow morphology. These embryos also frequently showed
8 severe hematomas near the developing brain and eyes (see Supplementary Material).
9 However, not all embryos showed an altered phenotype. This method was thus not sufficient
10 to demonstrate consistent phenotypic changes, as we could not judge whether the observed
11 variation was due to inconsistent treatment or down to other factors. Additionally, we could
12 not fully exclude confounding factors from the clay or foil.

13 **4.3. Enhanced Precision: Controlled Hypoxia Confirms Phenotypes**

14 Using a custom incubator to maintain oxygen at 10.5%, approximately halving atmospheric
15 levels, produced consistent cardiac phenotypes. This confirmed that reduced oxygen, rather
16 than physical shell covering or materials, caused OFT remodeling defects characterized by a
17 persistent truncus arteriosus over a single ventricle.

18 **4.4. Oxygen Sensitivity and Phenotypic Outcomes**

19 Testing multiple oxygen concentrations suggests different, embryo-specific sensitivity to
20 oxygen depletion. At 10% oxygen, all but one surviving embryos failed to properly remodel
21 the OFT, whereas at 15% oxygen, most developed normally (Fig. 2). Most notably, no
22 intermediate phenotypes were observed. Instead, embryos followed a binary developmental
23 trajectory: either normal OFT division or retention of an undivided truncus arteriosus (Fig. 3).
24 This suggests that OFT remodeling is controlled by a developmental switch depending on
25 oxygen availability with individual differences in oxygen sensitivity.

26 **4.5. Apoptosis Links Oxygen Availability and OFT Remodeling**

27 Apoptosis markers confirmed that embryos undergoing normal OFT remodeling exhibited
28 characteristic apoptotic activity within the truncal myocardium. In hypoxia-treated embryos,
29 apoptosis was either clearly present or completely absent, directly correlating with whether
30 the OFT remodeled correctly or remained undivided (Fig. 4). Embryos lacking apoptosis were
31 generally in poor condition, but their cardiomyocytes specifically failed to initiate programmed
32 cell death, strengthening the specificity of the apoptotic marker.

1 **4.6. Summary of Findings**

- 2 - Oxygen reduction by limiting gas exchange produced atavistic cardiac phenotypes,
3 with persistent truncus arteriosus and single-outflow configurations.
- 4 - Controlled hypoxia (10.5% O₂) generated consistent phenotypes confirming that
5 oxygen, not physical interference, drives OFT remodeling defects.
- 6 - Dose-dependent effects revealed a binary developmental switch: below a threshold
7 oxygen level, apoptosis failed and OFT separation did not occur.
- 8 - Apoptosis assays confirmed that normal OFT remodeling coincides with apoptotic
9 activity, while hypoxia-treated embryos lacked apoptosis in the truncal myocardium.

10 Together, these results demonstrate that oxygen availability during a critical developmental
11 window regulates programmed cell death in the OFT, determining whether the heart develops
12 a double or single outflow tract

13 **5. Discussion**

14 The results presented here identify oxygen availability as a genuine difference-maker in
15 vertebrate cardiogenesis: manipulating oxygen predictably alters developmental outcomes by
16 regulating apoptosis in the outflow tract (Waters 2007). This demonstrates that an
17 environmental factor can function as part of the causal architecture of development rather than
18 as a mere external constraint (Gilbert 2012; Pelster and Burggren 2018; Brun-Usan et al.
19 2022). The oxygen dependence of this process also highlights the heart's sensitivity to
20 environmental perturbation during embryogenesis.

21 Reducing oxygen in chick development suppressed apoptosis in the truncal myocardium,
22 leading to incomplete separation of the outflow tract and a single-outlet phenotype reminiscent
23 of amphibian hearts. Based on other published data, this process is most likely under the
24 control of hypoxia-inducible factor 1 α (HIF1 α), which prevents apoptosis by upregulating cell-
25 protective pathways under hypoxic conditions. When oxygen fails to reach the level required
26 for HIF1 α degradation, these protective pathways remain active, apoptosis does not occur,
27 and truncal remodeling fails, resulting in malformations similar to congenital defects such as
28 persistent truncus arteriosus (Watanabe *u. a.*, 1998; Watanabe, Jafri und Fisher, 2001;
29 Sugishita *u. a.*, 2004; Burggren und Reyna, 2011). This provides a mechanistic explanation for
30 the well-documented link between developmental hypoxia caused by placental abnormalities
31 (Burton and Jauniaux 2018; Kalisch-Smith et al. 2024; Nijman et al. 2024), high altitude (Hasan
32 2016; Miao et al. 1988; Song et al. 2021), smoking (Lee and Lupo 2013; Wang et al. 2022;
33 Zhang et al. 2017), or maternal diabetes (Basu and Garg 2018; Choudhury et al. 2021; He et

1 al. 2024; Ibrahim et al. 2023; Morishima et al. 1996; Zhao et al. 2020; Zhao 2010) and
2 increased risk of CHDs.

3 At a broader level, oxygen's role in heart development exemplifies how consistent
4 environmental conditions can become constitutive features of developmental systems. The
5 evolution of placentation in mammals, and of chorioallantoic respiration combined with
6 changes in the timing of egg deposition in birds and crocodilians, transformed oxygen into an
7 organizing condition, shifting it from being causally relevant to being constitutively necessary.
8 This historical embedding of environmental factors within development explains both the
9 stability and the vulnerability of the system: cardiogenesis proceeds reliably only within the
10 physico-chemical conditions that shaped its evolution (Calcott 2009; Gilbert 2012).

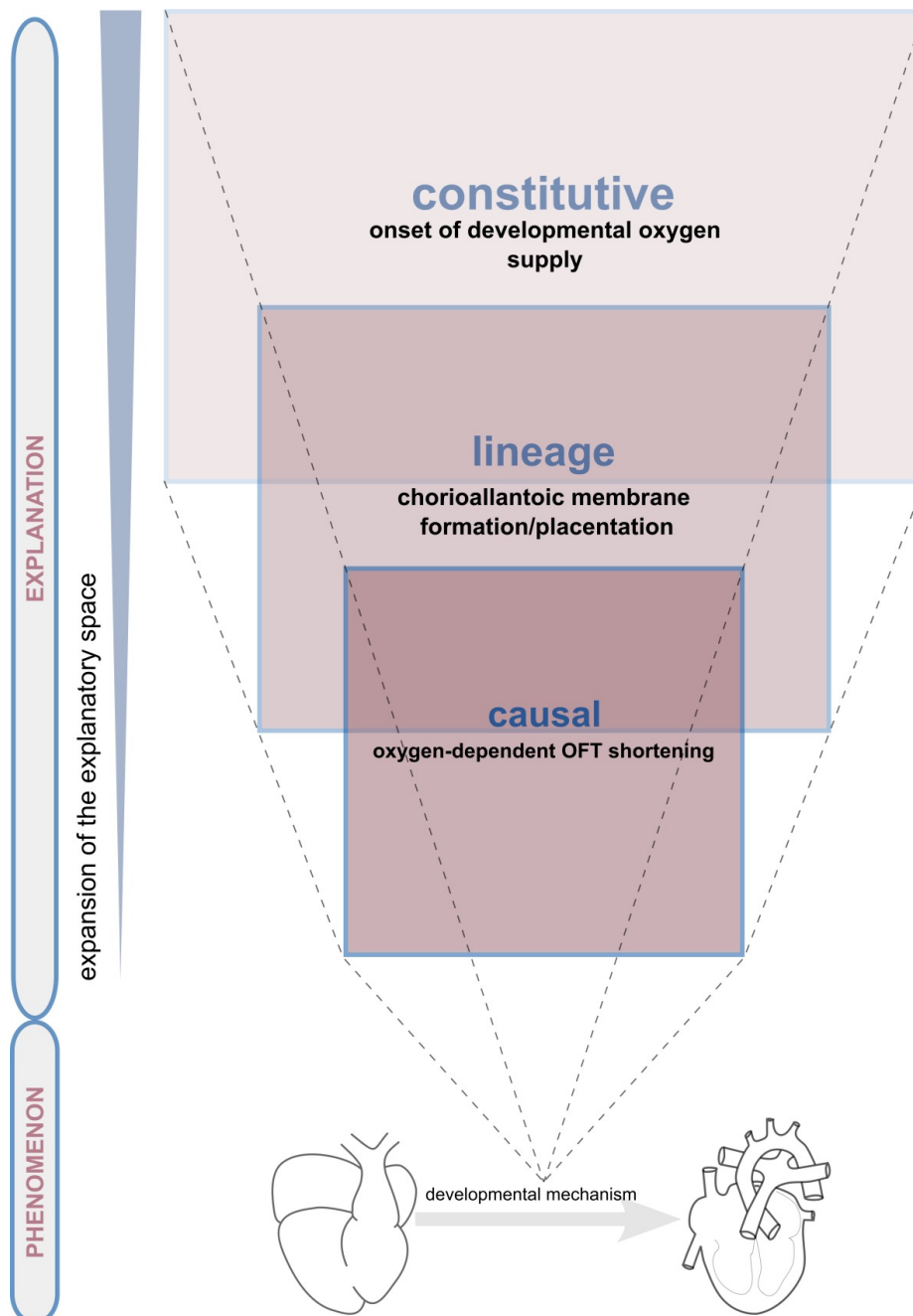
11 This transformation also illustrates the connection between mechanistic-extended causal and
12 mechanistic-extended constitutive explanations (Love 2015). Experimental manipulation
13 reveals oxygen's causal relevance, while a comparative, evolutionary framework shows how
14 this causal dependency became built into the mechanism's organization. Following Fagan's'
15 (2012) causal explanation thus provides the first step toward a deeper constitutive
16 understanding – one that explains how environmental factors are integrated into the structure
17 of developmental systems.

18 Within this perspective, developmental robustness and evolvability are two sides of the same
19 coin (Wagner 2007; Wagner and Altenberg 1996; Masel and Siegal 2009; Masel and Trotter
20 2010). The heart's morphogenetic program is robust under normal conditions, despite being
21 reliant on environmental inputs, because it evolved to accommodate those inputs. Yet
22 perturbations of these parameters reveal that this robustness includes evolved dependencies.
23 Notably, we observed individual differences in sensitivity to oxygen deprivation in our
24 experiments, raising the intriguing possibility that variation in HIF genes underlies these
25 differences. Interestingly, mutations in HIF-pathway genes that modulate oxygen sensitivity
26 are enriched in high-altitude populations and potentially mitigate this sensitivity (Bigham et al.
27 2009; González-Andrade 2020; Crawford et al. 2017). We posit that this could connect
28 mechanistic understandings of development with population genetics: while the oxygen-
29 dependent switch that controls apoptosis (and thus OFT remodeling) is an indispensable part
30 of the developmental system, populations can still accumulate mutations that fine-tune the
31 sensitivity or reaction norm of that switch (Lynch 2007; Pigliucci 2008; Love and Wagner 2022).

32 In sum, oxygen acts as both cause and constituent of vertebrate heart development. It is a
33 manipulable environmental variable that reveals how consistent environmental conditions
34 become integrated into developmental mechanisms over evolutionary time (Kaiser 2021;
35 Kaiser and Krickel 2017; Fagan 2012; Love 2015; 2008). Understanding this continuity

1 between cause and constitution clarifies how evolution reshapes not only phenotypes but the
2 mechanisms that generate them, and why the same dependencies that once enabled
3 innovation can persist as sites of developmental vulnerability (Laubichler 2009; Brun-Usan et
4 al. 2022; Gilbert 2012)..

5 **6. Figures**
6



1
2 **Figure 1: Integrating causal, lineage, and constitutive explanations of OFT shortening.**
3
4 The developmental step of interest (oxygen-dependent outflow-tract shortening) can be explained at different levels
5 of biological organization. A causal explanation identifies the immediate mechanism that changes the phenotype.
A lineage explanation accounts for how this mechanism became part of embryonic development through the

1 evolution of the chorioallantoic membrane and placentation. A constitutive explanation describes the present
2 configuration of the developmental system that now depends on embryonic oxygen supply and integrates these
3 historical components. Explanatory levels broaden from causal to constitutive, illustrating how development
4 incorporates both proximate processes and their evolutionary origins.

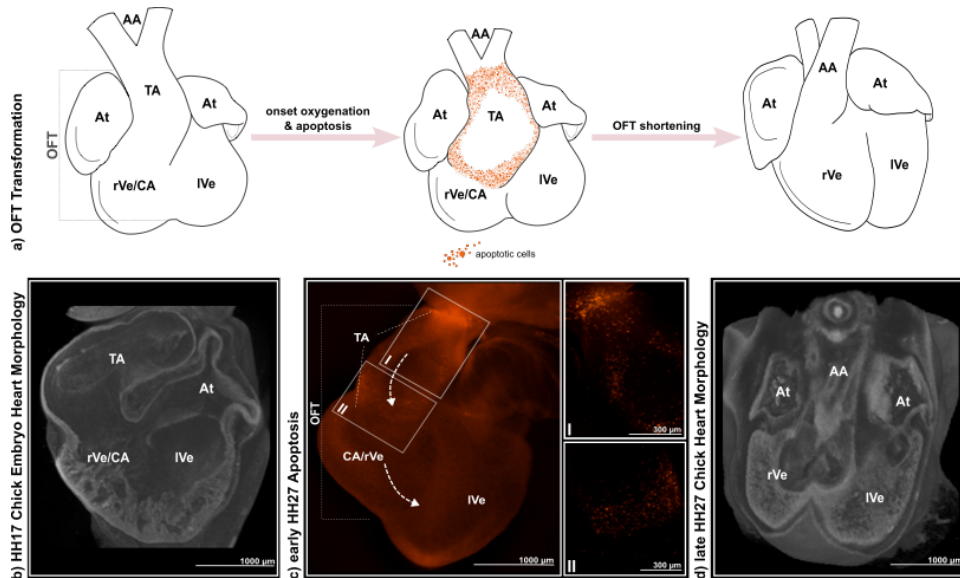


Figure 2: Hypothesized Role of Oxygen-Dependent Apoptosis in OFT Remodeling and Evolution of OFT Division.

9 Our hypothesis regarding the role of oxygen in OFT remodeling. (a) In normal amniote development, sufficient
10 oxygen availability triggers apoptosis in the OFT. This apoptosis facilitates OFT remodeling, ultimately reducing the
11 common trunk shared by the aorta and pulmonary artery. (b) Volume renderings of micro-CT scan of chick hearts
12 at HH17. (c) Apoptosis visualized using LysoTracker during normal OFT development of a HH27 chick embryo
13 (20.9% O₂). OFT remodeling is visualized using the fluorescent apoptosis marker LysoTracker, demonstrating how
14 the transition from a single-ventricle heart with a truncus arteriosus to a fully separated heart occurs. This
15 transformation involves both the incorporation of the conal portion of the OFT into the existing ventricle and
16 apoptosis-driven truncal shortening, eliminating the common trunk of the aorta and pulmonary artery. I and II show
17 4x magnifications of the main sites of apoptosis in the OFT, the junction between conal and truncal portion of the
18 OFT and at the side of aorta and pulmonary artery branching out of their shared truncus. (d) Volume renderings of
19 micro-CT scan of chick hearts at HH2. Together with (b), it confirms that heart morphology follows the transitions
20 predicted by our model. At Atrium, CA Conus arteriosus, OFT Outflow tract, TA Truncus arteriosus, (r/l)Ve (right/left)
21 Ventricle. Drawings made based on microCT scans, Kardong 2019 and Moharkar and Bharati 2023.

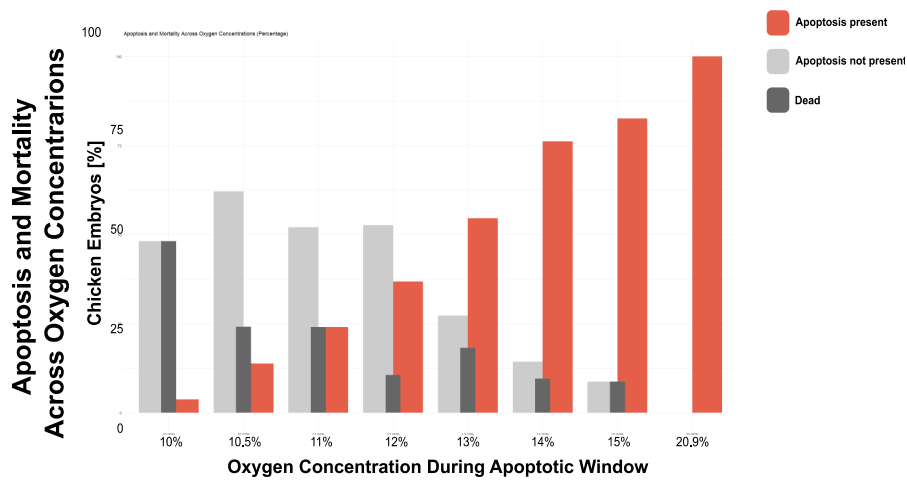


Figure 3: Oxygen Concentration During the Apoptotic Window Determines OFT Apoptosis in Chicken Embryos.

Bar plot of the percentage of chicken embryos exhibiting OFT apoptosis (red), lacking apoptosis (gray), or dying (black) is shown across different oxygen concentrations during the apoptotic window. Lower oxygen levels (10–12%) result in a high proportion of embryos lacking apoptosis or dying, whereas higher oxygen concentrations ($\geq 13\%$) progressively increase the proportion of embryos exhibiting apoptosis. At atmospheric oxygen levels (20.9%), all embryos show apoptosis, supporting the hypothesis that oxygen availability regulates OFT remodeling via apoptosis.

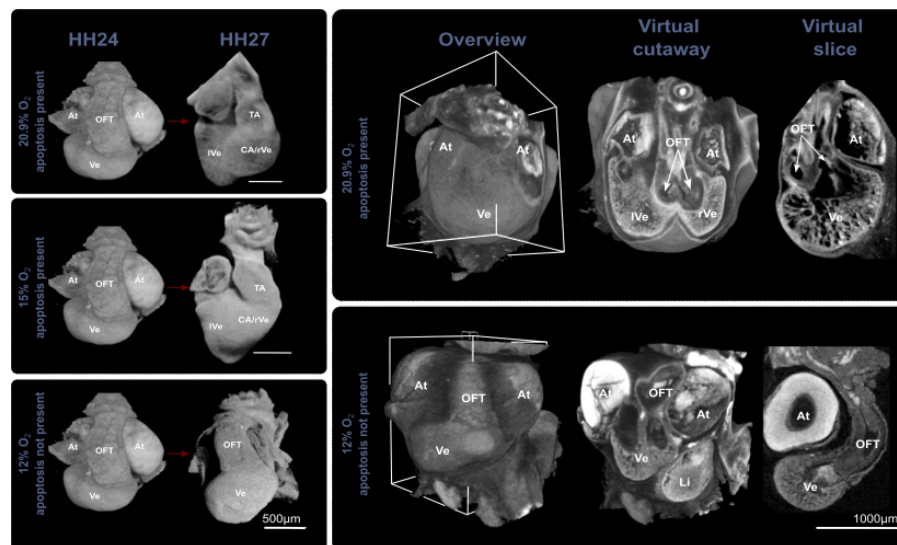
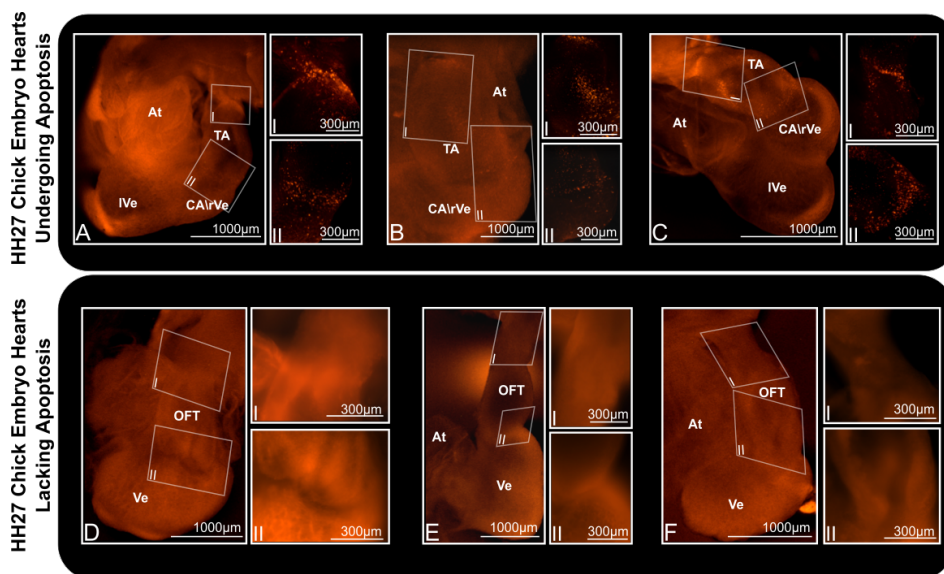


Figure 4: 3D Reconstructions of Chick Embryo Hearts Showing Structural Differences at Different Oxygen Conditions.

1 (Left panel) Representative HH24 and HH27 chick embryo hearts at different oxygen concentrations. Red arrows
2 indicate the temporal transition between the developmental stages. At atmospheric (21%) oxygen concentrations
3 and at 15%, apoptosis was induced, leading to a reduction of the truncal portion of the OFT. However, at 12%
4 oxygen, apoptosis is most often absent, and the truncal portion persists.

5 (Right panel) 3D reconstructions of HH27 chick embryo hearts without apoptosis, imaged using X-ray
6 microtomography. The overview shows external heart morphology, while the virtual cutaway and virtual slice reveal
7 internal structures, including the atria (At), ventricle (Ve), and OFT. Hearts developing in 21% and 12% oxygen
8 exhibit distinct structural features. This illustrates that when oxygen levels are high enough to support apoptosis,
9 this transition results in the aorta and pulmonary artery sitting directly over the now-separate ventricles (arrows). In
10 contrast, when oxygen levels are too low for apoptosis to occur, the aorta and pulmonary artery remain part of a
11 long common trunk. White boxes around the overview mark the cutting plane of the virtual cutaways. Note that
12 hearts in the right panel were not fully dissected from their embryonic membranes, as dissection often damaged
13 atria. All hearts were stained with 2% PbOAc.

14 At Atrium, CA Conus arteriosus, Li Liver, OFT Outflow tract, TA Truncus arteriosus, (r/l)Ve (right/left) Ventricle.



15 **Figure 5: Apoptosis in HH27 Chick Embryo Hearts Under Different Oxygen Conditions.**
16 (B–D) Representative HH27 chick embryo hearts undergoing apoptosis, visualized using LysoTracker staining.
17 Apoptotic regions appear as punctate fluorescence in the outflow tract and adjacent structures. Insets (I, II) show
18 higher-magnification views of the boxed regions.

19
20 (E–G) Representative HH27 chick embryo hearts lacking apoptosis, also stained with LysoTracker. These hearts
21 show no punctate fluorescence in corresponding regions. Insets (I, II) show higher-magnification views of the boxed
22 areas. At Atrium, CA Conus arteriosus, OFT Outflow tract, TA Truncus arteriosus, (r/l)Ve (right/left) Ventricle.
23

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7. Discussion

By identifying oxygen as a genuine difference-maker for cardiac phenotypes, this work connects mechanistic and lineage explanations, showing how changes in the physico-chemical substrate of development have historically reshaped the architecture of the vertebrate heart (see Papers II and III). What evolves, therefore, is not merely a structure but the mechanism that produces it. Or, in Gilbert's (2012) terms: "Change the environment, and you can change the effect."

Although the empirical analysis presented in Paper III focuses on a single experimental system, the broader explanatory claim is motivated by comparative considerations. Surveying developmental and molecular studies across fish, amphibians, reptiles, birds, and mammals reveals patterns that often appear contradictory when considered in isolation. However, when these findings are reinterpreted in light of differences in developmental mode and associated oxygen availability, a consistent pattern emerges: similar oxygen regimes tend to be associated with similar developmental responses (Paper II). This convergence suggests that the phenomenon examined here reflects a conserved sensitivity of cardiogenesis to oxygen, rather than a lineage-specific peculiarity of the chick model. The relevance of the present results thus lies not in their taxonomic scope, but in their capacity to illuminate a general dependency structure that recurs across vertebrate development.

In Love's (2015) framework, this study integrates mechanistic-extended causal (mEx-causal) and mechanistic-extended constitutive (mEx-constitutive) explanation. Experimentally, oxygen manipulation functions as an mEx-causal intervention: varying an environmental parameter produces systematic and reproducible changes in morphogenesis. In the chick embryo, reduced oxygen availability suppresses apoptosis in the truncal myocardium, leading to impaired outflow tract remodeling and single-outlet phenotypes, a process plausibly mediated by hypoxia-inducible signaling pathways. These results suggest oxygen as a causal difference-maker in cardiogenesis and point towards future experiments to further investigate the proposed mechanisms experimentally.

Following Fagan's (2012) analysis, causal explanations can be understood as preliminary steps toward more complete constitutive explanations that reveal how a mechanism actually works through its internal organization (Illari and Williamson 2012; Machamer et al. 2000). In the present case, the experimental manipulation of oxygen identifies it as a causal difference-maker (mEx-causal). At the same time, a broader evolutionary perspective invites a constitutive interpretation. The emergence of placentation in mammals, and of chorioallantoic respiration together with shifts in the timing of egg deposition in birds and crocodilians,

transformed oxygen supply from a contingent external condition into an organizing feature of development. Through these historical changes in developmental mode, oxygen became embedded within the regulatory architecture of cardiogenesis itself. The explanatory focus therefore shifts from oxygen as something that merely acts on the system to oxygen as something that partially constitutes the system—a transition from mEx-causal to mEx-constitutive understanding (Kaiser 2021; Kaiser and Krickel 2017; Fagan 2012; Love 2015; 2008; Paper III).

Seen in this light, developmental systems constitute a distinct level of biological organization with their own historical dynamics. Their evolution cannot be reduced to changes in gene frequencies but must be understood as the reconfiguration of causal architectures that link genes, cells, and environments. As Laubichler (2009) argued, comparative analyses across taxa provide the necessary integration between development and evolution: phenotypic evolution proceeds through modifications of developmental organization, while population-level processes such as selection act on the outcomes of these reorganizations.

This view also informs the discussion in Paper III, which suggests that mutations in hypoxia-inducible factors (HIFs) influencing oxygen sensitivity, common in high-altitude populations, may shape susceptibility to congenital heart defects (CHD). Although such variants can enhance oxygen delivery, they do not remove developmental vulnerability, as CHD prevalence remains elevated in these populations (Bigham et al. 2009; González-Andrade 2020; Crawford et al. 2017). Importantly, this vulnerability does not imply that oxygen acts merely as an external disturbance. Rather, it reflects the fact that oxygen availability has become historically embedded in the organization of cardiogenesis itself. Developmental mechanisms have evolved under relatively stable oxygen regimes, such that oxygen now functions as a constitutive condition for their proper operation, even though it can still vary externally. External perturbations therefore act by destabilizing an internalized dependency, not by imposing an entirely exogenous cause. In this way, adaptive genetic responses modulate sensitivity to oxygen while remaining constrained by a conserved developmental architecture, connecting evolutionary, developmental, and clinical explanations of variation (Lynch 2007; Pigliucci 2008).

This framework also reframes the relationship between evolutionary biology and medicine. Both are sciences of variation: evolutionary biology investigates how variation arises and changes over time, while medicine focuses on the subset of variation that compromises function. Congenital disease is therefore not an anomaly distinct from normal variation but a manifestation of the same developmental dependencies that once enabled evolutionary innovation (Pavlicev and Wagner 2022). As articulated in the EDDI framework presented in

Paper II, the mechanisms that once allowed environmental induction of novel traits persist in modern ontogeny. Evolution has not removed these dependencies but has rendered them more constrained. This preserves the need for specific environmental conditions and creates potential sites of vulnerability that are consistent with the heart's well-known sensitivity to perturbation during development.

Developmental repatterning triggered by new environmental inputs thus likely provides the mechanistic basis for evolutionary novelty in the vertebrate heart. This view describes how developmental systems reorganize to generate new forms and how these reorganizations themselves become subject to selection. The evolution of form is, in this sense, the evolution of the mechanisms that produce it (Laubichler 2009; Brun-Usan et al. 2022; Gilbert 2012). By situating the environment within these mechanisms, the comparative study of development offers an integrated account of evolution, development, and disease: a unified science of variation that connects evolutionary history to present-day ontogeny and its pathologies.

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*a few words together and don't try
to make them elaborate, this isn't
a contest, but a doorway*

into thanks (...)

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