

Biofidelic simulations of embryonic joint growth and morphogenesis

Unravelling the cell-level dynamics and the role of mechanical forces

By

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Philosophy

‘What we call little things are merely the causes of great things’

Henri Frederic Amiel

1821-1881

Josepha Godivier

Biofidelic simulations of embryonic joint growth and morphogenesis, © December 2022

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Declaration of Originality

I declare that this thesis, and the research presented within, is the product of my own work. Any ideas or quotations from the work of other people, published or otherwise, or from my own previous work are fully acknowledged in accordance with the standard referencing practices of the discipline.

Part of the data processed in this thesis originates from previous work from this group ([Huang 2022](#)), and is acknowledged accordingly where appropriate.

This thesis was carried out between October 2018 and December 2022 under the supervision of Prof. Niamh C. Nowlan in the Department of Bioengineering at Imperial College London, UK, and the co-supervision of Dr Chrissy L. Hammond from the University of Bristol, School of Physiology, Pharmacology & Neuroscience, Bristol, UK.

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Abstract

During skeletal development, the opposing surfaces in the joint mould into interlocking and reciprocal shapes in a process called morphogenesis. Morphogenesis is critical to the health and function of the joint, and yet, little is known about the process of joint morphogenesis. For example, how do different joints acquire their specific shapes? Which cellular processes underlie joint shaping and how are they regulated? However, it is known that fetal movements are critical to joint development, with alterations or absences of movement being implicated in multiple pre- and post-natal musculoskeletal conditions. This doctorate explored the cell-level dynamics governing joint growth and the implication of movements in regulating them, using novel biofidelic and mechanobiological models of joint growth.

Cell-level data from wild type zebrafish larvae were tracked and synthesised in a biofidelic simulation of zebrafish jaw joint growth. Growth characteristics were quantified revealing a strong anisotropy (Chapter 3). Next, zebrafish larvae were immobilised using drug treatment. The material properties of the zebrafish jaw cartilage were measured using nano-indentation in the presence or absence of movement showing a delay in cartilage stiffening in immobilised larvae (Chapter 4). Then, I developed a novel mechanobiological model of zebrafish jaw joint growth, which identified a correlation between growth characteristics and the dynamic patterns of mechanical stimuli experienced by joint elements over jaw motion (Chapter 5). Finally, local growth rates were characterised in the mouse elbow in the presence or absence of skeletal muscles. Spatial heterogeneity in the growth rates correlated with the emergence of specific shape features at the level of the condyles. Immobilisation led to disruption of the local growth rates correlated with failed shape differentiation of the condyles. The relative contribution of key cell activities to growth such as cell volume expansion, cell number increases and extracellular matrix expansion, were shown to vary over time in both wild types and muscleless-limbs and to be altered in the absence of skeletal muscles (Chapter 6).

This research offers avenues for improvement in simulations of joint development and potentially other organs. It provides fundamental advance in our understanding of mechanoregulation in the developing joint and increases our understanding of the origins of musculoskeletal abnormalities.

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List of Acronyms

AFM	Atomic Force Microscopy
AMC	Arthrogryposis Multiplex Congenital
ANOVA	Analysis of Variance
AP	Anteroposterior
DDH	Developmental Dysplasia of the Hip
dpf	day post fertilisation
e	embryonic day
ECM	Extracellular matrix
FADS	Fetal Akinesia Deformation Sequence
FE	Finite Element
LMP	Low Melting Point
MC	Meckel's Cartilage
ML	Mediolateral
MS222	tricaine methanesulphonate
OCT	Optimum Cutting Temperature
PBS	Phosphate-Buffered Saline
PQ	Palatoquadrate
ROI	Region of interest
TRPV4	Transient Reception Potential Vanilloid type 4
TS	Theiler Stage
VD	Ventrodorsal
YM	Young's Modulus

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Chapter 1

1. Introduction

1.1. Research gaps

Synovial joints, which are the most common joints of the skeletal system and which are characterised by the presence of a fluid-filled cavity, ensure stability and mobility to the body ([Juneja et al. 2022](#)). During prenatal development, the ends of the two opposing cartilaginous skeletal elements of a synovial joint mould into reciprocal and interlocking shapes in a process called morphogenesis ([Pacifici et al. 2005](#)). Incomplete or abnormal morphogenesis has important consequence for joint prenatal- and long-term health and may be debilitating ([Nowlan 2015](#), [Vaquero-Picado et al. 2019](#)). Currently, the mechanisms underlying joint morphogenesis are still unclear and the drivers for joint shaping at the cellular level have been poorly quantified.

Clinical and experimental evidence has shown that prenatal movement is a critical factor for joint development. Absent or abnormal movements have been correlated with common congenital conditions such as developmental dysplasia of the hip (DDH) or arthrogryposis ([Nowlan 2015](#)). These conditions, sometimes lethal, are associated with a substantial reduction in quality of life and treatments are limited. Malformations also impair joint long-term health by inducing overloads which participate to the development of early osteoarthritis ([Zeng et al. 2016](#), [Vaquero-Picado et al. 2019](#)). For example, DDH is the main cause of total hip replacement in young people and represents an economic burden to public health systems ([Vaquero-Picado et al. 2019](#), [Arthritis Research UK 2021](#)). The current literature has shown that cell-level activities involved in joint shaping adapt to changes in the mechanical environment ([Roddy et al. 2011a](#), [Shwartz et al. 2012](#)), however, how the mechanical stimuli arising from prenatal movement may trigger these changes is still unclear. Unravelling these mechanisms are valuable to deepen our understanding of joint morphogenesis and the aetiology of clinical conditions.

Gathering biological data into an understanding of the mechanisms driving dynamic shape changes at the macro-scale is a challenge that computational techniques can help overcoming. Computational techniques enable the synthesis of experimental data and a means to test hypotheses via simulation. Computational models of prenatal joint development have notably given insights on how joint growth may be triggered by mechanical stimuli (Heegaard et al. 1999, Giorgi et al. 2014). However, existing models of prenatal joint development used simplified or extrapolated cell activities which poorly represent the complex dynamics which occur during morphogenesis. A more precise quantification of cell-level dynamics combined with computational simulations of joint growth would help us to clarify which factors dominate joint morphogenesis.

This thesis addresses the following scientific gaps: (1) The need for an accurate method of quantification of joint growth based on cell-level data. (2) Identifying which characteristics of growth dominate joint morphogenesis and the underlying cell activities driving them. (3) Identifying which aspects of joint growth are mechanoregulated and which stimuli arising from fetal movement drive these changes. Being able to translate co-ordinated cell behaviours into quantitative descriptions of joint growth and morphogenesis by the means of computational models will give insights of the underlying biological and mechanobiological mechanisms of joint shaping.

1.2. Aims and hypotheses

In this doctorate, my objective was to investigate the role of dynamic cell activities and mechanical forces to joint morphogenesis using simulations of embryonic joint growth. The following aims were set and the chapter in which they are addressed is annotated:

- Aim 1: Developing a method for growth analyses of high-resolution cell-level scans in the developing larval zebrafish jaw joint (*Chapter 3*)
- Aim 2: To identify which characteristics of growth dominate zebrafish joint growth, testing the hypothesis that growth rate heterogeneity and orientation are both critical to zebrafish jaw joint morphogenesis (*Chapter 3*)
- Aim 3: Investigating how larval zebrafish jaw cartilage material properties are influenced by jaw movements. Estimated biomechanical moduli are necessary for later computational models (*Chapter 4*)
- Aim 4: Investigating how patterns of joint growth differ in the absence of jaw movements and testing hypotheses regarding to how mechanical stimuli arising from jaw movements may direct these changes (*Chapter 5*)

Aim 5: Generalising my methods to a larger animal model system, the murine distal humerus, and quantifying growth in several time windows in the presence or absence of skeletal muscles to identify which aspects of growth are mechanically regulated (*Chapter 6*)

Aim 6: Quantifying the respective contributions to growth and morphogenesis of critical cell activities in distal humeri of normal and muscleless-limb mice to understand the underlying biological and mechanobiological mechanisms of murine joint morphogenesis (*Chapter 6*)

The use two animal model systems with different advantages and limitations enabled me to test diverse hypotheses using analogous methods adapted to each model system.

The structure of the thesis is as follows:

Chapter 2: Literature review

Chapter 3: Methods development for zebrafish joint growth quantification and investigation of growth characteristics in free-to-move zebrafish jaw joints (*Aims 1 and 2*)

Chapter 4: Investigation of the material properties of the zebrafish jaw cartilage under normal and altered mechanical environments over time (*Aim 3*)

Chapter 5: Investigation of the mechanoregulation of growth in the zebrafish jaw joint (*Aim 4*)

Chapter 6: Methods adaptation to the mouse and analysis of the underlying mechanisms of murine joint morphogenesis (*Aims 5 and 6*)

Chapter 7: Global discussion gathering the main findings of this thesis and their contributions to the field of developmental biomechanics along with perspectives for future research.

Chapter 2

2. Background

2.1. Joints in the skeletal system

Three types of joints ensure stability and mobility to the skeletal system among which synovial joints are the most common. Unlike fibrous joints (e.g. the skull sutures) or cartilaginous joints (e.g. pubic symphysis), synovial joints allow a great range of movements between connected bones. Diverse body motions are achieved by distinct joint structures and thus synovial joints were classified into six different types: hinge (e.g. elbow), ball and socket (e.g. hip), saddle (e.g. carpometacarpal joint), pivot (e.g. between C1 and C2 vertebrae), plane (e.g. between tarsal bones in the foot) and condyloid (e.g. between radius and carpal bones of the wrist) (Juneja et al. 2022). In synovial joints, a fluid filled cavity separates out opposing bones. It is encapsulated by a fibrous capsule and a membrane which produces the lubricating synovial fluid. Opposing bone epiphysis are capped with articular cartilage, an avascular connective tissue with unique load-bearing and load-transferring properties (Juneja et al. 2022). These synovial joint components—cavity, fibrous capsule, membrane and articular cartilage—are shown in Figure 2.1. Articular cartilage is composed of chondrocytes embedded into an extracellular matrix

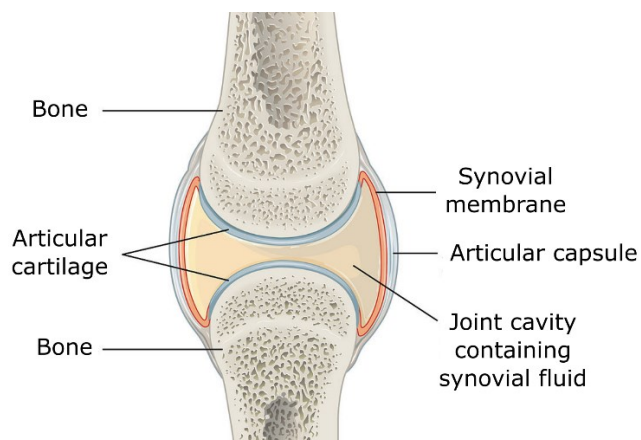


Figure 2.1: **Schematic of a human synovial joint and its main components.** From Synovial Joints, OpenStax (Betts et al. 2013).

(ECM) mainly made of collagen, proteoglycans and water (Fox et al. 2009, Camarero-Espinosa et al. 2016). Along its depth, articular cartilage has a layered architecture which enables it to withstand both tensile and compressional forces through the action of collagen fibres and fluid entrapment, protecting the long bones (Fox et al. 2009, Camarero-Espinosa et al. 2016).

2.2. Limb skeletal development

2.2.1. Skeletal development and joint formation

Synovial joints development starts prenatally with the aggregation of mesenchymal progenitor cells at the location of the future bones in the fourth week of gestation in humans (O'Rahilly and Gardner 1975, Goldring et al. 2006, Yang 2013). Mesenchymal aggregates, or condensations, are at first uninterrupted and the future skeletal elements are undistinguishable (Figure 2.2a). Then, cell progenitors in the mesenchymal condensations differentiate into chondrocytes and form a cartilage template, or anlagen, for the future bones (Figure 2.2b) (Goldring et al. 2006, Yang 2013). The separation of the skeletal elements occurs through the appearance of a zone of compact and interconnected mesenchymal cells marking the location of the future joint, called the interzone (Figure 2.2b) (Pacifici et al. 2005, Chijimatsu and Saito 2019, Rux et al. 2019). The physical separation of the skeletal elements is then marked by the formation of a cavity at the level of the interzone around the

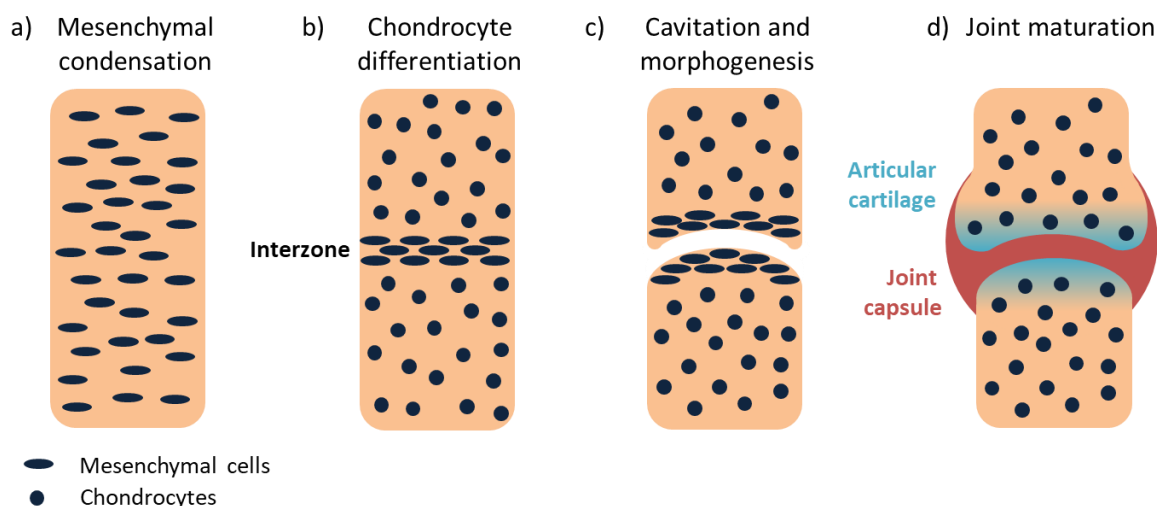


Figure 2.2: **Schematic of a synovial joint at key stages of limb development.** a) Uninterrupted mesenchymal condensations form at the location of the future bones. b) A zone of compact and interconnected mesenchymal cells forms at the location of the future joint, this is the interzone, whereas cell progenitors differentiate into chondrocytes. c) Joint cavitation initiates and the two opposing surfaces start to mould into reciprocal and interlocking shapes. d) The final shape of the joint is obtained, cartilage at the end of the epiphysis matures into articular cartilage and the joint capsule develops.

seventh week of gestation in humans (Figure 2.2c) (Gardner and O'Rahilly 1968, Pacifici et al. 2005, Chijimatsu and Saito 2019). The cavity emerges from the accumulation of hyaluronan in the extracellular matrix provoking cell detachment (Chijimatsu and Saito 2019, Kim et al. 2022) and the apparition of microcavities which eventually merge into a single, joint-wide cavity (Kim et al. 2022). Cell apoptosis in the interzone remains sparse, rather, cells migrate to the epiphyseal cartilage (Pacifici et al. 2006, Chijimatsu and Saito 2019). During joint maturation, the two opposing joint surfaces mould into reciprocal and interlocking shapes, in a process called joint morphogenesis which starts prior cavitation (Figure 2.2c) (Pacifici et al. 2006, Nowlan and Sharpe 2013). In long bones, endochondral ossification takes place to replace the cartilage anlagen with bony tissue (Nowlan et al. 2010b, Camarero-Espinosa et al. 2016, Chijimatsu and Saito 2019). This process, which starts at around the same time as joint cavities emerge, carries on after birth. Cartilage located at the end of the bone epiphyses remains to form the articular cartilage of the joint (Figure 2.2d) (Nowlan et al. 2010b, Chijimatsu and Saito 2019).

Of the key steps in joint development described above, morphogenesis is one of the least understood processes of joint formation and the cellular dynamics underlying it are still poorly characterised. Studies on the mouse embryo showed that rapid joint growth is sustained by a continuous influx of progenitor cells from the surrounding tissues into the joint region (Shwartz et al. 2016, Zhang et al. 2020). An uneven influx of progenitor cells was observed between the two opposing joint elements in the mouse knee and elbow with influx of new cells into the humerus and femur being more pronounced than in the opposing bones (Shwartz et al. 2016, Zhang et al. 2020). Asymmetric distributions of chondroprogenitors along the ventrodorsal axis cells were also observed within the murine femoral and humeral epiphyseal cartilages (Zhang et al. 2020) and murine interphalangeal joints (Koyama et al. 2008). Chondrocytes' maturation exhibited a similar ventrodorsal asymmetry in all skeletal elements with the distribution of col2-positive cells being spatially uneven (Zhang et al. 2020). Such ventrodorsal asymmetry was not observed in the diaphyseal cartilage (at the level of the shaft of the bone) indicating that spatial asymmetry is characteristic of joint morphogenesis (Zhang et al. 2020). Patterns of cell proliferation in the avian embryonic knee joint showed a mediolateral asymmetry with the number of proliferating cells being higher in the medial condyle relative to the lateral condyle (Roddy et al. 2011a). The number of proliferating cells in avian embryos varied with time, being significantly higher at embryonic day e7.5 compared to e6.5 and e8 when cavitation occurs (Roddy et al. 2011a). These studies provided insights that the emergence of interlocking joint shapes is driven by asymmetrical growth mechanisms along all three axes. Additionally to cell influx, maturation and proliferation, other active cell processes such as cell size, orientation and rearrangements evolve as joint morphogenesis proceeds (Shwartz et al. 2012, Brunt et al. 2015).

Despite these observations, there is a lack of understanding of the impact that these cellular dynamics have on growth and morphogenesis at the tissue scale and what drive these mechanisms.

2.2.2. Mechanobiology of limb skeletal development

2.2.2.1. Altered movement is a risk factor for prenatal conditions

Abnormal fetal movements during prenatal development can affect the development of skeletal elements and the shape of joints. In humans, most fetal movements have started by the tenth week of gestation (Nowlan 2015). They include general body movements, isolated limb movements, breathing movements and so on. Abnormal to absent fetal movements can arise from neuromuscular conditions. Fetal movements can also be restricted by an insufficient amount of intrauterine space and/or of amniotic fluid, and inadequate fetal positioning, with for example breech fetal positioning shown in Figure 2.3 (Nowlan 2015). Several pre- and post-natal musculoskeletal conditions have been associated with reduced fetal movements: the most commons, in order of incidence, being development dysplasia of the hip (DDH), arthrogryposis multiplex congenital (AMC) and fetal akinesia deformation sequence (FADS) (Nowlan 2015). In DDH, hip joint shape is altered which reduces joint stability with partial or complete joint dislocation as illustrated in Figure 2.4A. Its incidence varies from 0.1 to 6.6 per 1,000 depending on the geographical location (Nowlan 2015, Pollet et al. 2017, Harsanyi et al. 2020). Arthrogryposis designates the presence of congenital contractures in multiple joints as shown in Figure 2.4B. It affects 1 per 2,000 to 5,000 new-borns (Niles et al. 2019). A lethal form of arthrogryposis known as fetal akinesia deformation sequence (FADS) has an incidence of 1 per 15,000. It is characterised by multiple joint contractures and other defects such as bone hypomineralisation, craniofacial anomalies, and pulmonary and cardiac deficiencies (Chen 2012, Nowlan 2015). These congenital conditions, by altering joint shape, also impair joint long-term health; joint malformations lead to altered stress distributions and overloads which participate to the early onset of osteoarthritis (Mavcic et al. 2008, Allen and Golightly 2015, Zeng et al. 2016, Vaquero-Picado et al. 2019). Substantial

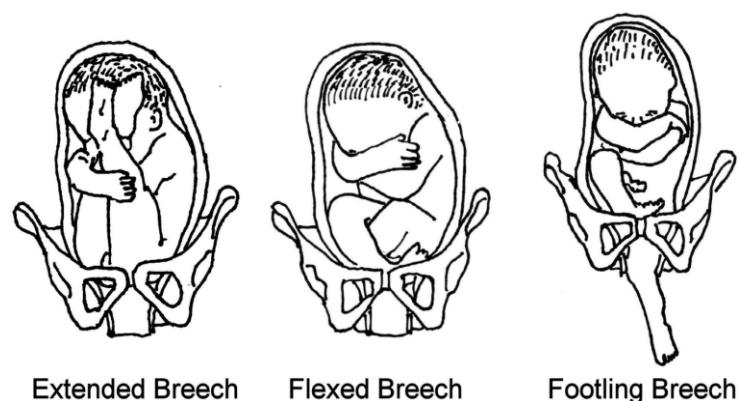


Figure 2.3: **Fetal breech positions.** From (Nowlan 2015)

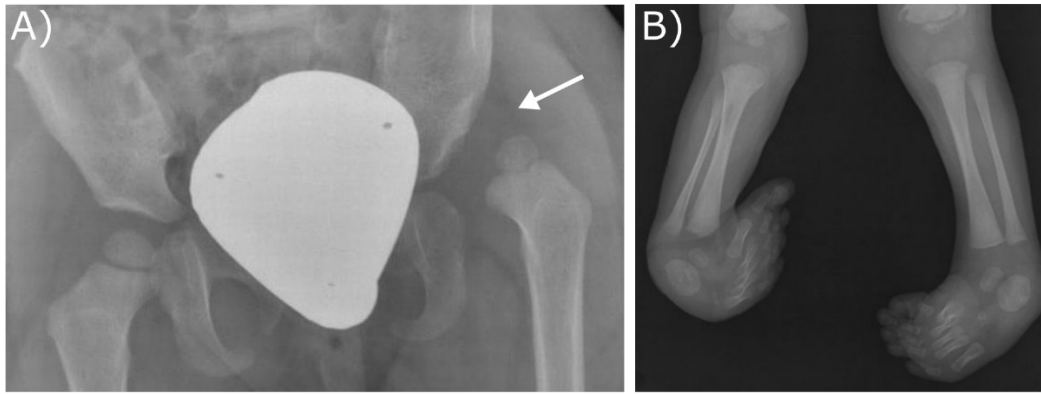


Figure 2.4: **Musculoskeletal conditions associated with reduced fetal movement.** A) 2 years old pelvic radiograph showing abnormalities of the left hip (white arrow) characteristic of DDH (dislocation and acetabular deformity). From (Sewell et al. 2009). B) Radiograph showing lower limb joint contractures typical for AMC taken at the age of 10 days old. From (Binkiewicz-Glinska et al. 2013).

reduction in quality of life was reported as a consequence of arthritis (Arthritis Research UK 2021). Treatment options are limited and mainly consist of pain relief medication and joint replacement surgery. DDH is responsible for as much as 29% of total hip replacements in people aged 60 or younger (Furnes et al. 2001, Engesaeter et al. 2011).

2.2.2.2. Impact of muscle contractions on endochondral bone formation and joint development, insights from animal models

A complex relationship exists between skeletogenesis and the mechanical environment. As reviewed previously, there is an established link between reduced movements and congenital musculoskeletal conditions in humans showing that mechanical forces are involved in the regulation of endochondral bone formation as well as joint development. A range of animal models have been used to understand the role of mechanical loading on skeletal development and have shown that absent muscle activity impacts bone and joint formation at multiple levels, as detailed below.

Abnormal fetal movement alters endochondral bone formation

The formation of limb skeletal elements is impacted in the absence of fetal movement. Prenatal bones in immobilised chicks (Osborne et al. 2002, Lamb et al. 2003, Nowlan et al. 2010b) and muscleless-limb mice (Gomez et al. 2007, Nowlan et al. 2010a, Sotiriou et al. 2022) are significantly shorter. Bone eminences formation is upregulated by muscle contraction. For example, the size of the humerus tuberosity is significantly smaller in embryonic muscleless-limb mice as shown in Figure 2.5a (Gomez et al. 2007, Nowlan et al. 2010b). In the absence of muscle contraction, bone mineralisation is reduced in both the chick and the mice (Figure 2.5b) (Nowlan et al. 2010a, Khatib et al. 2021, Sotiriou et al. 2022).

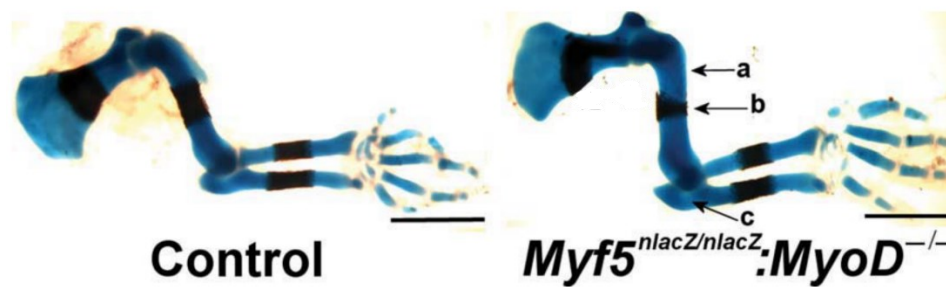


Figure 2.5: **Absent musculature results in multiple skeletal alterations in mice forelimbs:** (a) loss of humeral tuberosities, (b) decrease in ossification centres' size, (c) failed cavitation of the elbow at Theiler stage TS23 (embryonic day e14.5). Left: control; right: muscleless-limb. Scale bars 1mm. From (Nowlan et al. 2010b).

Joint formation is significantly altered in the absence of muscle contraction

If joint site determination is unaffected by absent muscle activity (Mitrovic 1982, Nowlan et al. 2010b, Bridglal et al. 2020), joint morphology is significantly altered with the loss of complementary and interlocking joint surfaces in all animal models. Observations include a loss of joint shape features with often failed cavitation leading to joint fusion in murine (Figure 2.5c) and avian embryos (Mitrovic 1982, Osborne et al. 2002, Nowlan et al. 2010a, Nowlan et al. 2010b, Nowlan et al. 2014, Sotiriou et al. 2019, Bridglal et al. 2020) and overlapping of jaw joint cartilage elements in zebrafish larvae (Brunt et al. 2015, Roddy et al. 2017). It should be noted that joints are differentially affected by the absence of muscle contraction; for example, in muscleless-limb mice embryos, the elbow joint is more severely impacted than the knee (Nowlan et al. 2010a). It has been hypothesised that biophysical stimuli arising from passive movements from the mother, or the littermates, could compensate for the lack of direct muscle contraction (Nowlan et al. 2012). However, these stimuli are unequally applied between the forelimb and hindlimb resulting in differential alterations between the knee and the elbow (Nowlan et al. 2012). Upon maturation, joint embryonic cartilage material properties are also altered as a result of absent mechanical stimuli. In chick embryos, knee cartilage glycosaminoglycan and hydroxyproline contents are altered when specimens are immobilised, resulting in a significant reduction of the instantaneous modulus up to 50% (Mikic et al. 2004).

The development of normal and functioning joints depends on the timing and duration of movement

The timing and duration of movement has differential impacts on joint development. Early movements are crucial for the separation of joint elements with failed cavitation being observed in paralysed chicks whereas short periods of immobilisation later in development have a small influence on joint morphology (Bridglal et al. 2020). Long periods of immobilisation, even after cavitation has taken place, result in marked shape changes which can lead to joint fusion in most extreme cases in chick limbs and

larval zebrafish jaws (Osborne et al. 2002, Roddy et al. 2017, Bridglal et al. 2020, Rolfe et al. 2021). Increased periods of loading applied *in vitro* to cultured fetal chick knees promotes bone mineralisation, joint growth and morphogenesis (Khatib et al. 2021).

Cell activities adapt to changes in the mechanical environment

Morphological changes in the skeletal elements in the absence of fetal movement are triggered by changes in cell activities. During skeletal development, bone elongation is promoted by the intercalation of chondrocytes into columns. In the absence of muscle contraction, skeletal element shortening is accompanied by marked changes in cell morphology and organisation with the partial loss of this columnar organisation. Abnormal staking patterns are observed in paralysed larval pharyngeal zebrafish cartilage with cells being smaller and more circular (Figure 2.6) (Shwartz et al. 2012, Brunt et al. 2016b). In muscleless-limb mice embryos, cell columns in humeral and femoral growth plates are shorter and exhibit an oblique alignment which is correlated with altered cell intercalation (Shwartz et al. 2012).

At the level of the joint, chondrocyte maturation is a key element to joint morphogenesis and is influenced by movements. Cells in paralysed zebrafish jaw joints and muscleless-limb mice elbows are generally smaller and rounder than controls and have an altered orientation, signs of cell immaturity (Kahn et al. 2009, Brunt et al. 2015, Brunt et al. 2016b, Roddy et al. 2017). The maintenance of cell fate in the interzone is essential for normal joint cavitation and morphogenesis but is altered in the absence of muscle activity. Interzone cells prematurely differentiate into chondrocytes in muscleless-limb mice elbow joints (Kahn et al. 2009) and in paralysed chick knee joints (Roddy et al. 2011b). It is accompanied by a significant decrease in cell proliferation in mutant mice embryos (Kahn et al. 2009), paralysed chicks (Roddy et al. 2011b) and paralysed zebrafish larvae (Brunt et al. 2017). This maintenance of joint progenitor cell fate is regulated by Wnt/ β -catenin signalling pathway which acts downstream to muscle activity in both animal models (Kahn et al. 2009, Brunt et al. 2017).

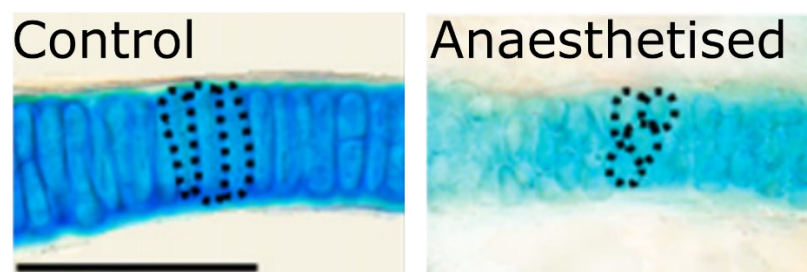


Figure 2.6: **Cell morphology and organisation change in the absence of muscle contraction.** Alcian blue stained control (left) and tricaine-paralyzed (right) pharyngeal cartilage at 5 day post fertilisation with individual cells delimited with dashes lines. Scale bar 50 μ m. Adapted from (Shwartz et al. 2012).

Patterns of biomechanical stimuli arising from movements have been characterised using finite element methods which have shown that elevated rates of cell proliferation and maturation co-localise with areas of higher biomechanical stimuli (Roddy et al. 2011a, Brunt et al. 2015, Brunt et al. 2016b). For example, the medial condyle in the embryonic avian femur grows more than the lateral condyle, which correlates with a higher proportion of proliferating cells and with increased magnitudes of biophysical stimuli (Roddy et al. 2011a).

In summary, absent muscle contractions impact skeletogenesis at multiple levels. In the absence of movement, the skeletal elements are shorter, bone eminence formation is compromised, ossification is reduced, and joint cavitation and morphogenesis are altered as summarised in Figure 2.7. These abnormalities are triggered by changes in cell behaviours which act downstream to muscle activity. Despite evidence that cells adapt in response to loads, we lack understanding of how and which biophysical stimuli direct cell activities and how these activities drive growth at the tissue level. Unravelling the impact of fetal movements on the cell-level dynamics involved in joint morphogenesis would help us to gain a better understanding as to why different joints have different shapes and of the mechanisms which have driven these shape specificities.

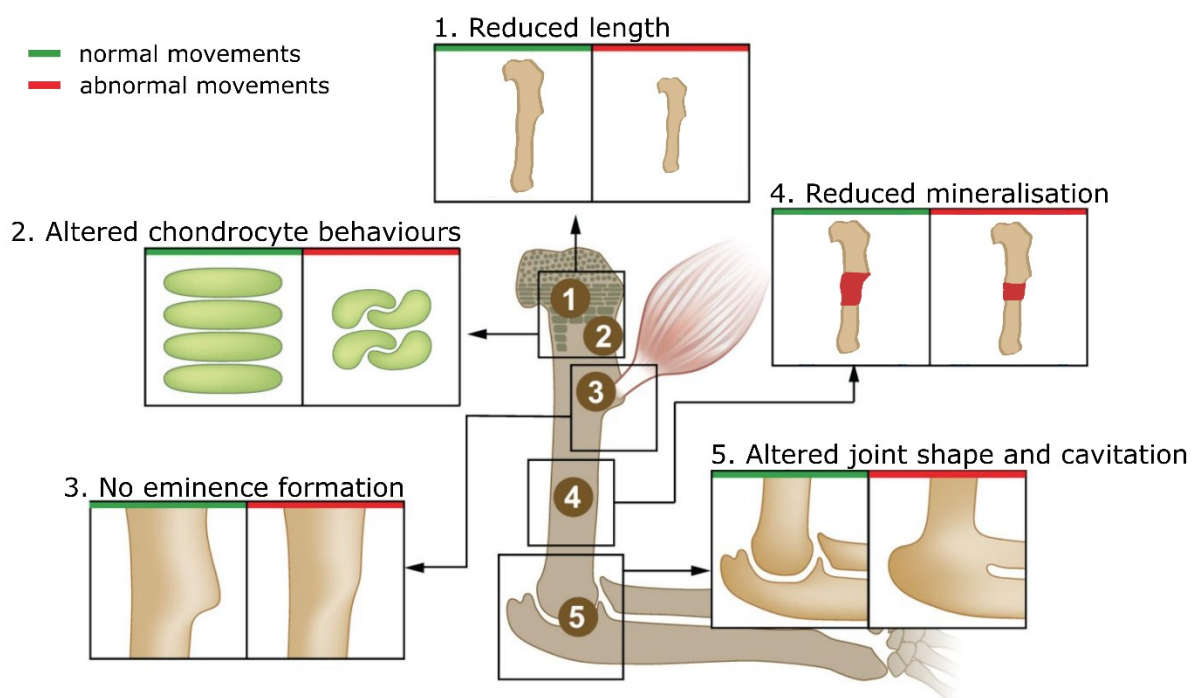


Figure 2.7: **Muscle contractions regulate the formation of limb skeletal elements at multiple levels.** In the absence of muscle activity, bone elongation is altered (1), chondrocytes change behaviour (reduced proliferation, reorganisation and maturation) (2), bone eminences' formation is compromised (3) mineralisation decreases (4) and joint formation is impaired and may result in joint fusion (5). Adapted from (Felsenthal and Zelzer 2017).

2.2.2.3. Impact of loads on chondrocytes, insights from cartilage tissue engineering

In humans, the relative motion between the two opposing joint surfaces generates complex mechanical stimuli in which compression, hydrostatic pressure (HP), shear and tension simultaneously exist (Li et al. 2017). The response of chondrocytes to these mechanical stimuli remains generally unclear. Since prenatal joints are cartilaginous tissues, such knowledge could give us insights on the mechanoregulation of joint growth. In the field of cartilage tissue engineering, mechanical stimulation is used as a strategy for the development of biomimetic tissues intended for the treatment of degenerative conditions such as osteoarthritis (Freeman and McNamara 2017). Researchers have designed increasingly sophisticated bioreactors to study the impact of different loading regimes on chondrocytes. The synthesis of their results is challenging because of the lack of standardised study design. There is a wide variation in the culture conditions, types of scaffolds used, cell sources, loading regimes and types of analyses conducted (Anderson and Johnstone 2017). A summary of the effects of different loading regimes to chondrogenesis is provided here. Chondrocyte phenotype, biomechanical moduli, proteoglycan and collagen contents were the most commonly assessed outcomes.

Joint motion causes compression of articular cartilage. In adult knees, static loading causes tibial cartilage deformation of in average 12% of its thickness when 47.1% of body weight is applied (Halonen et al. 2014). In cartilage tissue engineering, the effects of compression on chondrogenesis were commonly investigated. Dynamic compression positively influences proteoglycan production. Significant increases in glycosaminoglycan (GAG) content were reported when dynamic compression was applied to juvenile bovine chondrocytes (Mauck et al. 2000, Kisiday et al. 2004). Increased production of collagen is less evident. A review compiling results from 63 studies showed that a greater proportion of studies reported no increase or even decrease in the collagen content when dynamic compression was applied compared with studies measuring a positive effect (Anderson and Johnstone 2017). The expression of the chondrocyte phenotype shows upregulation of several genes, including *col2a1*, following compressive dynamic loading (Wang et al. 2009, Anderson and Johnstone 2017). A great number of studies have shown that compressive dynamic loading has a positive effect on tissue material properties (Mauck et al. 2003, Anderson and Johnstone 2017, Salinas et al. 2018). Wang et al. (2009) also reported increased chondrocyte proliferation compared with free-swelling controls. It is worth mentioning that the duration of loading is a key parameter for the positive effect of dynamic compression on chondrogenesis, with long-duration studies tending to find increased GAG or collagen contents compared to short-duration studies (Anderson and Johnstone 2017). In contrast with dynamic loading, static compression has a degenerative effect on chondrocyte metabolisms with for example decreased GAG content (Grodzinsky et al. 2000, Davisson et al. 2002, Sharma et al. 2007).

During joint motion, shear exists simultaneously to compression through sliding of one joint surface with respect to the other (Wong et al. 2008). The addition of low magnitude shear to dynamic compression in cartilage tissue explants has a positive impact on chondrocyte gene expression, extracellular matrix synthesis and tissue mechanical properties (Gemmiti and Guldberg 2009, Schatti et al. 2011). It is however unclear whether this upregulation is due to shear or increased nutrient perfusion (Salinas et al. 2018).

Fluid entrapment exposes articular cartilage to hydrostatic pressure during joint motion. Cyclic hydrostatic loading significantly increases ECM synthesis with upregulation of GAG and collagen productions (Elder and Athanasiou 2009, Kraft et al. 2011, Correia et al. 2012). The effect of static hydrostatic loading on chondrocytes is less clear. Some studies reported downregulation in the expression of several cartilage genes compared to unloaded controls (Smith et al. 1996, Correia et al. 2012) when others observed increased GAG and collagen contents (Elder and Athanasiou 2009).

In cartilage tissue-engineering, tension loading is another mechanical stimulation strategy, but few studies have investigated the impact of this loading regime on chondrocytes. Static tension results in marked increases in tensile biomechanical moduli (Lee et al. 2017) and increased proteoglycan content (Fan and Waldman 2010) compared with unstimulated controls. Chondrocytes from different cartilage zones differentially respond to dynamic tension with superficial zone chondrocytes being the most impacted (Vanderploeg et al. 2008).

In conclusion, dynamic loading positively influences chondrogenesis through upregulation of the expression of the chondrocyte phenotype, the extracellular matrix synthesis, and chondrocyte proliferation. Amongst multiple loading regimes, dynamic compression and cyclic hydrostatic pressure loading were the most extensively investigated and a consensus exists in the literature about their positive effects on chondrocytes.

2.3. Animal model systems of skeletal development

The study of embryonic skeletal development is a challenge that relies on model systems. Here I introduce three prevalent animal models (chick, mouse, and zebrafish) that have been used to examine skeletal development in normal or altered mechanical environments.

2.3.1. Chick

The chick embryo is a well-established model system for the study of skeletal development especially in an altered mechanical environment. The chick embryo develops externally in an egg which enables external manipulation such as drug-treatment or surgery. Several neuromuscular blocking drugs can

be administered to induce rigid (where muscles are contracted) or flaccid (where muscles are relaxed) paralysis of the chick embryo. For example, decamethonium bromide/iodide and pancuronium bromide are commonly used blocking drugs (Nowlan et al. 2010b). Immobilisation of specific regions can be performed through surgery. Other external manipulations include externally induced joint flexion-extension which has been performed *in ovo* to study the rescue of immobilised chick limbs by induced movements (Bridglal et al. 2020). *In vitro* studies have also been performed in which embryonic limbs were harvested then cultured in a bioreactor which contained a media supplying nutrients and which induced flexion-extension to the joint (Chandaria et al. 2016). Such bioreactors have been used to assess the impact of different loading patterns, altering the frequency and duration of loading during development (Khatib et al. 2021), but also investigating the activity of mechanosensitive channels which could be upregulated by movements (Parisi et al. 2018).

2.3.2. Mouse

Mouse embryos have been long used as a model system to study the musculoskeletal system. Mice, as mammals, are genetically close to humans (Pennacchio 2003) and have been broadly used to study gene regulation during embryogenesis (Taft 2008). Several genotypes have been established in which limb muscle contractions were eliminated. In the Splotch-delayed ($Pax3^{Sp-d/Sp-d}$) genotype, Pax3 transcription factor, responsible for the migration of muscle progenitor cells into the limb bud, is absent, resulting in muscleless-limb embryos (Bober et al. 1994, Bismuth and Relaix 2010). Knocking out both MyoD and Myf5 genes necessary for muscle differentiation, results in a complete absence of skeletal muscles in embryos ($Myf5^{nlacZ/nlacZ} \cdot MyoD^{-/-}$) (Rudnicki et al. 1993, Bismuth and Relaix 2010). It is worth noting that reduced muscles are seen in littermates with a functional copy of Myf5 ($Myf5^{nlacZ/+} \cdot MyoD^{-/-}$) (Rudnicki et al. 1993). Muscular dysgenesis (*mdg*), caused by a lethal mutation which eliminates excitation-contraction coupling, is characterised by the absence of contraction in any skeletal muscle (Pai 1965). Finally, in the absence of Dock1, fusion of the myoblasts is impaired resulting in dramatic muscle defects (Laurin et al. 2008). The use of *in vitro* load-inducing systems similar to those used with chicks can help further unravelling the impacts of movements on joint development and its signalling pathways (Khatib et al. 2023).

The aforementioned models are characterised by a complete lack of skeletal muscle ($Pax3^{Sp-d/Sp-d}$ and $Myf5^{nlacZ/nlacZ} \cdot MyoD^{-/-}$), reduced skeletal muscles ($Myf5^{nlacZ/+} \cdot MyoD^{-/-}$) or a lack of muscle contraction (*mdg*). The *mdg* mutation represents a strong model of extreme forms of human muscular defects being similar to diseases such as human muscular dystrophy. On the opposite, there is no human diseases in which skeletal muscles are absent altogether. In the absence of skeletal muscles, disturbed skeletogenesis could result from altered mechanical forces, or from missing factors from muscle cells

themselves. However, it is likely that effects on ossification and joint formation are due to the lack of mechanical stimuli rather than missing clues from adjacent muscle cells. Phenotypic analyses performed in mouse mutant limbs were similar whether muscles were immobile (Kahn et al. 2009), reduced (Nowlan et al. 2010a) or absent (Nowlan et al. 2008, Kahn et al. 2009). Similar disturbances were also observed in immobilised chick embryos in some of the joints (Roddy et al. 2011b, Singh et al. 2018).

2.3.3. Zebrafish

The zebrafish is a model system for skeletal development with numerous advantages including rapid development, small size, and availability of numerous transgenic lines. Many genes involved in mammalian skeletal development have orthologs in zebrafish (Howe et al. 2013, Valenti et al. 2020). Zebrafish larvae develop externally and are optically translucent, which enable *in vivo* imaging of live specimens throughout development. Drug treatments and laser surgery are also facilitated by these characteristics (Roddy et al. 2017). Over the last decade, it has been proven that the zebrafish possesses a true synovial joint in the jaw (Askary et al. 2016), establishing it as a model for human musculoskeletal disorders including arthritis and DDH (Roddy et al. 2017, Lawrence et al. 2018). Adult zebrafish jaw joint is located between the anguloarticular and quadrate bones (Figure 2.8 A & B) and resembles a hinge joint allowing motion in a single plane. It possesses a fluid-filled cavity (appearing around 14 days post fertilisation (dpf) and being consistently present by 28 dpf) encapsulated by a minimal fibrous capsule (Figure 2.8B). In adult zebrafish, layers of articular chondrocytes line the cavity similar to articular cartilage in mammals (Figure 2.8B). Lubricating proteins were also found to act as homologs to critical lubricants of mammal synovial joints (Askary et al. 2016). However, contrary to mammals, joint cavitation (at 14 dpf in the zebrafish) occurs long after movements have started (at 3 dpf) and most morphological changes have taken place (at 6 dpf, see Figure 2.8C).

2.4. Computational techniques

Experimental studies have provided insights on the cellular behaviours involved in joint growth and morphogenesis as reviewed in Sections 2.2.1 and 2.2.2. Gathering such complex data, and of multiple types, into an understanding of the dynamic shape changes at the macro-scale is a challenge that computational techniques can help overcoming. Applications of these methods to morphogenesis with a focus on biomechanics when existing are detailed below.

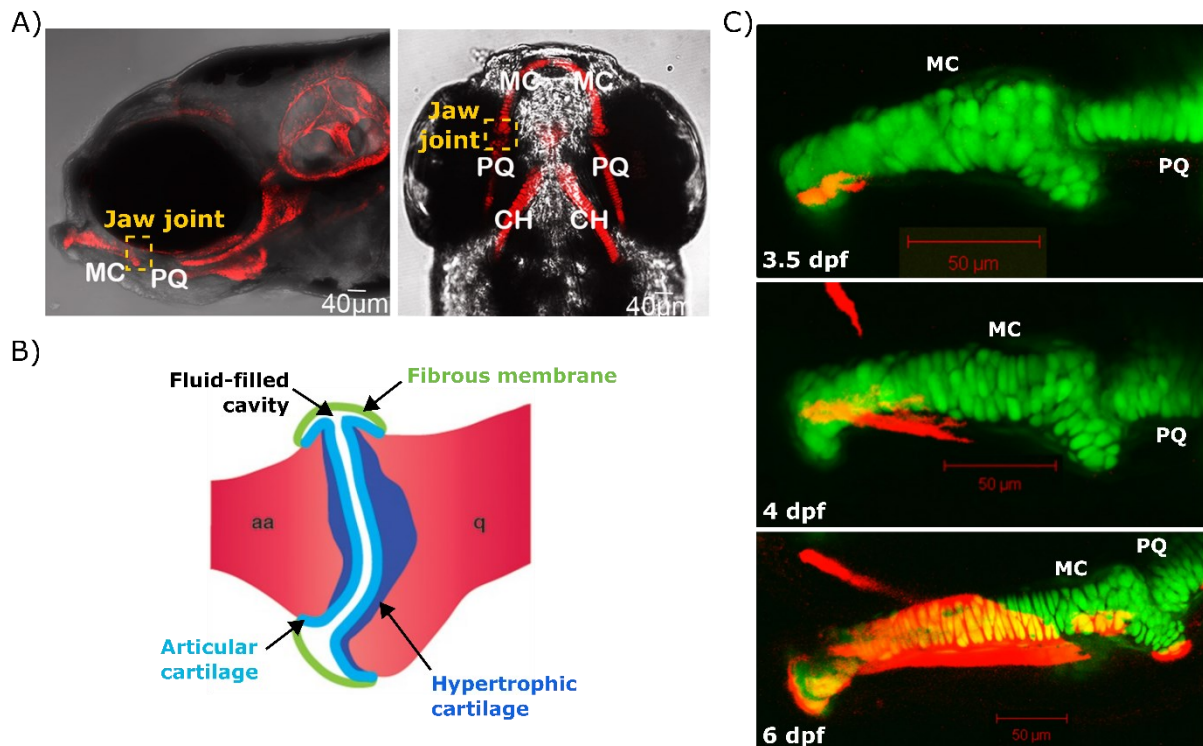


Figure 2.8: **Zebrafish synovial joint and its development.** A) Brightfield lateral and ventral views of 5 dpf zebrafish expressing *Tg(Col2a1aBAC:mcherry)* cartilage marker showing the location of the synovial joint in the jaw. Adapted from (Brunt et al. 2015). B) Schematic of the adult zebrafish synovial jaw joint and its main components. Adapted from (Askary et al. 2016). C) Confocal lateral views of Alizarin red-stained *sox9a:EGFP* jaws at 3.5 , 4 and 6 dpf. MC anteriorly elongates and ventroposteriorly projects at the level of the joint. Chondrocytes intercalate to form organised cell stacks visible by 6 dpf. Adapted from (Eames et al. 2013). MC: Meckel's cartilage, PQ: palatoquadrate, CH: ceratohyal, aa: anguloarticular, q: quadrate.

2.4.1. Quantifying cellular dynamics involved in growth and morphogenesis

There is a growing body of research on quantifying cellular dynamics involved in growth and morphogenesis using analytical computational methods on large quantities of biological data. Rubin et al. (2021) built 3D maps of cell morphologies from light-sheet images of the embryonic murine tibia.

These maps revealed that cell volume expansion switches from isometric to allometric growth between distinct zones of the growth plates, showing that differential growth strategies underlie tissue morphogenesis of the developing growth plates. Stern et al. (2022) quantified cell dynamic behaviours in the epithelial sheet of the *Drosophila* embryo and found that cell-division, with subsequent cell area expansion, contributes up to 30% of tissue growth and that a direct relationship exists between intercalation frequency and rate of tissue elongation. Heller et al. (2016) developed an automated image analysis toolkit for epithelial tissues called EpiTools which enables spatial and temporal morphometric analyses of time lapse images taken at high temporal and cellular resolution. Applied

to the *Drosophila* wing imaginal disc, this toolkit showed that cells were more mobile than previously thought with numerous rearrangements being recorded. Overall, in the model systems described above, cell volume expansion, proliferation and rearrangements are key factors driving tissue growth and morphogenesis.

Recently, in our group, cell morphometric analyses were performed in the murine distal humerus at several developmental stages and showed that increases in cell size and number are important drivers of joint morphogenesis (Huang 2022). Analyses conducted on Splotch-delayed ($\text{Pax3}^{\text{Sp-d/Sp-d}}$) muscleless-limb mouse elbows showed that cell size and number are disturbed in the absence of skeletal muscle (Huang 2022), reinforcing that the cellular dynamics involved in joint shaping adapt in response to mechanical loading with consequences for joint morphology. To my knowledge, no other computational analysis has been undertaken on developing joints. Qualitatively studying cell morphological behaviours in developing synovial joints is a means to clarify which factors dominate joint growth and what the relative contributions of several dynamic cell behaviours are.

2.4.2. Characterising tissue deformation during limb growth (reversed engineering)

Another computational technique aims to capture the dynamic shape changes at the macro-scale and consists of directly characterising tissue growth using tissue landmarks data over time. This technique provides information on local growth rates and tissue deformation directions which can then be compared to cell- and molecular-level data. The spatiotemporal patterns of limb bud elongation in the mouse have been studied by combining analyses of limb bud morphologies over time and cell clonal- (Marcon et al. 2011) or cell cycle times- (Boehm et al. 2010) data sets in computational models. It showed that murine limb bud elongation is driven by anisotropic behaviours and cannot be explained by spatially heterogeneous isotropic rates of cell proliferation only. Another study used statistical tools to generate a map of chick limb bud deformation based on cell lineage tracing data (Morishita and

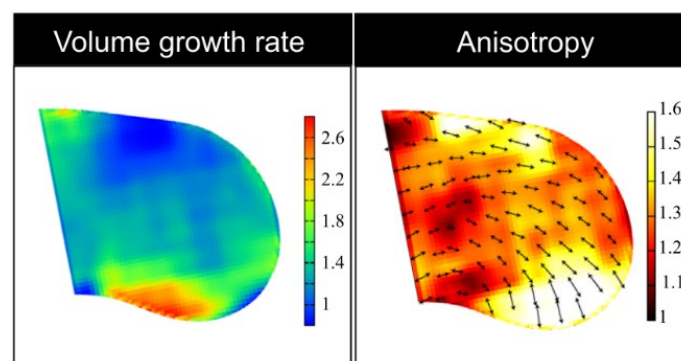


Figure 2.9: **Spatial patterns of early chick limb bud elongation during a twelve-hour time interval.** The black double-headed arrows indicate the directions of deformation. Adapted from (Morishita and Suzuki 2014).

Suzuki 2014, Morishita et al. 2015). Similar to the mouse model, chick limb bud elongation was shown to be driven by spatially heterogeneous volume growth patterns coupled with anisotropic behaviours as shown in Figure 2.9. In the *Drosophila* wing disc, folding initiation was studied by characterising patterns of growth from clonal data coupled with computational models of growth (Tozluoglu et al. 2019). Simulations showed that spatial and temporal growth heterogeneity is the main driver determining fold positioning. Altogether, these studies showed that spatially and temporally heterogeneous growth rates and growth anisotropy are key drivers of morphogenesis in multiple aspects of skeletal development.

2.4.3. Simulating growth in limb developmental biomechanics (direct engineering)

The interplay between growth patterns and mechanical loading has become an area of increased interest leading to the development of numerous computational models with applications to many biological systems such as arterial mechanics, embryo morphogenesis, plant growth, or tumour development. In limb developmental biomechanics, tissue growth has been modelled at the macroscale using continuum finite element (FE) models (Jones and Chapman 2012). Studies have notably sought to predict skeletal development patterns such as bone remodelling (Rodriguez et al. 1994), and ossification (Carter and Wong 1988, Carter et al. 1998), and highlighted the determining role that mechanical stimuli have on these processes. Predictions of bone and joint growth based on biological and mechanical factors have been obtained and are described hereafter.

Integration of biological and mechanobiological factors in endochondral growth simulations

In 1999, Carter and co-workers developed one of the first 2D model of endochondral growth which integrated both biological and mechanobiological factors to growth. They introduced a maturation rate which increased as development proceeds, and which incorporated mechanical stress components (Stevens et al. 1999). Longitudinal growth rate was set proportional to the maturation rate. When the maturation rate reached 1, the region was assumed to ossify and underwent remodelling through a densification of its material. Simulations successfully predicted the appearance and expansion of a second ossification centre, the maintenance of the growth plate, the formation of a layer of articular cartilage and bone elongation. Later, Shefelbine and Carter adapted these equations to model the progression of the growth front of the femoral head in 3D (Shefelbine and Carter 2004b, Shefelbine and Carter 2004a). Growth rates were modelled as the sum of biological and mechanobiological growth rates. The biological growth rates were assumed constant and the mechanobiological growth rates were prescribed as a linear combination of the maximum octahedral shear stress and of the minimum hydrostatic stress. Predictions showed that abnormal loading conditions, such as the one experienced in DDH, resulted in altered growth patterns with enhanced

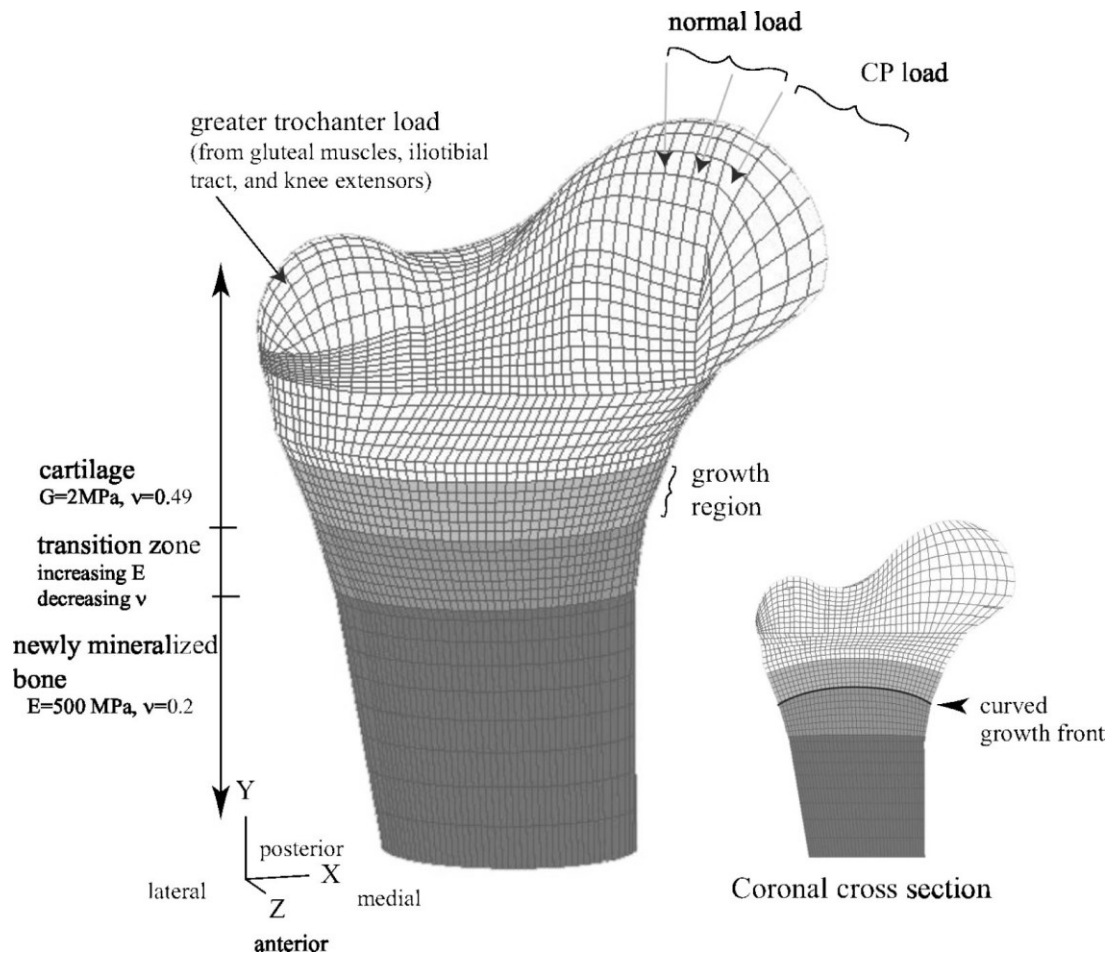


Figure 2.10: **FE simulations of the proximal femur growth under normal and cerebral palsy (CP) loading conditions.** The growth region consists of six rows of elements and represents growth plates' progression. Anteversion of the femoral head is obtained when CP conditions are applied. From (Shefelbine and Carter 2004a).

growth on the medial side of the rudiment leading to the formation of a shape feature characteristic of DDH (coxa valga) (Shefelbine and Carter 2004b). Alternative loading conditions seen in cerebral palsy was also applied to the model and resulted in the formation of an eminence corroborating with clinical observations as shown in Figure 2.10 (Shefelbine and Carter 2004a).

Mechanobiological models of prenatal joint development

The first computational study to focus on prenatal joint development was performed by Heegaard et al. (1999). On line with Carter's first model of endochondral growth, a growth law which linearly combined biological and mechanobiological contributions to growth was used to model interphalangeal joint morphogenesis in 2D. The biological contributions were modelled as a linear function of the local chondrocyte density, itself depending on the distance to the joint line. The biomechanical contributions were set proportional to the local peak hydrostatic stress. When a joint flexion up to 90° was simulated, the model successfully obtained reciprocal joint surfaces. Later, Giorgi

et al. (2014) developed a 3D mechanobiological model of joint morphogenesis. They hypothesised that static hydrostatic compression inhibits cartilage growth while dynamic hydrostatic compression promotes it and simulated both pre- (static loads) and post-cavital (dynamic loads) phases of joint development. Mechanobiological growth rates were set proportional to the compressive hydrostatic stresses averaged over joint motion and modulated by the chondrocyte density (under the assumption that tissue adaptation to mechanical loading is likely to be enhanced in areas of high cell density). No change was made to the biological contributions from Heegaard et al. (1999). The authors showed that the initial shape of the joint and the mode of motion applied to it were critical to the resulting shape of the joint as shown in Figure 2.11 (Giorgi et al. 2014, Giorgi et al. 2015). For example, when modelling the hip joint, the authors showed that reduced, absent or asymmetric movements resulted in shape alterations in both the femoral head and the acetabulum which were characteristic of congenital musculoskeletal conditions such as DDH (Giorgi et al. 2015). A later simulation using aspects of the same model investigated the impact of muscle mass and anatomy on the development of glenohumeral joint deformities to predict the consequences of brachial plexus birth injury on joint shape (Dixit et al. 2020). Another analogous model of the regenerating axolotl humerus used poroelastic instead of linear elastic material properties and correlated interstitial pressure driven from cyclic loading with joint growth (Comellas et al. 2022). A summary of how biological and mechanobiological growth factors were integrated into the mechanobiological models discussed in this Section is provided in Table 2.1.

One limitation of all of the reviewed simulations of joint morphogenesis is that they are based on simplified or extrapolated cell activities solely based on chondrocyte density. The biological contributions integrated in these simulations poorly represent the numerous cell morphological changes which occur during morphogenesis. Complex and intertwined cell activities are involved in

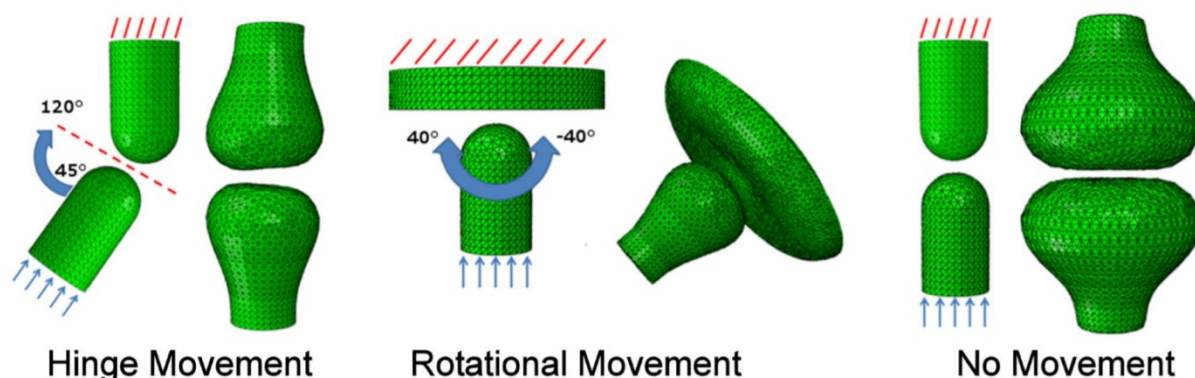


Figure 2.11: **FE mechanobiological models of joint shape morphogenesis.** The initial shape and the modes of motion determine the final shape of the joint. Source work (Giorgi et al. 2014). From (Nowlan 2015).

	Simulated aspect of skeletal development	Biological growth rates	Mechanobiological growth rates
(Stevens et al. 1999)	Endochondral growth and ossification (human humerus)	Function of time and maturation stage	Linear combination of the maximum octahedral shear stress and of the minimum hydrostatic stress
(Shefelbine and Carter 2004a)	Growth plates progression (human proximal femur)	Constant	Linear combination of the maximum octahedral shear stress and of the minimum hydrostatic stress
(Heegaard et al. 1999)	Joint morphogenesis (human phalangeal joint)	Proportional to the local chondrocyte density (varying longitudinally)	Proportional to local peak hydrostatic stress
(Giorgi et al. 2015) (Dixit et al. 2020)	Joint morphogenesis (human hip and shoulder joints)	Proportional to the local chondrocyte density (varying longitudinally)	Proportional to hydrostatic compression levels averaged over joint motion and modulated by chondrocyte density
(Comellas et al. 2022)	Joint morphogenesis (salamander elbow joint)	Proportional to the local chondrocyte density (spatially constant)	Proportional to the interstitial fluid pressure history over joint motion

Table 2.1: Mechanobiological models of skeletal development.

joint formation and need to be more precisely integrated for accurate simulations of growth. Simulations of joint growth based upon accurately tracked cell activities will also afford us more clarity on how alterations of cell behaviours impact shape at the macro-scale. Lastly, it is still largely unclear which mechanical stimuli promote or inhibit growth during joint formation. Hydrostatic pressure, octahedral shear stress and pore pressure have been considered as potential drivers for growth, and though it is clear that mechanical stimuli play a determining role in joint shaping, there is a lack of evidence pointing to the mechanisms at work.

2.5. Summary

This chapter, after presenting a review of prenatal limb skeletogenesis and synovial joint formation, has highlighted the importance of muscle contraction for endochondral bone and joint development. In the absence of muscle activity, joint growth and morphogenesis are dramatically altered, with consequences for both joint function and short- and long-term health. Experimental and computational studies have given insights on how the cell-level dynamics involved in joint shaping may be influenced by mechanical stimuli and have opened up avenues to a more precise characterisation of the processes driving joint morphogenesis. Since existing computational models of prenatal joint development integrated simplified or extrapolated cell activities, a more precise quantification of cell-level dynamics combined with computational simulations of joint growth is necessary to deepen our understanding of the mechanisms underlying joint shaping.

In this thesis, I translated cell-level observations into quantitative descriptions of joint growth and morphogenesis which were then integrated into growth simulations. The influence of mechanical loading to growth was explored by using cell-level data sets from both normal and immobilised specimens and by directly integrating patterns of biomechanical stimuli arising from movements in growth simulations. Such characterisations and simulations provided insights into the mechanisms underlying prenatal joint growth and morphogenesis. The specific role of major cell behaviours, such as cell volume expansion, was explored in the presence of normal or altered mechanical environments which helped me to further unravel dominant biological and mechanobiological factors to growth. I used two well-established animal model systems, zebrafish larvae and mice embryos, to examine how my methods and findings can be applied to model systems with different advantages and limitations.

Chapter 3

3. Quantification of larval zebrafish jaw joint growth characteristics using a biofidelic model of growth

3.1. Introduction

As described in Section 2.2, the mechanisms underlying the emergence of joint shape are poorly understood. Some studies on animal model systems have identified cell activities involved in joint growth and morphogenesis such as the migration of progenitor cells ([Shwartz et al. 2016](#)), cell proliferation ([Zhang et al. 2020](#)), cell size, orientation and rearrangements ([Shwartz et al. 2012](#), [Brunt et al. 2015](#)). These observations provide insights on the cellular dynamics driving joint morphogenesis but it is still unclear how a complex range of dynamic activities combine to enable the formation of specific shape features in synovial joints.

There is a growing body of research on quantifying cellular dynamics involved in tissue growth and morphogenesis using computational tools. Morphometric analyses of cells have been performed from high resolution images of embryonic murine tibial growth plates ([Rubin et al. 2021](#)) and *Drosophila* epithelial sheets ([Heller et al. 2016](#), [Stern et al. 2022](#)) and have shown that the differential expression of several cell behaviours, such as cell volume, orientation, proliferation and intercalation, influences morphogenesis ([Heller et al. 2016](#), [Rubin et al. 2021](#), [Stern et al. 2022](#)). Others have directly quantified tissue growth based on cell level data using lineage tracing ([Marcon et al. 2011](#), [Morishita et al. 2015](#), [Suzuki and Morishita 2017](#), [Tozluoglu et al. 2019](#)). Quantitative maps of tissue deformation coupling growth rates and anisotropy were obtained for chick and mouse limb bud elongation ([Marcon et al. 2011](#), [Morishita et al. 2015](#), [Suzuki and Morishita 2017](#)) and in the *Drosophila* wing disc ([Tozluoglu et al. 2019](#)). These studies showed that spatially and temporally heterogeneous growth patterns as well as growth anisotropy are key drivers of tissue morphogenesis in these model systems. In the joint, the emergence of different shapes has been explored using mechanobiological simulations which have highlighted the role of mechanical stimuli in driving joint growth ([Shefelbine and Carter 2004b](#), [Giorgi](#)

et al. 2014). These simulations of joint morphogenesis integrated simplified and extrapolated cell data which poorly represented the complex intertwinement of cell activities. In order to accurately simulate joint growth, in a way which faithfully models the biological mechanisms at play, cell-level activities need to be accurately characterised and combined in growth simulations.

In this research, I quantified key cellular dynamics involved in growth and morphogenesis in the zebrafish jaw joint and synthesised them in a computational simulation of joint development. Cells in larval zebrafish jaw joints labelled with cartilage markers were tracked over a forty-eight hour time window using confocal imaging. Changes in the relative positions between adjacent cell centroids, resulting from cell rearrangement, volume expansion and extracellular matrix deposition, were measured and used to calculate the rates and directions of local tissue deformations. I observed spatially and temporally heterogeneous growth patterns with marked anisotropy over the developmental period assessed. Synthesising the dynamic cell data into a finite element model of jaw joint development resulted in accurate simulated shapes demonstrating that zebrafish jaw joint growth can be reasonably approximated based on cell positional information over time. I also used the model to test whether growth characteristics—heterogeneity or orientation—are dominant influences on jaw joint morphogenesis.

3.2. Methods

3.2.1. Study design

In this research, the main objective was to quantify morphogenesis of the larval zebrafish jaw joint based on cell-level data over a two-day time window. Research methodology was as follow and is shown in Figure 3.1. Confocal images at the level of the joint were obtained in live zebrafish expressing cartilage markers at twelve-hour intervals from 3.5 day post fertilisation (dpf) to 5.5 dpf (Figure 3.1.1). Cells in the confocal image stacks were segmented (Figure 3.1.2) and tracked in successive timepoints (Figure 3.1.3). The relative position of cell centroids in successive timepoints was used to calculate growth in cubic regions of interests (ROIs) (Figure 3.1.4). The obtained growth maps were integrated into finite element (FE) models (Figure 3.1.5) to simulate growth in each twelve-hour time intervals (Figure 3.1.7). The objective when simulating growth was to determine whether the necessary drivers for shape determination were captured with the growth calculations. The initial shapes of the FE models were obtained from averaging the shapes of all fish jaws at each timepoint (Figure 3.1.6). Finally, the relative importance of two characteristics of growth (spatial heterogeneity and anisotropy) were assessed by suppressing each of these features in growth simulations (Figure 3.1.8).

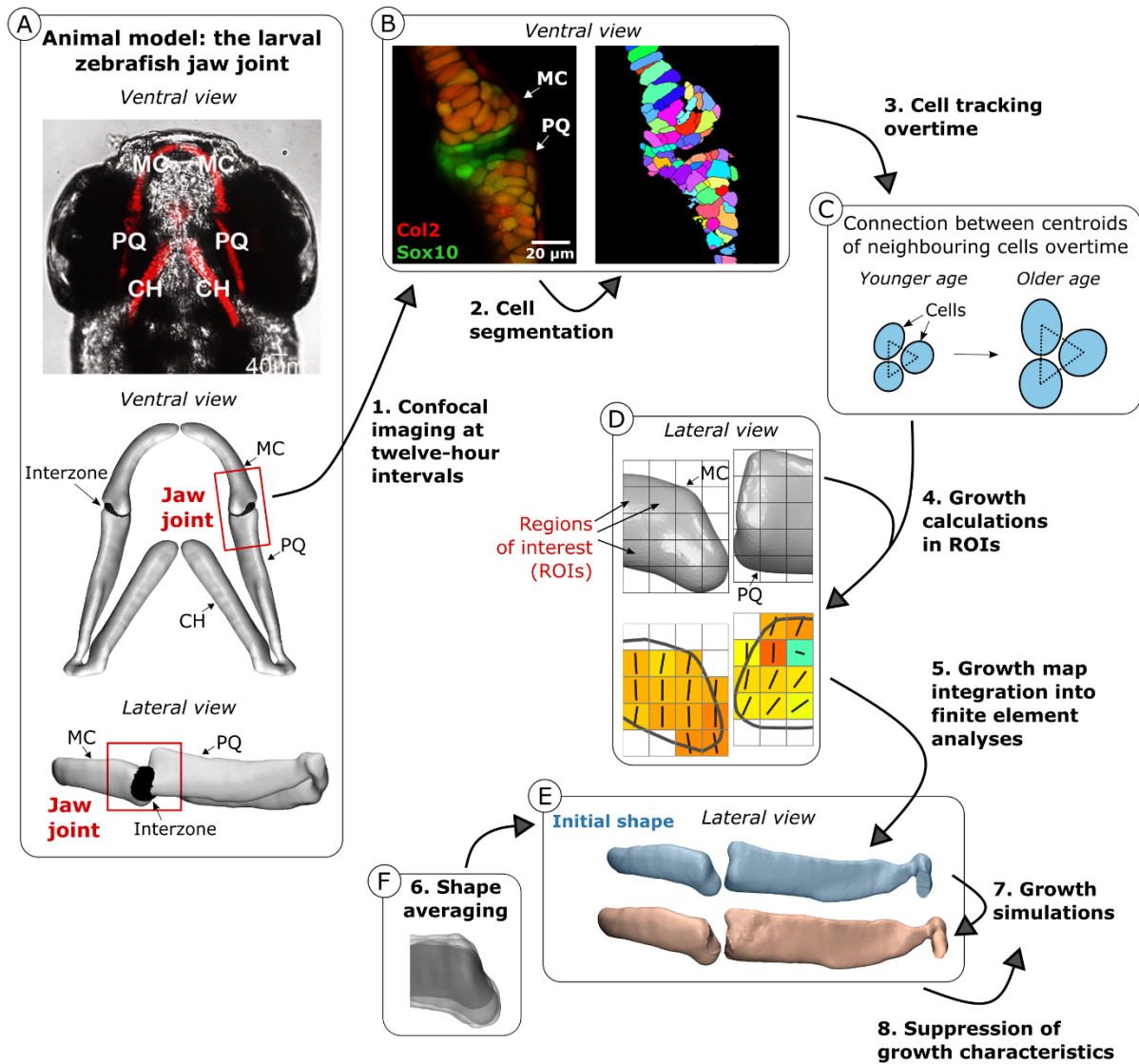


Figure 3.1: **Quantifying morphogenesis in the larval zebrafish jaw joint.** The main steps of research methodology are numbered. A) Brightfield ventral view of 5 dpf zebrafish expressing *Tg(Col2a1aBAC:mCherry)* cartilage marker (from (Brunt et al. 2015)) along with ventral and lateral views of 3D reconstructions of the lower jaw. The joint is marked with a red box. CH: ceratohyal, MC: Meckel's cartilage, PQ: palatoquadrate. B) Confocal ventral view of the jaw joint of a live 4.5 dpf zebrafish expressing the transgenic reporters *Col2a1aBAC:mcherry* (red) and *-4.9sox10:eGFP* (green) which mark cartilage chondrocytes. Cells in the Meckel's cartilage (MC) and the palatoquadrate (PQ) were segmented. C) 2D scheme of cell connectivity overtime. D) Top panel: Lateral view of a 3D reconstruction of the jaw joint showing the cubic regions of interest (ROIs) in which growth calculations were performed. Lower panel: Growth map example in which growth rates are represented by the squares' colours while the direction of growth is shown by solid black lines in the corresponding squares. E) An example of growth simulations using finite element (FE) methods in which the simulated shape (orange) resulted from integration of the calculated growth maps. F) Initial FE shapes (blue in E) were generated from averaging several zebrafish jaw shapes.

3.2.2. Zebrafish husbandry/Zebrafish lines

Fish were maintained as described in Aleström (2020). All experiments were approved by the local ethics committee (Bristol AWERB) and performed under a UK Home Office Project Licence. Transgenic lines *Tg(col2a1aBAC:mCherry)* (Mitchell et al. 2013) and *Tg(-4.9sox10:eGFP)* (Carney et al. 2006) have been previously described. These transgenic markers allow expression of fluorescent reporters for the immature chondrocytes in the interzone (sox10-positive and col2-negative) and the mature chondrocytes (positive for both sox10 and col2). Both reporters are expressed in the cytoplasm and as such label the whole cell. Fish husbandry was performed at University of Bristol with the help of Mat Green.

3.2.3. Characterising growth from cell-level data in zebrafish jaw joints

3.2.3.1. Zebrafish jaw joint live imaging

Ten jaw joints from double transgenic *Tg(col2a1aBAC:mCherry; -4.9sox10:eGFP)* larvae were imaged at twelve-hour intervals from 3.5 to 5.5 days post fertilisation (dpf) using a Leica SP8 confocal microscope with a temperature-controlled chamber set to 28°C. Images centred on the joint line, as marked by a red box in Figure 3.2A, were acquired with a 20x HCX PL APO lens at a resolution of 512 x 512 px. Confocal image stacks at twelve-hour intervals in which cells from the anterior Meckel's cartilage (MC) joint element express the cartilage markers are shown in Figure 3.2B. Prior to imaging, larvae were anaesthetised in 0.1 mg ml⁻¹ tricaine methanesulphonate (MS222) and mounted in a ventral orientation in warm 1% low melting point (LMP) agarose. Following imaging, the larvae were flushed from the agarose using Danieau's buffer, allowed to resume normal movement, and kept in separate wells of a 24-well plate between imaging timepoints. Imaging was performed by Dr. Elizabeth A. Lawrence and Mengdi Wang at University of Bristol.

3.2.3.2. Cell segmentation and tracking

Consecutive image stacks with *sox10:eGFP* chondrocyte marker were filtered in Fiji (Schindelin et al. 2012). 3D Fast Filters-OpenGray, 3D Edge and Symmetry and 3D Morphological filters from the 3D ImageJ Suite plugin (Ollion et al. 2013) were applied in this order with the parameters supplied in Table 3.1. An example of processed stack is shown in Figure 3.2C. Once filtered, morphological segmentation followed by Inertia Ellipsoid filtering using Fiji's MorpholibJ plugin (Legland et al. 2016) were performed to extract the 3D cell centroids' coordinates in the joint at each timepoint. An example of segmented stack with individually labelled cells following morphological segmentation is shown in Figure 3.2C. Segmentation results were then cleaned and used to manually track joint cells between images from two consecutive timepoints using manual labelling in MATLAB (R2018a, The MathWorks, Inc., Natick,

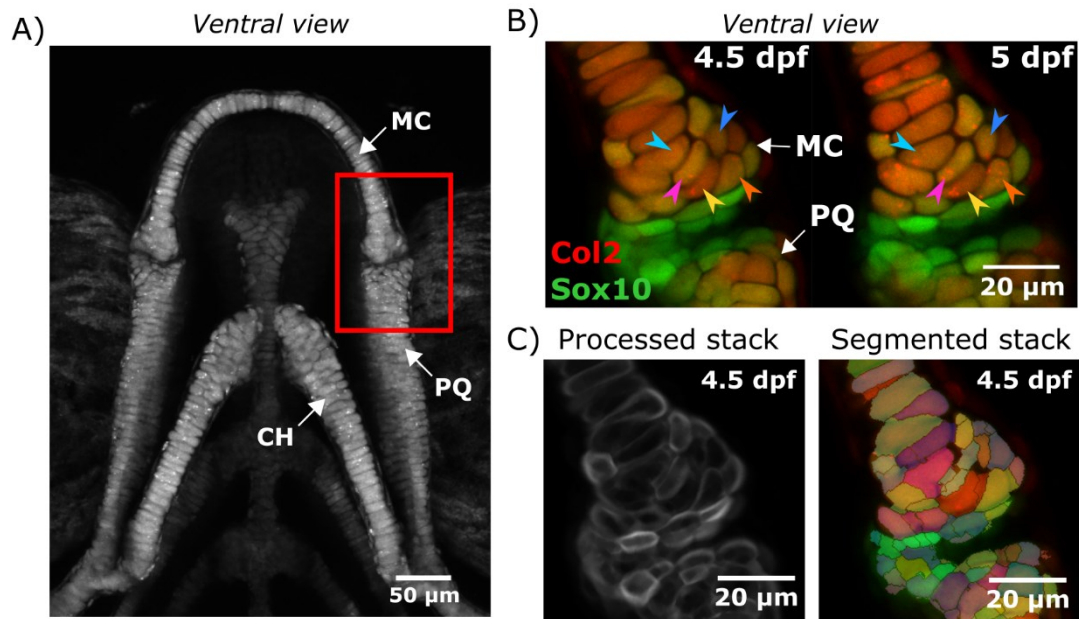


Figure 3.2: Cell segmentation in larval zebrafish jaw joint. A) Maximum projection of ventral confocal image stacks of the jaw from a larval zebrafish aged 5 dpf expressing *Tg(Col2a1aBAC:mcherry)* cartilage marker; red box shows the jaw joint for which morphogenesis is characterised in this study. B) Representative ventral stacks of the anterior jaw joint element of a live specimen aged 4.5 and 5 dpf expressing the transgenic reporters *Col2a1aBAC:mcherry* (red) and *-4.9sox10:eGFP* (green) marking cartilage in which cells can visually be identified over time. A few cells were marked by arrows as examples. Green cells are *sox10*+ve and *col2*-ve and therefore less differentiated than the more mature yellow cells co-expressing the two transgenes and which form the cartilaginous joint elements. C) Processed and segmented stack of the same representative specimen at 4.5 dpf. The processed stack is obtained after 3D Fast Filters-OpenGray, 3D Edge and Symmetry and 3D Morphological filters are applied to the original confocal stacks. In the segmented stack (after morphological segmentation was applied to the processed stack), cells are labelled and shown with different colours. CH: ceratohyal, MC: Meckel's cartilage, PQ: palatoquadrate.

Filters	Parameters
3D Fast Filters-OpenGray	Isotropic radius: 2 pixels
3D Edge and Symmetry	Canny: 0.6
3D Morphological Filter	Operation: closing
	Element shape: diamond
	Isotropic radius: 2 pixels

Table 3.1: Filters applied to larval zebrafish jaw joint image stacks before cell segmentation

Massachusetts, United States). An example of how cells were visually identifiable over time is shown in Figure 3.2B with arrowheads. Cells which were *col2*-negative (showing up as green in Figure 3.2B) were considered part of the interzone and not tracked. Due to image resolution and segmentation quality some image stacks were discarded from the analysis, and the final sample numbers per timepoint are detailed in Figure 3.8. At each timepoint, cells in the joint were counted to assess proliferation, and the volume occupied by the tracked cells was calculated to assess cell volume expansion.

3.2.3.3. Growth maps calculations

For each twelve-hour interval time window (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf), joint shapes were extracted from the consecutive image stacks with *col2a1:mcherry* chondrocyte marker in Mimics (Materialise NV, Leuven, Belgium) and aligned in 3-matic (Materialise NV, Leuven, Belgium). Any transformation which was applied to the joint shapes in 3-matic was consistently applied to the corresponding centroids in MATLAB. A cubic grid of side length fifteen microns was superimposed on the aligned joints to divide them into regions of interest (ROIs) as shown in Figure 3.3A. For each ROI, cells within the ROI's limits were detected and their adjoining cells were listed. Vectors linking the centroids of adjacent cells were created as shown in Figure 3.3B. The relative position of cell centroids in an ROI at a timepoint t forms a pattern which can be described by a texture matrix M calculated from the link vectors l connecting neighbouring cell centroids (equation 3.1) (Figure 3.3B) (Graner et al. 2008).

$$M = \langle l \otimes l \rangle \quad (3.1)$$

where l is a link vector connecting two neighbouring cells, $\langle \dots \rangle$ denotes averaging taking all links within an ROI into consideration, $\otimes$ is the outer (or tensor) product, M is the texture matrix within an ROI at time point t , in a fish. M has the dimension of $[m^2]$ and, in 3D, is a 3x3 matrix.

Over time the link vectors l may change in length and direction, changing the pattern's overall geometry (Figure 3.3B). Graner et al. (2008) defined the “statistical symmetrised velocity gradient” (matrix V) as a tool to describe at which rate and in which direction the pattern of the tissue deforms between consecutive timepoints t and $t+\Delta t$. This gradient quantifies local tissue distortions, such that if cells within an ROI grow or intercalate, or if extracellular matrix is built, the distance between cell centroids, and therefore the geometry of the tissue, change (Figure 3.3B). V , set by Graner et al (2008), writes as follows (equation 3.2):

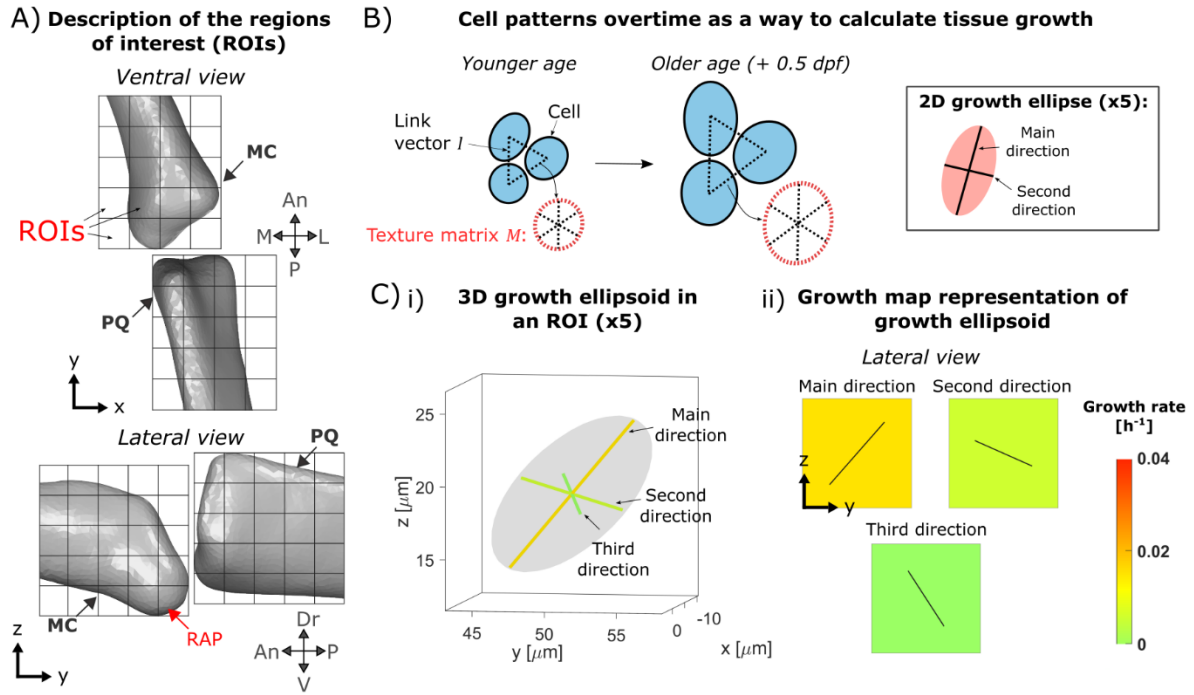


Figure 3.3: **Growth rate calculations.** A) A grid marks out the regions (ROIs) of the anterior MC and posterior PQ joint elements in which growth is characterised. Each cube side length is $15\mu m$. B) The position of cells with respect to each other forms a pattern described by a texture matrix M . This pattern evolves overtime which enables local growth characterisation. C) i) The growth rate calculated for each ROI is represented by an ellipsoid with orthogonal axes. ii) The ellipsoid's radii and the orientation of its axes are used to generate a growth map for each of the ellipsoid's radii in the lateral plane; growth rate is represented by the square's colour while the direction of growth is shown by solid black lines in the corresponding square. MC: Meckel's cartilage, PQ: Palatoquadrate, CH: ceratohyal, RAP: retroarticular process, An: Anterior, P: Posterior, L: Lateral, M: Medial, Dr: Dorsal, V: Ventral.

$$V = \frac{W + W^T}{2} = \frac{M^{-1}C + C^T M^{-1}}{2} \quad (3.2)$$

where T denotes matrix transposition and intermediate steps W and C write as:

$$C = \langle l \otimes \frac{dl}{dt} \rangle \quad (3.3)$$

$$W = M^{-1}C = \langle l \otimes l \rangle^{-1} \langle l \otimes \frac{dl}{dt} \rangle \quad (3.4)$$

where M is the texture matrix within an ROI, l is a link vector connecting two neighbouring cells, $\frac{dl}{dt}$ is the time derivative of a link vector l , $\langle \dots \rangle$ denotes averaging taking all links within an ROI into consideration, $\otimes$ is the outer (or tensor) product.

V has the dimension of a strain rate [s^{-1}] and is a statistical measurement of tissue changes based on discrete objects. In continuum mechanics, V is an equivalent to the velocity gradient's symmetrical part $\dot{\epsilon}$, that is, the strain rate (Graner et al. 2008), as explained below:

$$\dot{\epsilon} = \frac{\nabla v + \nabla v^T}{2} \quad (3.5)$$

where the velocity gradient ∇v is the spatial derivative of the velocity field, and ∇v^T is its transposed.

Under the affine assumption where the velocity field is considered continuous and linearly varying with position (Figure 3.4), consider a link $l = r_2 - r_1$ (3.6) which connects two objects of coordinates r_1 and r_2 . Its time derivative is:

$$\frac{dl}{dt} = v(r_2) - v(r_1) \quad (3.7)$$

where $v(\dots)$ is the velocity of each individual object (Graner et al. 2008). Under the affine assumption we have:

$$v(r_2) \sim v(r_1) + \nabla v^T \cdot (r_2 - r_1) \quad (3.8)$$

that is

$$\frac{dl}{dt} \sim \nabla v^T \cdot l \quad (3.9)$$

where ∇v^T is the transposed of the velocity gradient ∇v , l is a link vector connecting two objects of coordinates r_1 and r_2 , $v(\dots)$ is the velocity of each individual object (Graner et al. 2008).

From equation 3.3, the transposed of the matrix C is $C^T = \langle \frac{dl}{dt} \otimes l \rangle$ (10) (Graner et al. 2008).

Therefore, under the affine assumption, using equation 3.9:

$$C^T \sim \langle (\nabla v^T \cdot l) \otimes l \rangle \quad (3.11)$$

that is

$$C \sim M \nabla v \quad (3.12)$$

where ∇v is the velocity gradient, ∇v^T is transposed, M is the texture matrix within an ROI, l is a link vector, $\langle \dots \rangle$ denotes averaging taking all links within an ROI into consideration, $\otimes$ is the outer (or tensor) product (Graner et al. 2008).

$W = M^{-1}C$ (equation 3.4) is then a statistical equivalent of the velocity gradient ∇v in a discrete description, and V (equation 3.2) is a statistical equivalent to the strain rate (equation 3.5) (Graner et

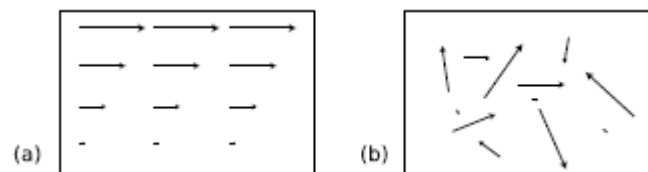


Figure 3.4: Affine (a) and non-affine (b) flow fields. From (Graner et al. 2008)

al. 2008). In each ROI, the gradient matrix V can be represented by an ellipsoid with orthogonal axes by computing the eigenvalues and eigenvectors of V , as illustrated in Figure 3.3Ci. The orientation of the axes (eigenvectors) and their associated radii (eigenvalues) correspond to the direction and rate of local tissue geometry deformation respectively. Maps of local strain rates (units $[s^{-1}]$) with the associated directions of deformation (main, second and third directions for growth, corresponding to the major, medium and minor axes of the ellipsoid respectively) were generated from each of the three ellipsoid's axes as shown in Figure 3.3Cii. These maps are referred to hereafter as growth maps. Growth maps were calculated for each fish, at each time window. Statistical tests were performed to identify significant differences ($p < 0.05$) between the mean growth rates in each direction for growth in all time windows. Kolmogorov-Smirnov test for normality revealed that groups were not normally distributed (Appendix 1.1). Friedman test followed by Wilcoxon signed rank test for paired comparisons with Bonferroni adjustments were performed. For each time point, growth maps were averaged across fish to obtain a unique growth map for each direction for growth. Within each ROI, strain rates that lay outside the interquartile range were removed from the averaging.

3.2.4. Simulating zebrafish jaw growth with a finite element model

3.2.4.1. Shape generation

Confocal image stacks of four to five larval zebrafish lower jaws (encapsulating the Meckel's cartilage, the palatoquadrate and the ceratohyal, Figure 3.2A) from the transgenic line *Tg(col2a1aBAC:mCherry)* were taken with a Leica SP8 confocal microscope at the "endpoints" of each time window (3.5, 4, 4.5, 5 and 5.5 dpf) using the methodology described above. Imaging was performed by Dr. Elizabeth A. Lawrence and Mengdi Wang. A 3D Gaussian grey filter with isotropic radius 3.0 pixels was applied to the image stacks in Fiji. These were imported in Mimics to be segmented and the resulting 3D surfaces were aligned. Only half-jaws (separated at the level of the midsagittal plane) were segmented, as shown in Figure 3.5A(i). The half-jaws were imported into MATLAB and were divided into slices in the transversal plane as shown in Figure 3.5A(i). For each slice, a shape outline was obtained for each specimen from the shape vertices and an average outline was generated as shown in Figure 3.5A(ii-iii). Averaged shape outlines were saved as image stacks and imported into Mimics where the resultant average half-jaw shape was generated. Also in Mimics, the interzone was added as a volume filling the gap between the two joint elements using Boolean operations, with the interzone's external boundaries approximated based on imaging data (Brunt et al. 2016a). Finally, a non-manifold assembly combining the half-jaw and the interzone was generated as shown in Figure 3.5B(i). In 3-matic, the non-manifold assembly was meshed with ten node tetrahedral elements and exported to Abaqus CAE (Dassault Systemes, 2019) where a model for each 12-hour time window was created.

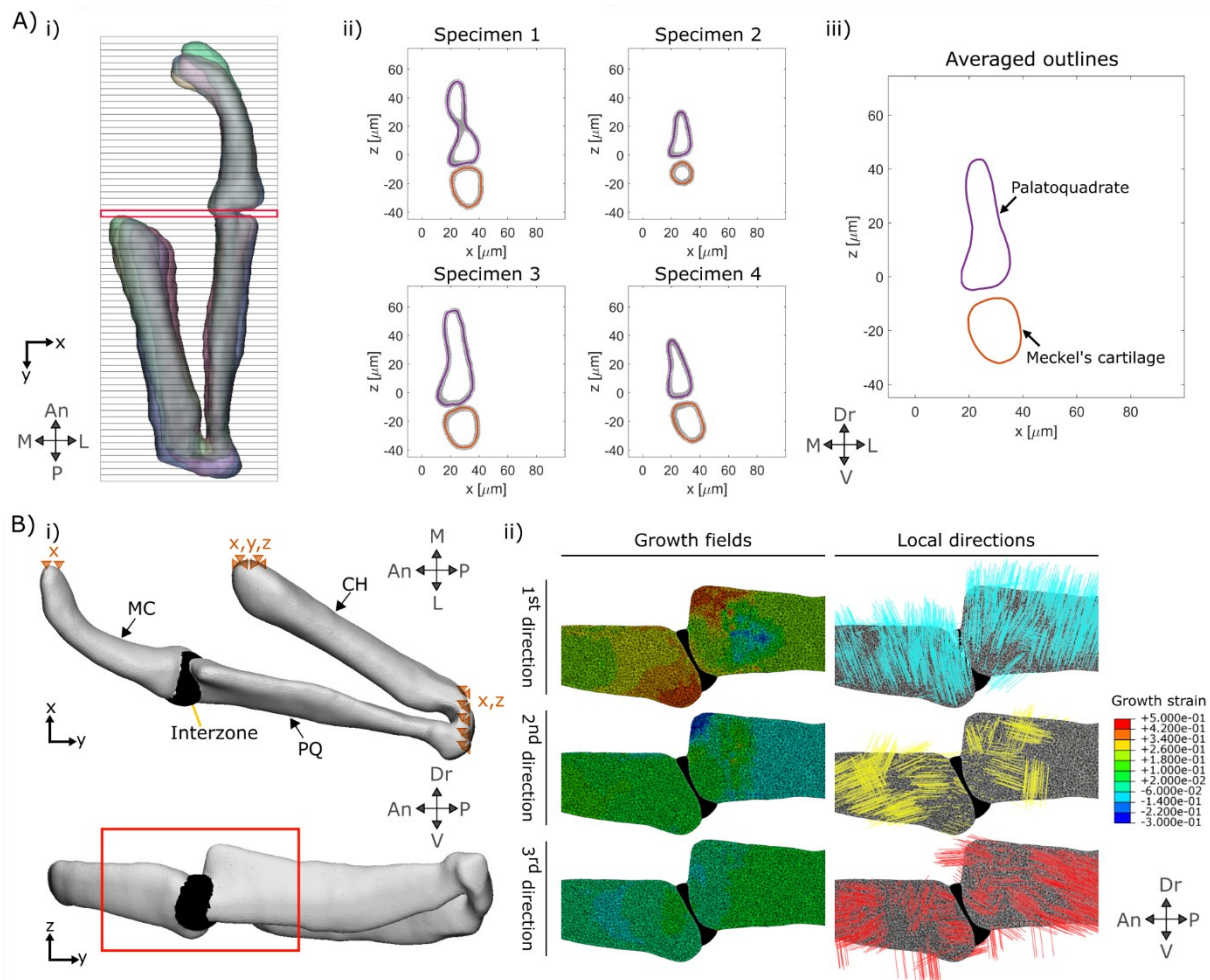


Figure 3.5: **Integration of the growth maps in a finite element model.** A) The first step in constructing my FE model is to obtain an average geometry for each timepoint (3.5, 4, 4.5, 5 and 5.5 dpf). For each timepoint, half jaw shapes are aligned and sliced transversally (i). For each slice, the shape outlines of each sample (four here) are obtained (ii) then averaged (iii). The slide marked in red in (i) is shown as an example in (ii) and (iii). B) i) An FE model is generated based on the averaged shape outlines; the joint interzone is added and the areas marked with dashed triangles are constrained in the specified dimensions (e.g., x). ii) Section of the joint in the lateral plane showing the growth fields which are applied to the model along with their associated directions. The view is marked by a red box in (i). MC: Meckel's cartilage, PQ: Palatoquadrate, CH: ceratohyal, An: Anterior, P: Posterior, L: Lateral, M: Medial, Dr: Dorsal, V: Ventral.

3.2.4.2. Material properties and boundary conditions

All cartilaginous regions (Meckel's cartilage (MC), palatoquadrate (PQ) and ceratohyal) were assigned homogeneous isotropic elastic material properties with Young's Modulus (YM) 54.8 kPa based on nano-indentation measurements taken on 5 dpf wild type zebrafish jaw joints (Lawrence et al. 2021). Poisson's ratio was assigned 0.3 based on published estimations of unmineralized cartilage material properties of mouse embryos (Tanck et al. 2000) and in accordance with previously published FE

models of the larval zebrafish jaw (Lawrence et al. 2021). The interzone was assigned isotropic elastic material properties with Poisson's ratio 0.3 and YM set at 0.25% of the cartilaginous YM based on Brunt et al.'s original study where this ratio between the two YM was found to facilitate physiological jaw displacements when muscle loading was applied (Brunt et al. 2015). The ceratohyal does not form part of the region of the jaw joint of interest (Figure 3.2A), but was needed for implementing physiological boundary conditions. The following boundary conditions were applied, as illustrated in Figure 3.5B(i). The anterior end of the ceratohyal was fixed in all directions and translations in the lateromedial direction of the anterior end of the Meckel's cartilage were prevented to maintain the symmetry with the missing half-jaw. Only anteroposterior translations of the posterior end of the palatoquadrate, where the lower jaw connects to the rest of the craniofacial skeleton, were allowed.

3.2.4.3. Growth maps integration

For each 12-hour period, strains derived from the growth maps were imported into Abaqus CAE as three distinct analytical mapped fields and applied to the model. The coordinates of the ROI centres were assigned the calculated strains and interpolation was performed between ROI centres to assign strains to each element lying within the ROIs' limits. Local material orientations matching the local directions for growth were assigned to the joint elements. Elements whose nodes' coordinates were contained within an ROI were all assigned the directions for growth of this ROI. Direction 1 is the main direction for growth (corresponding to the major axis of the statistical velocity gradient's ellipsoid), direction 2 is the second direction for growth (median axis) and direction 3 is the third direction for growth (minor axis). These directions differed from ROI to ROI. As an example, growth fields and their associated directions at the level of the joint for time window 4–4.5 dpf are shown in Figure 3.5B(ii). MC and PQ hypertrophic regions were not visible in the cell tracking data, but were included in the FE model of the half-jaw. For these hypertrophic regions, growth rates were set to the average of those of the adjacent proliferative cartilage (a 30 μm depth was considered). In the PQ hypertrophic cartilage, the material orientation of the adjacent proliferative region was used throughout. In the MC hypertrophic region, cell orientation varies along the length of the rudiment as shown in Figure 3.2A. Discrete measurements of cell orientation were taken in Fiji to which a linear regression of cell orientation with respect to joint line was fitted. Material orientations in the MC hypertrophic regions were therefore set as the material orientations of the adjacent cartilage accordingly rotated based on the linear regression. The Abaqus user subroutine UEXPAN was used to apply spatially varying expansion based on the strain fields along the corresponding material orientations to simulate growth for each time-window. Mesh convergence study was conducted on a representative time window showing that the chosen mesh was appropriate when modelling growth using the methodology described in this Chapter (Appendix 1.2).

3.2.4.4. Quantification of simulation performance

The simulated shapes were imported into 3-matic where they were aligned with the average jaw shapes of each of the “endpoints” of each time window. For each time-window, the initial shape is the shape average of the original samples at younger age (start point of the time windows), and the comparison shape is the shape average of the original samples at older age (end point of the time window). Views in the lateral and the ventral planes were exported to Fiji where shape outlines were extracted, and the following shape features were measured: anterior MC length, depth and width and posterior PQ length and depth, as shown in Figure 3.6. To assess the quality of the simulation for each shape feature, a percentage match of change was calculated as a) the difference between the simulated shape measurement and the initial shape measurement divided by b) the difference between the comparison shape measurement and the initial shape measurement. The following scores were then assigned based on the percentage match:

- less than 10% match: no simulated growth
- between 10% and 70%: undergrowth
- between 70% and 130%: accurate growth
- above 130%: overgrowth

3.2.4.5. Quantification of the relative roles of growth characteristics

To quantify the relative importance of growth heterogeneity versus growth direction, simulations were conducted in which each of these features was removed or kept constant. Spatial growth heterogeneity was removed in both the MC and PQ by averaging the growth ellipsoids, within the set of ROIs in each rudiment, at each time window. In each rudiment, the average growth ellipsoid was used to generate homogeneous growth maps along the three directions for growth (corresponding to the ellipsoid's axes) and applied to the model throughout the joint and hypertrophic regions. Orientation in the MC hypertrophic region was still adapted along its length. To remove the role of orientation, isotropic growth was used. Within each ROI in both the joint and hypertrophic regions, an average growth rate corresponding to the average of the three growth ellipsoids' radii was obtained and applied to the ROI. In other words, ROIs growth ellipsoids became spheres. The resultant shapes when either growth heterogeneity or growth direction were removed were compared to the “full” simulation and with each other.

Raw, filtered, and segmented confocal image stacks, along with MATLAB codes for cell tracking, growth rate calculations and shape averaging, Abaqus CAE models and real and simulated shapes are available at <https://doi.org/10.5281/zenodo.5769854>.

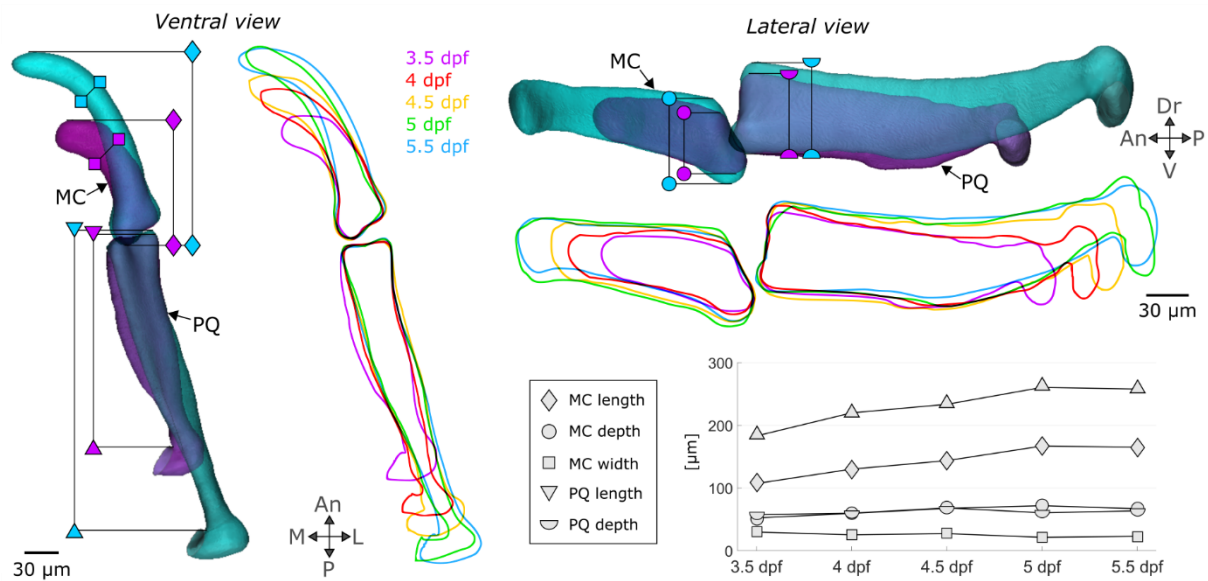


Figure 3.6: **Shape changes between 3.5 and 5.5 dpf in zebrafish jaws.** Superimposed 3.5 (purple) and 5.5 (turquoise) dpf 3D average shapes and 3.5 to 5.5 dpf average shape outlines in the ventral and lateral planes. The shape features which are observed to change as the jaw develops are marked with specific symbols (diamond: MC length, square: MC width, circle: MC depth, triangle: PQ length, semi-circle: PQ depth). MC: Meckel's cartilage, PQ: palatoquadrate, An: Anterior, P: Posterior, L: Lateral, M: Medial, Dr: Dorsal, V: Ventral.

3.3. Results

3.3.1. Growth in the zebrafish jaw joint exhibits spatial and temporal heterogeneity as well as marked anisotropy

Comparing shape feature measurements between 3.5 and 5.5 dpf revealed an overall volume expansion over time with a marked increase in Meckel's cartilage (MC) and palatoquadrate (PQ) length (Figure 3.6: diamond and triangle), a slight increase in MC and PQ depth (Figure 3.6: circle and semi-circle) and a slight contraction of MC width (Figure 3.6: square). In the anterior MC joint element, growth rates in the main direction for growth varied between time windows from 3.5 to 5.5 dpf. Growth rates in the main direction for growth for each fish and for each region of interests (ROIs) were computed showing significant growth rate increases ($p < 0.05$) between each time-windows from 3.5–4 to 4.5–5 dpf as shown in Figure 3.7A. Growth rates from 5–5.5 dpf were significantly lower than from 4.5–5 dpf but still significantly greater than from 4–4.5 dpf (Figure 3.7A). High sample variability was observed from 3.5–4 dpf due to the experimental challenges of imaging the youngest zebrafish larval jaw joints and a degree of natural variation in the onset and number of jaw movements (and therefore mechanical stimuli) at earlier stages. A negative average growth rate, meaning tissue contraction,

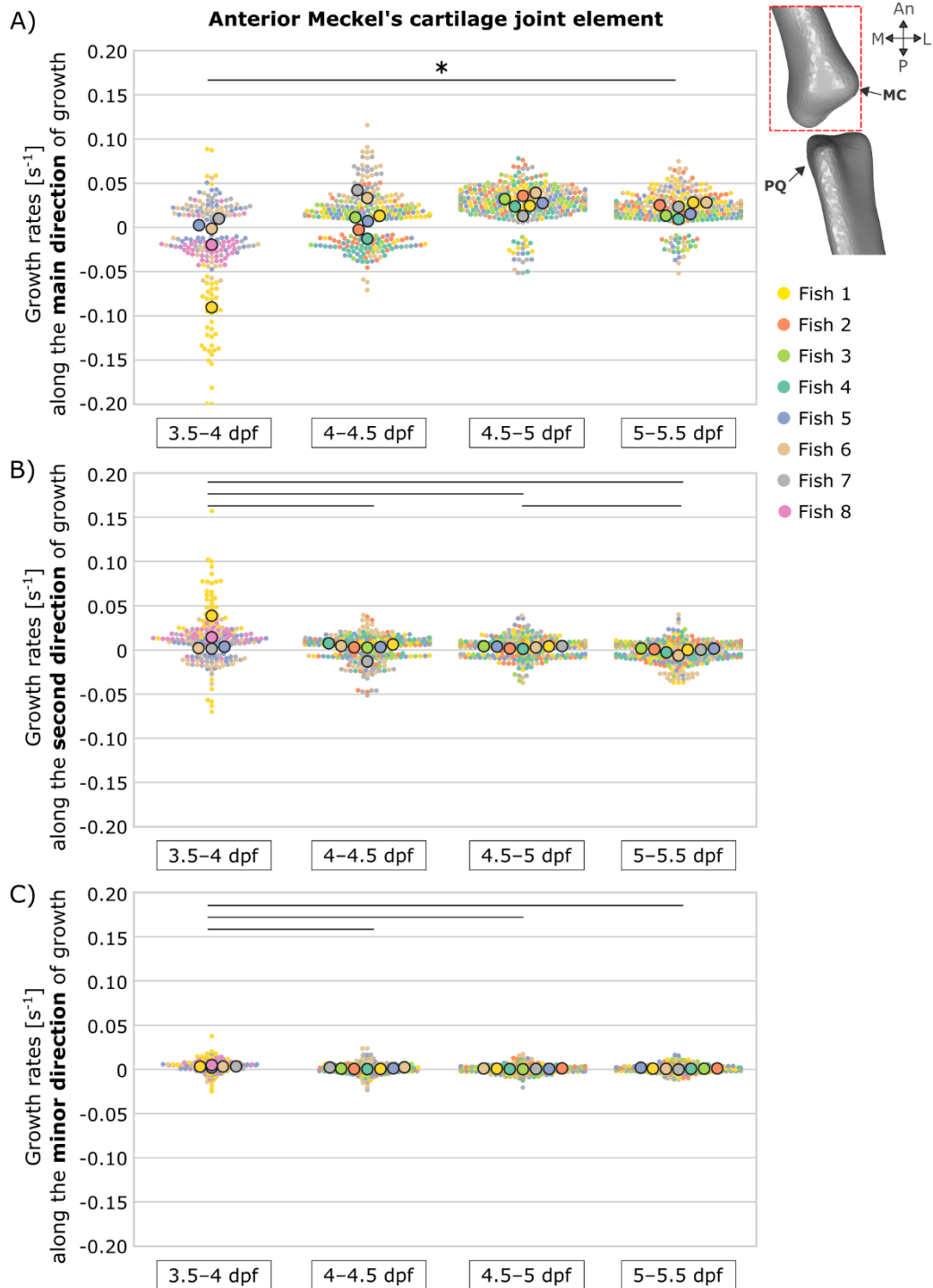


Figure 3.7: **Growth in the anterior Meckel's cartilage joint element vary in different time-windows and exhibits anisotropy from 3.5–5.5 dpf.** (A–C) Growth rates along the three axes of growth ellipsoids (major (A), medium (B) and minor (C)) for all time-windows (3.5–4 dpf, 4–4.5, 4.5–5 and 5–5.5). Each colour represents dataset from a specific fish. Each dot represents dataset for one ROI and for one fish. Large coloured dots represent the mean across ROIs for each fish. Solid bars indicate significant difference between means ($p < 0.05$). * indicates significant differences between all groups ($p < 0.05$). Top right shows from which joint element the datasets were collected (red dashed box) (unscaled). An: Anterior; L: Lateral; M: Medial; MC: Meckel's cartilage; P: Posterior; PQ: Palatoquadrate.

does not seem physiological at this developmental stage, was calculated from 3.5–4 dpf possibly as a consequence of sample variability with fish 1 (yellow in Figure 3.7A) creating a bias towards negative growth rates. A spatial map of average growth rates and directions across fish showed elevated growth rates in the main direction at the retroarticular process from 4–4.5 and 4.5–5 dpf as shown in Figure 3.8A with black arrowheads. Growth rates along the second and third directions for growth were much lower than those of the main direction as shown in Figure 3.7B, C, demonstrating growth anisotropy in the anterior MC joint element. The mean growth rates along the second direction for growth were for example 81% lower compared to those along the main direction from 4–4.5 dpf and 89% lower from 4.5–5 dpf. In the same time windows, the mean growth rates along the third direction for growth were 91% and 98% lower compared to those along the main direction respectively. Spatial growth maps in the second and third directions for growth are provided in Appendix 1.3. Growth orientations in the anterior MC element exhibited consistent alignment across ROIs; with time, the main direction shifted to align with the ventrodorsal axis from 4.5–5 and 5–5.5 dpf, as shown with solid black lines in Figure 3.8A.

In the posterior PQ element, growth rates along the main direction for growth did not vary between time windows (Figure 3.9A). Sample variability was higher than in the MC element (Figures 3.7A and 3.9A). Similarly to the MC element, growth anisotropy in the posterior PQ element was demonstrated. Growth rates along the second and third directions decreased compared to those along the main direction and were accompanied by a reduction in sample variability (Figure 3.9B, C). The main direction for growth in the posterior PQ element also tended to align with the ventrodorsal axis from 4.5–5 and 5–5.5 dpf as shown with the black lines in Figure 3.9B. Overall, growth rates and orientations in the developing jaw joint changed over the time period studied in both joint elements and elevated growth rates were observed at the retroarticular process of the MC demonstrating spatial and temporal growth heterogeneity. Marked growth anisotropy was observed in both joint elements.

Manual assessment of tracked cells over the time window studied revealed very low proliferation rates in the joint. The percentage of cells which underwent division in the joint over twelve hours was 2.42 ± 1.73 % in the MC and 0.50 ± 0.56 % in the PQ, suggesting that proliferation would only minorly impact joint growth. No intercalation of joint cells was observed over the twelve-hour timeframes during cell tracking (sample cell tracking over time shown in Figure 3.10). The volume occupied by tracked joint cells over the timeframe of interest increased substantially, with a mean relative volume expansion per twelve-hour period of 18.49 ± 20.44 % in the MC and 23.68 ± 23.92 % in the PQ. Because the extracellular matrix (ECM) forms a thin layer between adjacent cells (see Figure 3.10), it could not be accurately segmented and its volume was not directly quantified. However, the interstitial space between adjacent cells was consistently narrow, with no apparent increase over time (see example in

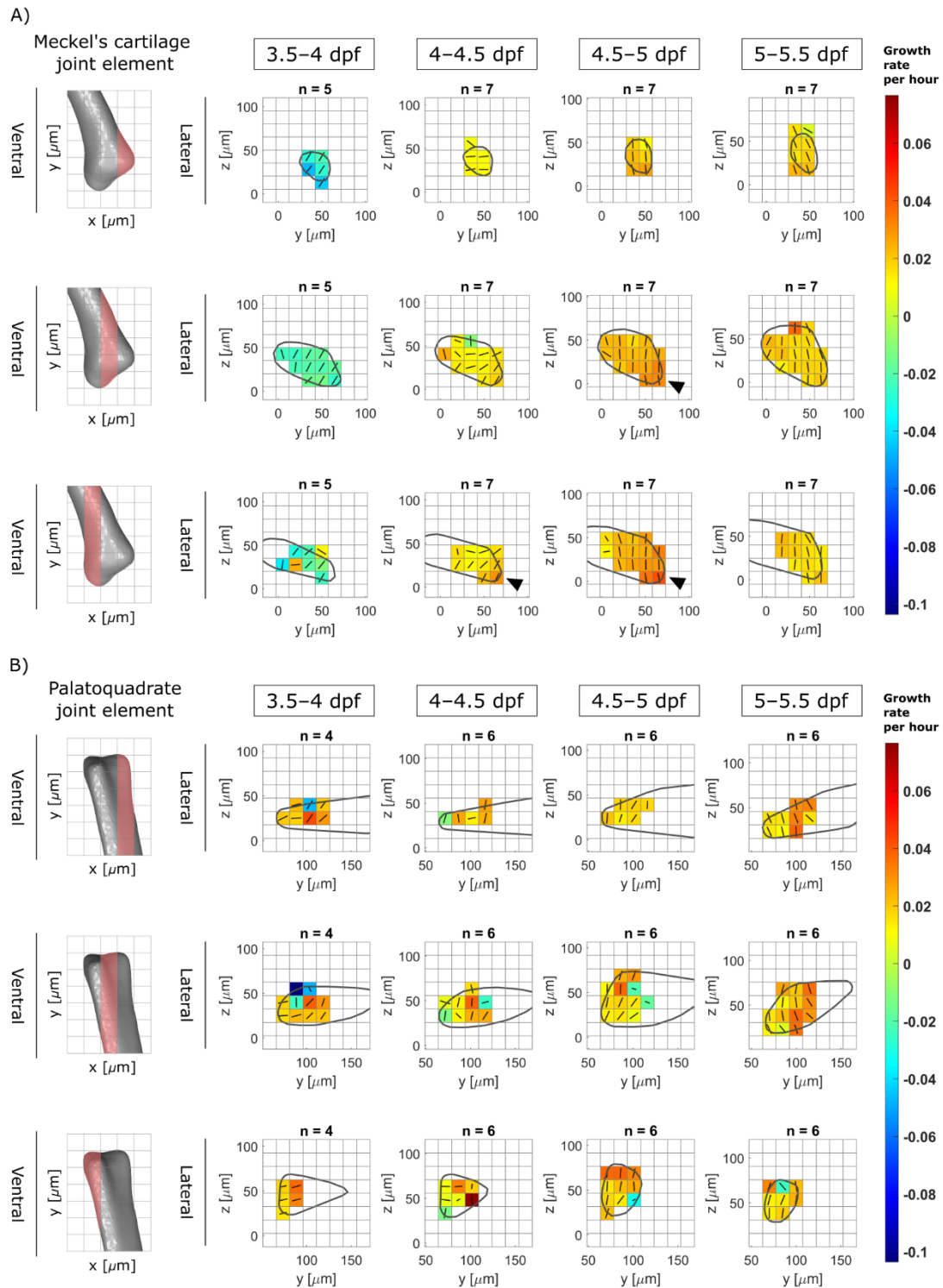


Figure 3.8: **Growth rates from 3.5 to 5.5 dpf in zebrafish jaw joint exhibits spatial and temporal patterns.**

Maps showing growth rates along the main direction for growth (major axis of the ellipsoid) and their associated directions for each time window (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf) in the anterior Meckel's cartilage (A) and posterior Palatoquadrate (B) joint elements in the lateral plane. Growth rates are represented by colours while the direction is shown by solid black lines. Results are displayed across the rudiment's width; views in the ventral plane of each section are displayed on the left panels. Black arrows in (A) show areas of elevated growth rates.

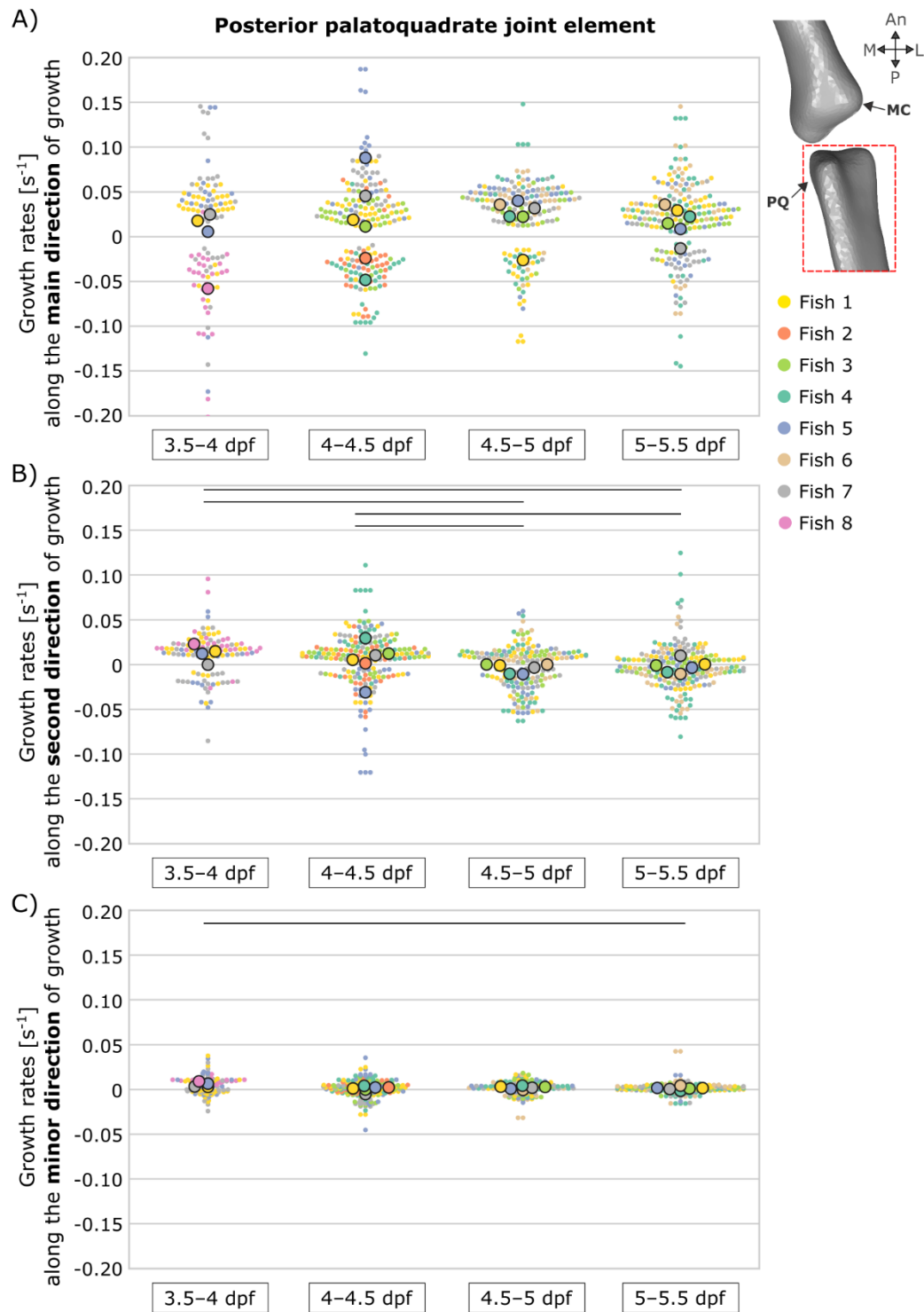


Figure 3.9: **Growth in the posterior palatoquadrate joint element exhibits anisotropy from 3.5–5.5 dpf.**

(A–C) Growth rates along the three axes of growth ellipsoids (major (A), medium (B) and minor (C)) for all time-windows (3.5–4 dpf, 4–4.5, 4.5–5 and 5–5.5). Each colour represents dataset from a specific fish. Each dot represents dataset for one ROI and for one fish. Large coloured dots represent the mean across ROIs for each fish. Solid bars indicate significant difference between means ($p < 0.05$). * indicates significant differences between all groups ($p < 0.05$). Top right shows from which joint element the datasets were collected (red dashed box) (unscaled). An: Anterior; L: Lateral; M: Medial; MC: Meckel's cartilage; P: Posterior; PQ: Palatoquadrate.

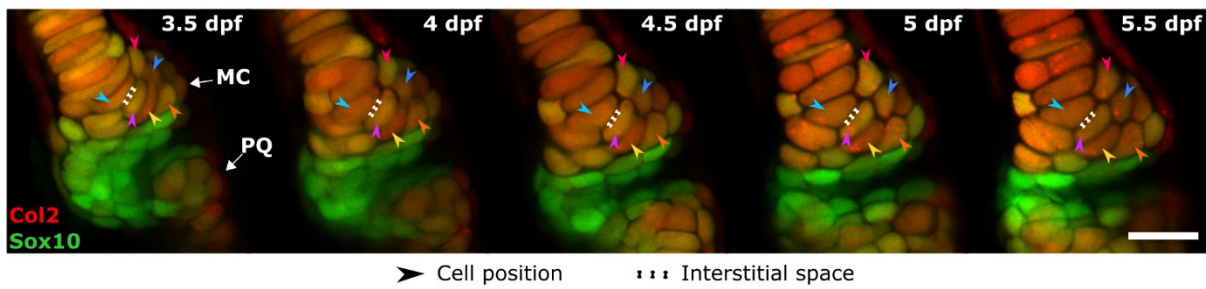


Figure 3.10: Cell intercalation and the extracellular matrix minimally contribute to jaw joint shaping.

Representative ventral stacks of the anterior jaw joint element of a live specimen aged 3.5, 4, 4.5, 5 and 5.5 dpf expressing the transgenic reporters *Col2a1aBAC:mcherry* (red) and *-4.9sox10:eGFP* (green) marking cartilage. Green cells are sox10+ve and col2-ve and therefore less differentiated than the more mature yellow cells co-expressing the two transgenes and which form the cartilaginous joint elements. No observation of cell intercalation is made with the cells being clearly identified over time (three cells marked by arrows as examples). The volume occupied by the interstitial space is minor compared to the volume occupied by cells. Scale bar: 20µm. MC: Meckel's cartilage, PQ: Palatoquadrate.

Figure 3.5). Therefore, my data indicate that increases in joint volume over the studied time window were primarily due to cell volume increases.

3.3.2. Cell positional information over time enables consistent simulations of zebrafish jaw morphogenesis

Growth for each of the time windows was computationally simulated based on the calculated growth maps, to determine whether the necessary drivers for shape determination were captured using cell positional information overtime. The shape features undergoing change between 3.5 and 5.5 dpf were used to assess the quality of the grown simulated shapes. For each time window, most observed shape changes were simulated, either accurately, or with some under- or over-growth, as highlighted with green, yellow and purple (respectively) symbols in Figure 3.11. Length change in both rudiments was accurate from 4–4.5 and 5–5.5 dpf (green triangles and diamonds in Figure 3.11B & D) but undergrowth was observed from 3.5–4 and 4.5–5 dpf (yellow triangle and diamond in Figure 3.11A & C). The change of depth in the lateral plane in both rudiments was mostly simulated (yellow and purple circles and semi-circles in Figure 3.11A, C & D) though only the 4–4.5 simulated shapes accurately matched the shape average of the original samples at older age (comparison shape) (green circle in Figure 3.11B). The decrease of MC width observed from 3.5–4 and 4.5–5 dpf in the ventral plane was not replicated in the growth simulations (red squares, Figure 3.11A & C). Overall, the simulated shapes were close to the staged-match shape average (comparison shape) (Figure 3.11), with the most

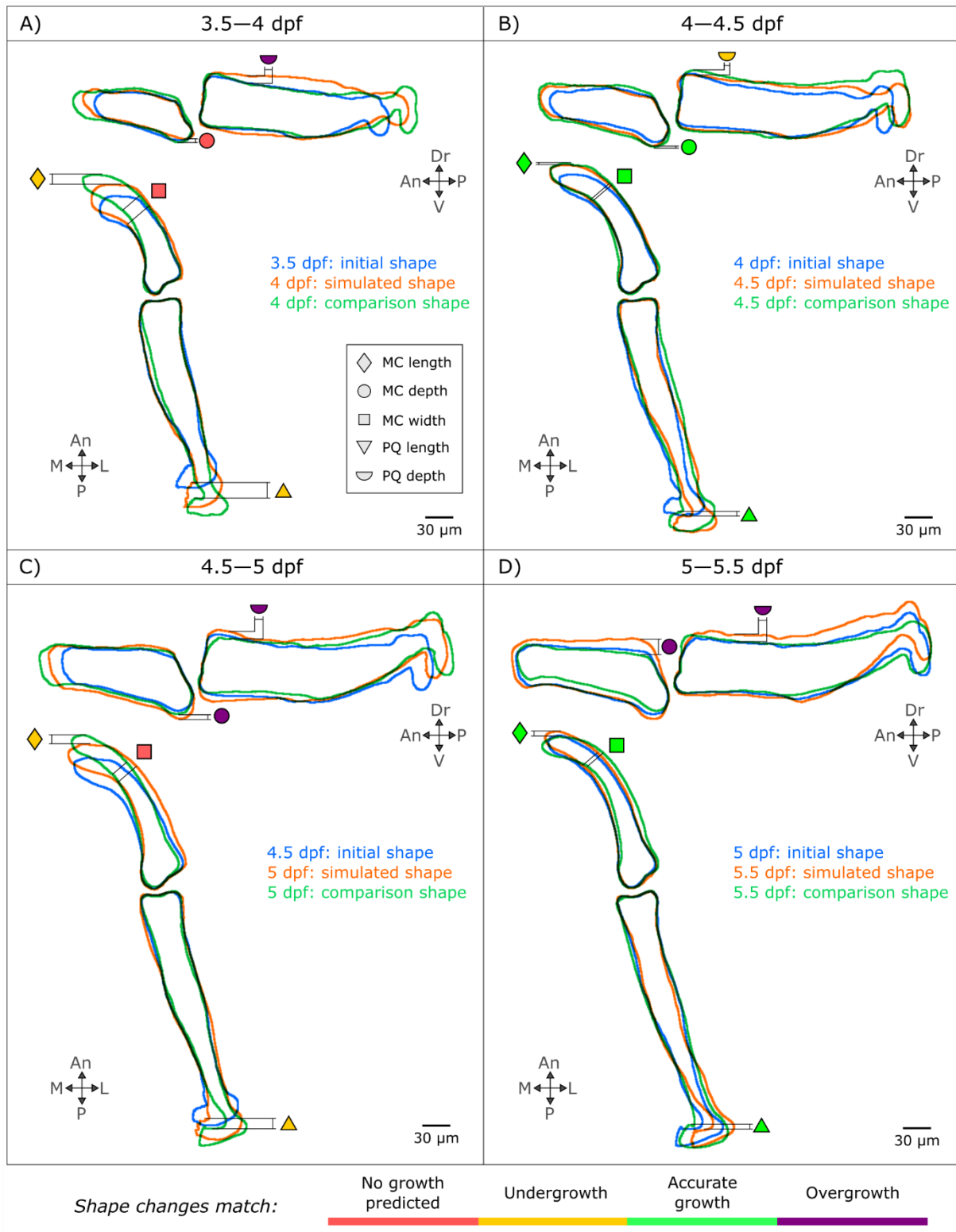


Figure 3.11: The integration of cell-based data in an FE model successfully simulates zebrafish jaw shape changes from 3.5 to 5.5 dpf, with most faithful simulations from 4 to 4.5 dpf. The shape outlines for each time window are superimposed (blue: initial shape (shape average of the original samples at younger age), green: comparison shape (shape average of the original samples at older age), orange: simulated shape) and the shapes features introduced in Figure 3.3 are compared (triangle: Palatoquadrate (PQ) length,

diamond: Meckel's cartilage (MC) length, square: MC width, semi-circle: PQ depth, circle: MC depth) and rated with a colour code explained in the bottom panel (red means no simulated growth, yellow means undergrowth though the pattern of change is correct, green means accurate shape changes and violet means overgrowth though the pattern of change is correct). An: Anterior, P: Posterior, L: Lateral, M: Medial, Dr: Dorsal, V: Ventral.

successfully simulated growth being from 4–4.5 dpf, in which all but one shape feature was scored with a green symbol (Figure 3.11B). The least successful growth simulations occurred from 3.5–4 dpf. In the 3.5–4 dpf timeframe, immature cells in the MC anterior tip rearrange and undergo morphological changes contributing to MC elongation and reduction in width, as shown in Appendix 1.4. Cell rearrangements at the anterior tip were not quantified in this study (due to the primary focus on the synovial jaw joint), which may have contributed to less accurate overall simulated growth. In conclusion, zebrafish jaw joint growth and shape change for the time window modelled can be reasonably approximated based on cell positional information over time, where that cell positional information derives mainly from cell rearrangements and volume expansion.

3.3.3. Growth orientation is more important for zebrafish jaw joint shaping than growth heterogeneity

The importance of growth heterogeneity and orientation was assessed in simulations in which each of these features was either removed or kept constant. Removing growth heterogeneity resulted in only minor shape changes: over the four time-windows, two features exhibited undergrowth compared to the “full” simulations (PQ length from 3.5–4 dpf and MC depth from 5–5.5 dpf as shown in Figure 3.12A). In contrast, when growth orientation was removed, several shape features were markedly altered compared to the “full” simulation. From 3.5–4 dpf, both MC and PQ length under isotropic growth exhibited marked undergrowth as seen in Figure 3.12B. No change was observed from 4–4.5 dpf. MC depth slightly undergrew from 4.5–5 dpf as shown in Figure 3.12B. From 5–5.5 dpf, MC and PQ both length and depth were markedly undergrown as shown in Figure 3.12B. Complete growth simulations for the four time-windows and both adjusted simulation types are provided in Appendix 1.5. These results indicate that growth orientation, and the cellular dynamics likely responsible for it, such as cell orientation and oriented cell division, are crucial to correct morphogenesis. Taken together, my findings suggest that cell volume expansion and orientation dominate joint growth and morphogenesis.

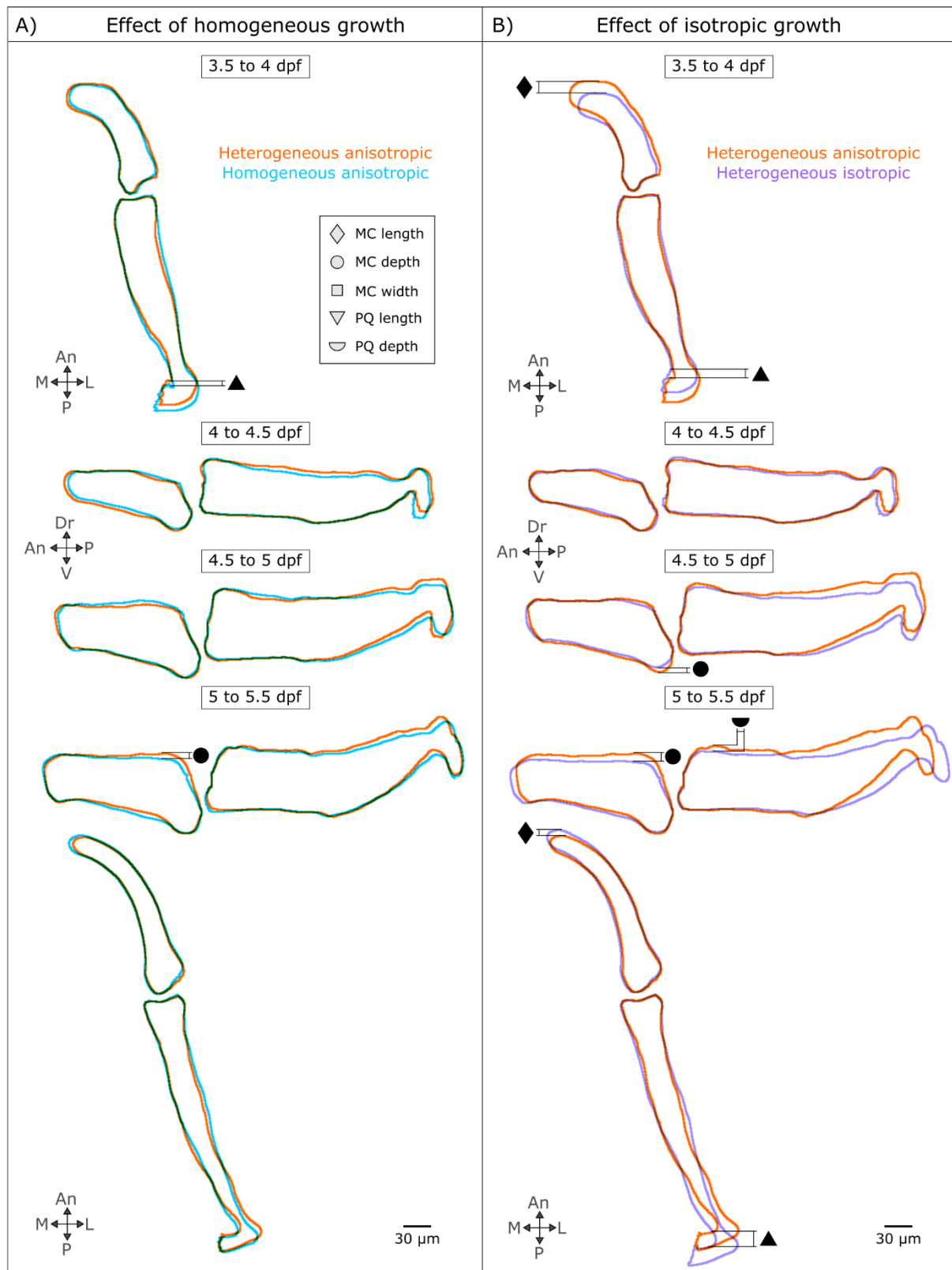


Figure 3.12: **Growth orientation plays an important role in jaw joint shaping whereas growth heterogeneity minorly impacts zebrafish jaw simulated shapes.** Growth simulations obtained from homogeneous anisotropic (A) and heterogeneous isotropic (B) growth fields are compared with the “full” simulations (heterogeneous anisotropic). Only the views where shape changes were observed in either

the homogeneous anisotropic or the heterogeneous isotropic growth simulations are displayed. The shape outlines in all views and time windows are displayed in Appendix 1.5. The black symbols denote the shape features which have been altered when either growth heterogeneity or orientation have been removed (triangle: Palatoquadrate (PQ) length, diamond: Meckel's cartilage (MC) length, square: MC width, semi-circle: PQ depth, circle: MC depth). An: anterior, Dr: dorsal, L: lateral, M: medial, P: posterior, V: ventral.

3.4. Discussion

In this research, growth in the larval zebrafish jaw joints was quantified based upon tracked cell-level data. Rates and directions of local tissue deformations were calculated at the cellular level revealing that heterogeneous growth rates and strong deformation anisotropy underlie jaw joint growth dynamics. Biofidelic growth simulations showed that zebrafish jaw joint growth can be reasonably approximated based on cell positional information over time. By tweaking the parameters of the simulations, I established that growth orientations rather than heterogenous growth rates dominate growth and morphogenesis in the developmental period assessed.

My analysis of zebrafish jaw joint cell dynamics revealed spatially and temporally heterogeneous growth patterns. Growth rates and orientations evolved over the time period studied and elevated growth rates were evident at the retroarticular process of the Meckel's cartilage, which is known to project ventro-posteriorly from the jaw joint during larval development ([Eames et al. 2013](#)). In other developing tissues, such as the developing chick limb bud ([Morishita et al. 2015](#), [Suzuki and Morishita 2017](#)) and the drosophila wing disc ([Tozluoglu et al. 2019](#)), spatial and temporal growth heterogeneity was shown to be a key driver of morphogenesis, and in simulations, uniform growth rates did not lead to correct shape predictions ([Tozluoglu et al. 2019](#)). In contrast to the limb bud and wing disc, my data indicate that spatial growth heterogeneity is not a dominant influence on zebrafish jaw joint shape for the time windows investigated. Rather, growth orientation was more important for jaw joint growth and morphogenesis in the timeframe studied. My analysis of zebrafish jaw joint cell dynamics revealed a marked growth anisotropy for the time period studied, and in simulations, isotropic growth led to pronounced shape alterations. This observation is in line with Boehm et al.'s work ([2010](#)) in which a parameter optimisation approach on murine limb bud development revealed that growth orientation was critical for accurate shape prediction. Altered cell orientation and increased cell sphericity has been shown to be correlated with altered zebrafish jaw shapes which could indicate a link between cell orientation and growth orientation ([Brunt et al. 2015](#), [Brunt et al. 2016b](#), [Lawrence et al. 2018](#)).

My quantification of cell dynamics was derived from cell rearrangements, cell volume expansion and ECM deposition, demonstrating that joint growth and morphogenesis can be reasonably approximated

based on these behaviours. Analysis of cell numbers indicated that proliferation is unlikely to be a dominant influence in the joint over the timeframe examined, despite the fact that proliferation has been highlighted in the more mature regions of the developing cartilage elements and in the interzone (Kimmel et al. 1998, Brunt et al. 2017). I also propose that cell intercalation is not likely to have a very strong influence on jaw joint growth in the timeframe and region examined, while acknowledging that cell stacking and convergent extension are key features of more mature regions of the developing cartilage elements (Kimmel et al. 1998, Schwartz et al. 2012, Eames et al. 2013, Mork and Crump 2015, Brunt et al. 2016b). As previously reported (Kimmel et al. 1998, Brunt et al. 2016b, Brunt et al. 2017), I found that cell volume expansion is likely a key contributor to joint growth. In the present research ECM volume increases could not be quantified due to insufficient segmentation quality. In a recent study conducted on the juvenile zebrafish pharyngeal skeleton, ECM volume increases were found to be negligible (Heubel et al. 2021), but, to my knowledge, such quantification was not performed in the larval zebrafish.

Some failures in growth simulations were observed in my results. Cell contraction in the hypertrophic regions of the Meckel's cartilage has not been accurately simulated due to the specific cell arrangements; in the Meckel's cartilage, cells stack into a single column in the antero-posterior axis. Because the algorithm for growth quantification does not directly account for cell shape, a medio-lateral contraction of cells in such a columnar arrangement cannot be captured. Another imprecision in my models arose from the lack of quantification of cell morphological changes and rearrangements at the MC anterior tip which occur from 3.5–4 dpf and which participate to the rudiment's growth. In some other timeframes, under- or over-growth of the Meckel's cartilage and the palatoquadrate length and depth arose from the small number of cells in the zebrafish jaw. The use of a model using a discrete number of cells, such as vertex models in which each cell is represented by a polygon (Alt et al. 2017), would have likely increased my control of the impact of each individual cell shape over time. A major application of cell-based models is the study of epithelial morphogenesis, notably in the *Drosophila* wing (Rauzi et al. 2008, Fletcher et al. 2017, Ioannou et al. 2020). An advantage of my modelling approach compared to cell-based models is the capacity to apply it to organisms with much greater numbers of cells. Because my model focusses on macro-scale shape changes and does not simulate individual cell behaviours, it ensures computational simplicity and practicability, whereas cell-based models are constrained to a limited number of cells.

My method as presented here is optimal for specimens in which live imaging can be performed. A straightforward application is to quantify growth patterns in epithelial tissues using high cellular resolution images obtained through fluorescence microscopy combined with automated tools for cell segmentation and tracking like EpiTools (Heller et al. 2016). Modelling axolotl joint growth using my

approach is also feasible. The axolotl is often used as a model for limb development ([Nye et al. 2003](#), [Hutchison et al. 2007](#)) and progress has been made in visualising cells at high resolution during live imaging ([Masselink and Tanaka 2021](#)). The existence of rainbow transgenic lines also facilitates cell tracking and visualisation and was used in the past to study digit tip regeneration ([Currie et al. 2016](#)). Though live imaging is optimal, it may not be critical to track individual cells with larger numbers of cells. Comparisons between local tissue geometry at successive timepoints may be sufficient to model joint growth and morphogenesis, which I am exploring in Chapter 6.

In conclusion, my findings show that cell volume expansion and orientation are key drivers of larval zebrafish jaw joint growth and morphogenesis. These new insights on what drives joint growth and morphogenesis was facilitated through growth simulations based upon precise and specific cell-level characterisation of growth. This work opens new avenues towards exploring the underlying processes of joint morphogenesis and in Chapter 5 I examine the role of mechanical loading.

Chapter 4

4. The material properties of the larval zebrafish jaw cartilage: time evolution and impact of movements

4.1. Introduction

There has been little quantification of cartilage material properties at the embryonic stage. With the rise of finite element models of joint growth which integrate mechanical forces ([Giorgi et al. 2015](#)), the characterisation of embryonic cartilage material properties is valuable and enables realistic estimations of the mechanical stimuli which are experienced during development. Studies have used various animal models and testing techniques which have engendered a broad variability of results. For example, larval zebrafish jaw Young's modulus was recently measured using Atomic Force Microscopy (AFM) ([Lawrence et al. 2021](#)), but values were about fifty times higher than previous estimations made on chick knee cartilage samples subjected to unconfined compression ([Mikic et al. 2004](#)). It is also known that fetal movements impact the quality of cartilage. Abnormal mechanical environments result in alterations of the extracellular matrix content which have direct consequences on cartilage biomechanical performances ([Mikic et al. 2004](#), [Lawrence et al. 2021](#)). Decreased or increased loading conditions have respectively resulted in reduced or elevated biomechanical moduli in the embryonic chick limb or the larval zebrafish jaw ([Mikic et al. 2004](#), [Lawrence et al. 2021](#)). In the present study, jaw cartilage biomechanical moduli are calculated after nano-indentation testing on free-to-move and immobilised larval zebrafish at several developmental stages. Nano-indentation was chosen over AFM because it enables indentation at a larger scale. Results show a delayed cartilage stiffening in the absence of movement with altered viscoelastic properties. The obtained Young's modulus values are used in the next chapter in a finite element (FE) model of jaw movements.

4.2. Methods

4.2.1. Indentation testing

Jaw cartilage material properties in hypertrophic and immature regions were estimated in both wild type free-to-move and immobilised specimens at 3, 4 and 5 day post fertilisation (dpf) using nano-indentation. Immobilisation was achieved via drug treatment by subjecting larvae to 0.1 mg.ml⁻¹ tricaine methanesulphonate (MS222) from 3 dpf, before the start of recorded jaw movements (Kimmel et al. 1995). The solution was refreshed twice daily. The indentation methodology was previously published in Lawrence et al. (2021) in which I performed nano-indentation testing on fish exposed to hypergravity. Whole larvae were fixed in 4% PFA and stored in 100% MeOH. Prior to nano-indentation, samples were rehydrated to 1 x PBS before being stored in 30% sucrose in PBS. The samples were then submerged in a 1:1 mix of 30% sucrose and optimum cutting temperature (OCT) at room temperature until the samples sunk to the bottom of an Eppendorf tube. Samples were then embedded in fresh 30% sucrose and OCT mix and flash-frozen on dry-ice. Embedded samples were sectioned coronally at a thickness of 10 µm using an NX70 Cryostat (CryostarTM, ThermoFisher). Nano-indentation was performed on sections featuring the Meckel's cartilage (hypertrophic cartilage), the ceratohyal (hypertrophic cartilage) and/or the jaw joint (immature cartilage) using a Chiaro nanoindenter (Optics 11) as shown in Figure 4.1A. All measurements were taken in PBS at room temperature. A spherical nano-indentation probe with an 8 µm radius and stiffness of 0.49 N/m was used. Indentation was performed to a depth of 1 µm with velocity of 1 µm/s, and the tip held at a constant depth for 10s (see Figure 4.1B). Nano-indentation was performed across all sections containing relevant cartilage regions,

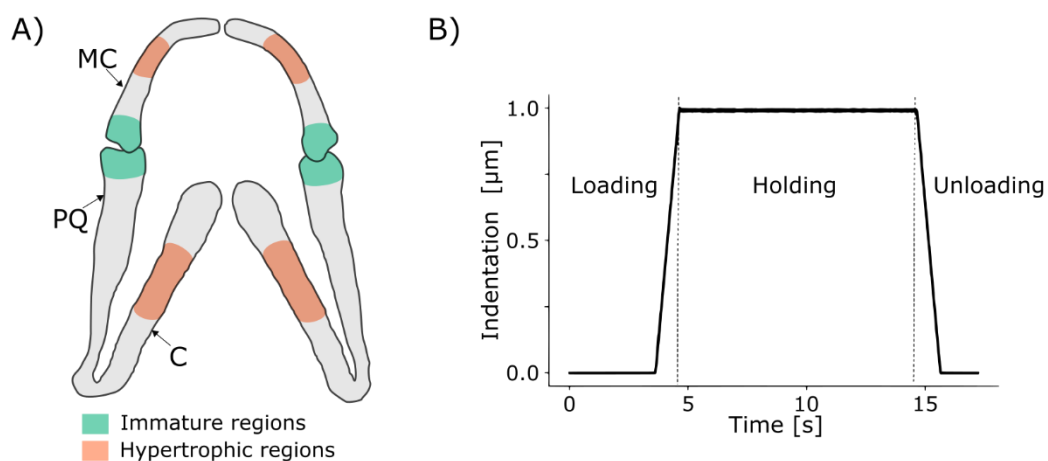


Figure 4.1: **Indentation testing.** A) Location of measurements in immature (green) and hypertrophic cartilaginous regions (orange) in the lower jaw. MC: Meckel's cartilage, PQ: palatoquadrate, C: ceratohyal. B) Indentation profile consisting of a loading phase during which a depth of 1µm was reached, a holding phase of 10 s and an unloading phase.

with one measurement collected per region of interest in each section. Sample numbers are provided in Table 4.1.

	3 dpf	4 dpf	5 dpf
Before movements	7	-	-
Free-to-move	-	6	6
Immobilised	-	7	6

Table 4.1: Sample numbers for nano-indentation testing

4.2.2. Elastic and viscoelastic analyses

Young's moduli were firstly estimated using the elastic Hertzian contact model. The governing equation for spherical indentation is:

$$E_{Hertz} = \frac{3}{4\sqrt{R}} P_{max} \frac{(1 - \nu^2)}{h_{max}^{3/2}} \quad (4.1)$$

where R is the radius of the probe, ν is the Poisson's ratio, h_{max} is the maximum indentation depth (indentation depth during holding phase), P_{max} is the peak force at maximum indentation.

We assumed a Poisson's ratio of 0.3, value which was previously used for AFM testing of the larval zebrafish jaw cartilage (Lawrence et al. 2021). The resulting Young's Moduli E_{Hertz} were averaged for each region across multiple sections of each fish.

Since relaxation was observed in all load-time curves (Figure 4.2A), viscoelastic analyses were also performed using elastic-viscoelastic correspondence, following previous work on prenatal vertebral murine tissues (Levillain et al. 2021). Viscoelastic operators were substituted for elastic constants in the elastic solution giving a relationship between the load P and the displacement h :

$$P = \frac{4\sqrt{R}}{3} \frac{E}{(1 - \nu^2)} h^{3/2} = \frac{8\sqrt{R}}{3} \frac{G}{(1 - \nu)} h^{3/2} \quad (4.2)$$

where R is the radius of the probe, ν is the Poisson's ratio, E is the material elastic modulus, and G is the shear modulus.

The displacement-time conditions for ramp-hold relaxation experiments in displacement control are:

$$\begin{aligned} h(t) &= kt, 0 \leq t \leq t_R \\ h(t) &= kt_R, t \geq t_R \end{aligned} \quad (4.3)$$

where $h(t)$ is the displacement-time condition, k is the ramp rate (constant rate loading) and t_R is the duration of the ramp.

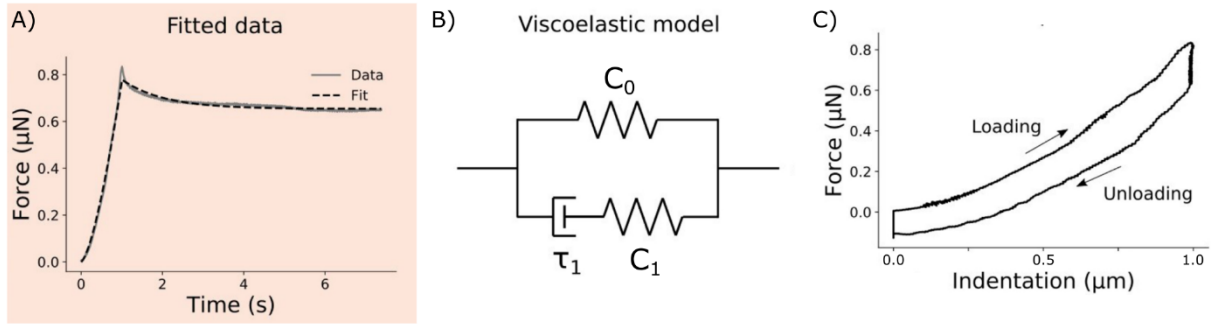


Figure 4.2: Analyses of nano-indentation measurements. A) The relaxation function associated with the viscoelastic model is fitted to experimental data to characterise the material's behaviour during load- and hold-phases. B) The viscoelastic model consists of a spring (relaxation coefficient C_0) in parallel to a spring (relaxation coefficient C_1) and a dashpot (time constant τ_1) in series. C) Output force-indentation curve displaying machine hysteresis. Adapted from (Levillain et al. 2021).

A Boltzmann integral method was used with the generalized Boltzmann integral expression for spherical indentation under displacement control being (Mattice et al. 2006):

$$P(t) = \frac{8\sqrt{R}}{3(1-\nu)} \int G(t-u) \left[\frac{d}{du} h^{3/2}(u) \right] du \quad (4.4)$$

where $P(t)$ is the load-time function, $G(t)$ is the shear relaxation modulus, $h(t)$ is the displacement-time condition, R is the probe's radius and ν is the Poisson's ratio.

The material was represented by a spring in parallel to a Maxwell solid (Figure 4.2B) which has a relaxation function of the form:

$$G(t) = C_0 + C_1 \exp\left(-\frac{t}{\tau_1}\right) \quad (4.5)$$

where $G(t)$ is the shear relaxation modulus, C_0 and C_1 are the relaxation coefficients of the viscoelastic model (Figure 4.2B), and τ_1 its time constant (Figure 4.2B).

The numerical integration of equation 4.4 for the conditions given by equation 4.3, results in a load-relaxation solution of the form (Mattice et al. 2006):

$$P(t) = B_0 + B_1 \exp\left(-\frac{t}{\tau_1}\right) \quad (4.6)$$

where B_0 and B_1 are fitting parameters and τ_1 the material's time constant

This function was fitted directly to the experimental load-time relaxation data to determine the fitting parameters B_0 , B_1 and τ_1 . An approximate relationship was proposed by Mattice et al. (2006) between the fitting parameters B_k and the material parameters C_k using a ramp correction factor (RCF) which “defines the difference between the observed values for analysis assuming ramp loading assumption”:

$$C_0 = \frac{B_0}{h_{max}^{\frac{3}{2}} \frac{8\sqrt{R}}{3(1-\nu)}} \quad (4.7)$$

and

$$C_1 = \frac{B_1}{RCF h_{max}^{\frac{3}{2}} \frac{8\sqrt{R}}{3(1-\nu)}} z \quad (4.8)$$

where

$$RCF = \frac{\tau_1}{t_R} \left[\exp\left(\frac{t_R}{\tau_1}\right) - 1 \right] \quad (4.9)$$

where R is the probe's radius, ν is the Poisson's ratio, h_{max} is the maximum indentation depth (indentation depth during holding phase), C_0 and C_1 are the relaxation coefficients (Figure 4.2B), and τ_1 its time constant (Figure 4.2B), t_R is the duration of the ramp, B_0 and B_1 are the fitting parameters of the experimental load-time relaxation function and RCF the ramp correction factor.

From the relaxation coefficients C_0 and C_1 , the instantaneous and long-time shear moduli G_{ins} and G_{eq} , (and from them the instantaneous and equilibrium moduli E_{ins} and E_{eq} which characterise the material behaviours during load- and hold-phases respectively (Figure 4.2C)), were computed:

$$G_{ins} = G(0) = C_0 + C_1 \quad (4.10)$$

$$G_{eq} = G(\infty) = C_0 \quad (4.11)$$

$$E_{ins} = 2G_{ins}(1 + \nu) \quad (4.12)$$

$$E_{eq} = 2G_{eq}(1 + \nu) \quad (4.13)$$

As before, a Poisson's ratio of 0.3 was assumed (Lawrence et al. 2021). The resulting instantaneous and equilibrium moduli were averaged for each region across multiple sections of each fish. The extent of viscous behaviour of the material was quantified with the elastic fraction $f = \frac{E_{eq}}{E_{ins}}$ (4.14). A null elastic fraction corresponds to a purely viscous material; an elastic fraction of 1 describes a purely elastic material (Mattice et al. 2006, Oyen 2006, Levillain et al. 2021). Machine hysteresis was systematically detected in the output force-indentation curves as shown with the straight lines in Figure 4.2C resulting in negative force output at the end of the unloading phase. Analyses in this Chapter were conducted using outputs from loading and holding phases which were a priori unaffected by the observed machine hysteresis (null force measurements at the start of indentation (Figure 4.2))

4.2.3. Statistical analysis

Statistical tests were performed in SPSS (IBM Corp. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY, USA). The Shapiro-Wilks test for normality was performed on each group. To test for significant differences between the hypertrophic and immature regions within each age and larva type group, paired t-tests were performed in groups which were normally distributed, and Wilcoxon signed-

rank tests were used in groups which were not normally distributed. Since no significant difference was observed between the hypertrophic and immature regions at any age (see Appendix 2.1), tests for significant differences between 3, 4 and 5 dpf free-to-move and immobilised groups were only performed on measurements taken from immature regions. In groups which were normally distributed, One-way Analysis of Variance (ANOVA) with Levene's test for homogeneity of variance was used. Because the data did not meet the assumption of homogeneity of variance, Games Howell post hoc test was performed to identify groups which differed significantly (significance level 5%). In groups which were not normally distributed, the non-parametric Kruskal-Wallis test was used with Dunn's post-hoc test and Bonferroni adjustment for multiple comparisons.

4.3. Results

4.3.1. Zebrafish jaw cartilage stiffening is delayed in the absence of jaw movement

I characterised lower jaw cartilage material properties for both free-to-move and immobilised larvae at 3, 4 and 5 dpf using nano-indentation. At 3dpf, jaw movements have not yet started. Measurements were taken at the jaw joint, where chondrocytes are not fully differentiated, and in the more mature regions, containing resting hypertrophic chondrocytes. The elastic modulus E_{Hertz} , useful for later FE analyses, was estimated using the Hertzian contact model. No significant difference nor trend was observed between measurements taken at the level of the joint compared to measurements taken in hypertrophic regions for all groups (see Appendix 2.1). From hereafter, only measurements taken at the jaw joint are discussed. In free-to-move larvae, there was a pronounced increase of E_{Hertz} from a mean of 28.1 ± 12.3 kPa at 3 dpf to 148.2 ± 31.3 kPa at 4 dpf as shown in Figure 4.3. No significant difference was observed between 4 dpf and 5 dpf measurements in free-to-move larvae. In immobilised larvae, E_{Hertz} at 4 dpf was found to be lower than for free-to-move specimens with a mean of 82.9 ± 26.6 kPa (Figure 4.3). By 5 dpf no difference was observed between immobilised and free-to-move larvae. It seems that, in the absence of movement, larval zebrafish jaw cartilage stiffening was delayed before compensatory mechanisms took place. However, it is likely that the cartilage composition and structure of immobilised 5 dpf larvae are different from the material properties of free-to-move larvae based on previous work from our group which showed that muscle movements are necessary for the onset and maturation of multiple collagens in the prenatal murine cartilage (Ahmed et al. 2022).

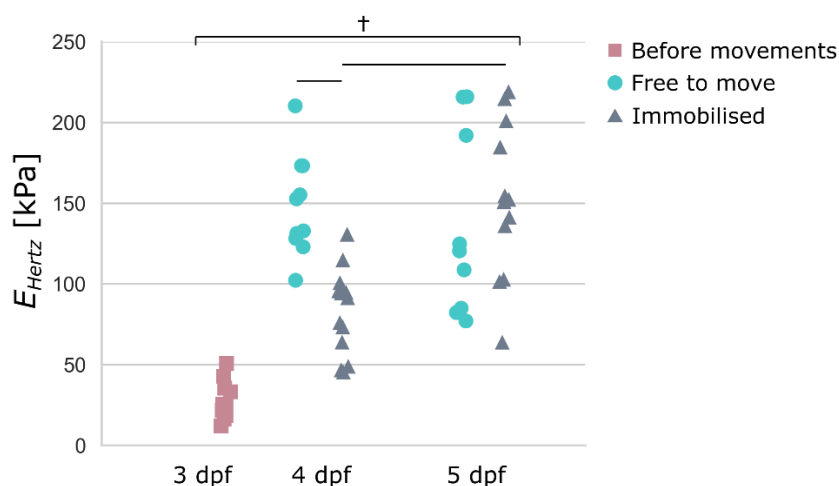


Figure 4.3: **Zebrafish jaw cartilage stiffening is delayed in the absence of movement.** Elastic moduli E_{Hertz} of free-to-move and immobilised larvae at 3, 4 and 5 dpf obtained from nano-indentation measurements taken at the level of the jaw joint. Solid bars indicate significant difference ($p < 0.05$) between groups. † indicates significant difference ($p < 0.05$) between 3 dpf group and all other groups.

4.3.2. Viscoelastic properties are not recued in immobilised larvae despite cartilage stiffening by 5 dpf

An additional viscoelastic analysis, more representative of embryonic material properties seeing the hysteresis in the measured load-displacement curves, was conducted and the instantaneous E_{ins} and equilibrium E_{eq} moduli along with the elastic fraction $f = \frac{E_{eq}}{E_{ins}}$ were calculated. A similar delay in stiffening due to immobilisation was observed in the equilibrium modulus E_{eq} as shown in Figure 4.4A. 4 dpf immobilised E_{eq} was significantly lower than 4 dpf free-to-move E_{eq} , whereas no difference was found between 5 dpf immobilised and free-to-move larvae. The instantaneous material properties however displayed another pattern. The free-to-move E_{ins} increased from 23.6 ± 9.4 to 189.0 ± 130.8 kPa between 3 dpf and 4 dpf as shown in Figure 4.4B. No significant difference was observed between 4 dpf and 5 dpf measurements in free-to-move larvae. 4 dpf immobilised E_{ins} fell within the same order of magnitude as 4 dpf free-to-move, as shown in Figure 4.4B. However, immobilised E_{ins} at 5 dpf was significantly higher than free-to-move measurements. Therefore, immobilisation led to an increased stiffening of instantaneous material properties but not of long-term properties. As a consequence of this differential stiffening of material properties in the absence of movement, the elastic fraction, which is an indication of a material's viscosity, was significantly reduced in the 5 dpf immobilised fish compared to all other groups indicating an increase in viscosity (Figure 4.4C). An elastic fraction of 0 represents a purely viscous material while an elastic fraction of 1 represents a purely elastic material. In immobilised fish, a trend of increased viscosity was visible at 4 dpf compared to free to move fish, with significant decrease in the elastic fraction being achieved between 4 dpf immobilised and 5 dpf free to move groups (Figure 4.4C). The differential stiffening of material

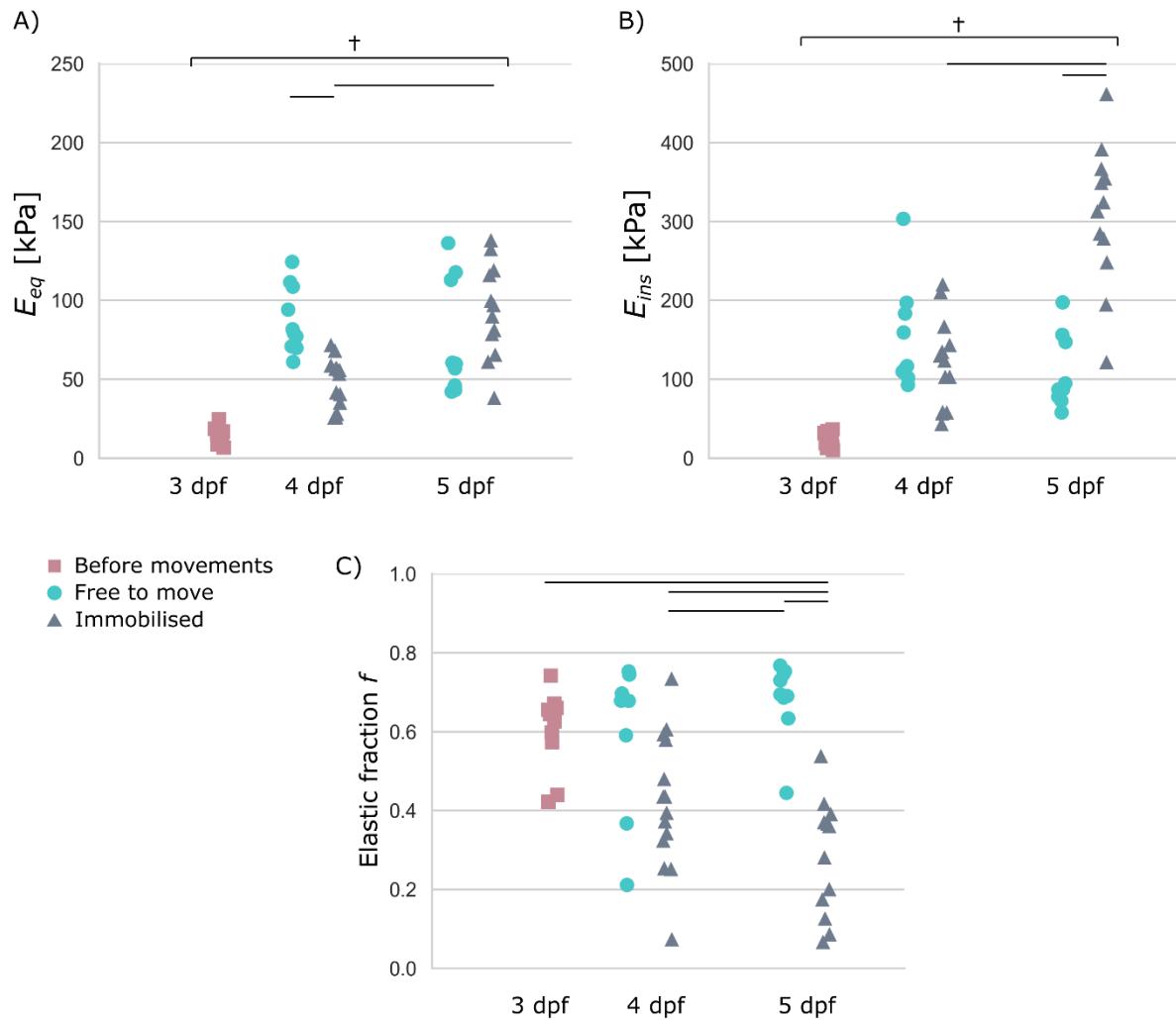


Figure 4.4: **Long- and short- term stiffening patterns are altered in the absence of movement with consequences for material viscoelasticity.** (A–C) Biomechanical moduli ((A) equilibrium moduli E_{eq} , (B) Instantaneous moduli E_{ins} and (C) elastic fraction f) of free-to-move and immobilised larvae at 3, 4 and 5 dpf obtained from nano-indentation measurements taken at the level of the jaw joint. Solid bars indicate significant difference ($p < 0.05$) between groups. † indicates significant difference ($p < 0.05$) between 3 dpf group and all other groups.

properties in the absence of movement and the increase in viscosity may be an indication of altered cartilage composition and structure.

4.4. Discussion

Zebrafish jaw cartilage material properties were quantified using nano-indentation in the presence or absence of movement during morphogenesis. Results showed a delay in cartilage stiffening in immobilised larvae compared to free-to-move larvae with normal Young's Moduli being measured at

5 dpf. Despite normal Young's Moduli at 5 dpf, cartilage viscoelastic properties were abnormal and exhibited increased viscosity in immobilised larvae at 5dpf compared to free-to-move larvae.

Little is known about transient cartilage material properties. In the literature, the variability in the estimated biomechanical moduli is broad due to the use of various animal model systems and testing techniques. Bending experiments on unmineralized embryonic mouse ribs calculated an average Young's modulus of 1.11 ± 0.62 MPa (Tanck et al. 2004). AFM measurements on murine growth plates from embryonic day e13.5 to birth reported Young's moduli ranging from 20 to 120 kPa (Prein et al. 2016). Stress-relaxation tests on the chick knee transient cartilage estimated an equilibrium modulus of 0.08 MPa (Mikic et al. 2004) when AFM measurements reported a softer Young's modulus of 0.54 ± 0.07 kPa (Roddy et al. 2011a). My estimations of larval zebrafish jaw joint cartilage Young's modulus fell within the range of values previously reported for embryonic cartilage with a mean of 0.15 ± 0.03 MPa for wild type larvae aged 4 dpf. Conversely, Lawrence et al. (2021) calculated an average Young's modulus of 4.2 MPa for 5 dpf wild type zebrafish using AFM in regions close to the jaw joint. These AFM measurements were performed after staining with Alcian blue and Alizarin red which may have stiffened the cartilage material properties.

Upon maturation, transient cartilage's composition and structure vary in response to mechanical forces with consequences for material properties. A study on chick embryonic knee cartilage revealed that glycosaminoglycan and hydroxyproline contents were altered when specimens had been immobilised during their development (Mikic et al. 2004). This change in cartilage content directly impacted the material properties of the tissue with the instantaneous modulus being reduced up to 50% (Mikic et al. 2004). My measurements showed that in the larval zebrafish jaw joint, immobilisation led to a reduction of the Hertzian and equilibrium moduli at 4 dpf. Cartilage stiffness recovered in the immobilised fish at 5 dpf, however, the viscoelastic properties were still abnormal. Opposite to the effects of immobilisation on joint cartilage, increased loads upregulate its biomechanical properties. Submitting larval zebrafish to hypergravity during development resulted in cartilage stiffening of the lower jaw (Lawrence et al. 2021). This change in material properties was associated with disturbed chondrocyte maturation and slight changes to the ECM (reduction in proteoglycan content and modified collagen fibre organisation) (Lawrence et al. 2021). In cartilage tissue engineering, dynamic loads commonly resulted in increased biomechanical moduli associated with increased accumulation of collagen type II and proteoglycans (Anderson and Johnstone 2017, Salinas et al. 2018). In this research, cartilage content was not investigated. Despite recovery of the equilibrium modulus by 5 dpf, the instantaneous response to loads in immobilised larvae suggests that the collagen and proteoglycan contents and/or organisation are disturbed. Differences in ECM content in the presence or absence of movements are triggered by cells mechanosensory behaviour. Mechanical cues are

perceived by cells through mechanosensitive membrane-anchored receptors which form at the contact interface between cells and the ECM and between cells and cells and which biochemically relay mechanical inputs across the intracellular actomyosin cytoskeleton ([Chen et al. 2017](#), [Mierke 2022](#)). Examples of such receptors include stretch-activated ion channels and integrin- and cadherin-based adhesion complexes ([Chen et al. 2017](#), [Mierke 2022](#)). In response to the transduced mechanical loads, cell biochemical signalling changes, thus influencing changes to the ECM but also cell behaviours such as cell migration, adhesion, division and growth ([Phan et al. 2009](#), [Chen et al. 2017](#), [Mierke 2022](#), [Khatib et al. 2023](#)). In the larval zebrafish jaw, chondrocytes are known to be smaller and rounder in the absence of movement ([Shwartz et al. 2012](#), [Roddy et al. 2017](#)), showing that cell growth in the embryonic cartilage is influenced by sensing of mechanical cues. This point will be further explored in the next Chapters.

In conclusion, a new estimation of larval zebrafish material properties has been obtained using nano-indentation. The zebrafish jaw joint cartilage's biomechanical properties vary in response to mechanical forces arising from muscle activity. The measured values are used in finite element models of zebrafish jaw movements in the next chapter.

Chapter 5

5. Mechanobiological model of zebrafish jaw joint identifies dynamic patterns of mechanical stimuli correlated with growth patterns

5.1. Introduction

There are numerous clinical and experimental studies showing that prenatal movement is critical to healthy joint development. Abnormal fetal movement is a known risk factor for several pre- and post-natal musculoskeletal conditions in which both joint function, and short- and long-term health, with the onset of early osteoarthritis, are compromised ([Nowlan 2015](#), [Vaquero-Picado et al. 2019](#)). Developmental dysplasia of the hip (DDH), arthrogryposis and fetal akinesia deformation sequence are common examples of such conditions ([Nowlan 2015](#)). Experimental studies on animal models have shown that the absence of muscle contraction often results in a loss of complementary and interlocking joint shape features and failed cavitation in the most extreme cases ([Roddy et al. 2017](#), [Sotiriou et al. 2019](#), [Bridglal et al. 2020](#)). At the cellular level, it appears that several cell activities involved in joint shaping adapt to local changes in the mechanical environment. Cell proliferation, maturation and reorganisation have been shown to be impaired in the absence of movement ([Shwartz et al. 2012](#), [Roddy et al. 2017](#)). Despite insights at the tissue and cellular level, the mechanisms by which fetal movements influence joint growth are still unclear, and the complex process of joint morphogenesis mechano-regulation needs to be unfolded.

Since prenatal joints are cartilaginous tissues, insights on joint growth mechano-regulation can be obtained by studying the effects of loads on chondrocytes using cartilage explants. In the field of tissue engineering, researchers have investigated the impact of different loading regimes on chondrogenesis. A consensus exists that dynamic loading, either through direct compression or hydrostatic pressure loading, promotes chondrocyte gene expression, extracellular matrix synthesis and tissue material properties ([Anderson and Johnstone 2017](#), [Salinas et al. 2018](#)). To investigate how mechanical stimuli

influence prenatal joint morphogenesis, mechanobiological models of joint growth have been developed and showed that hydrostatic stress distributions arising over joint motion can predict the emergence of shape features seen under normal or altered loading conditions (Shefelbine and Carter 2004b, Shefelbine and Carter 2004a, Giorgi et al. 2015, Dixit et al. 2020, Comellas et al. 2022). For example, when simulating hip joint growth, reduced, absent or asymmetric movements resulted in shape alterations in both the femoral head and the acetabulum which were characteristic of congenital musculoskeletal conditions like DDH (Giorgi et al. 2015). However, existing mechanobiological models were limited by the use of simplified shape and cell inputs. With the progress made in characterising cellular dynamics involved during tissue growth as detailed in Section 2.4.1, the complex relationship between joint morphogenesis and mechanical loading can now be further explored.

In this research, experimental data of the zebrafish jaw joint were synthesised using computational models. I quantified zebrafish jaw joint growth in the presence of normal and altered mechanical environments using the methodology described in Chapter 3. Comparisons between free-to-move and immobilised larvae showed that growth rates are differentially altered along different anatomical axes. A novel mechanobiological model of zebrafish jaw joint growth integrating the quantified cell-activities was used to test hypotheses regarding how biomechanical stimuli arising from movement promote growth pattern changes. It emerged that, rather than average tensions or compressions, the most likely stimulus for mechano-regulated growth is the dynamic changes from tension to compression experienced over joint motion.

5.2. Methods

5.2.1. Characterising zebrafish jaw joint growth in the absence of muscle loading

Growth patterns in the larval zebrafish jaw joint were characterised from 3.5 to 5.5 days post fertilisation (dpf) in free-to-move and immobilised larvae to investigate the impact of muscle loading on joint morphogenesis. Three analyses were performed as illustrated in Figure 5.1. First, qualitative observations of joint growth were made on average jaws over time (Figure 5.1A). Next, growth rates were quantified from tracked cell-level data (Figure 5.1B and C). Finally, growth simulations were performed (Figure 5.1D). In immobilised specimens, cells in the posterior palatoquadrate (PQ) joint element could not be reliably segmented and tracked due to a weaker expression of fluorescent markers. Growth analyses were therefore performed solely on the anterior Meckel's cartilage (MC) joint element. Free-to-move shape averaging, growth rates calculations and growth simulations were performed as described in Chapter 3 and methods in this Chapter focus on the immobilised data set.

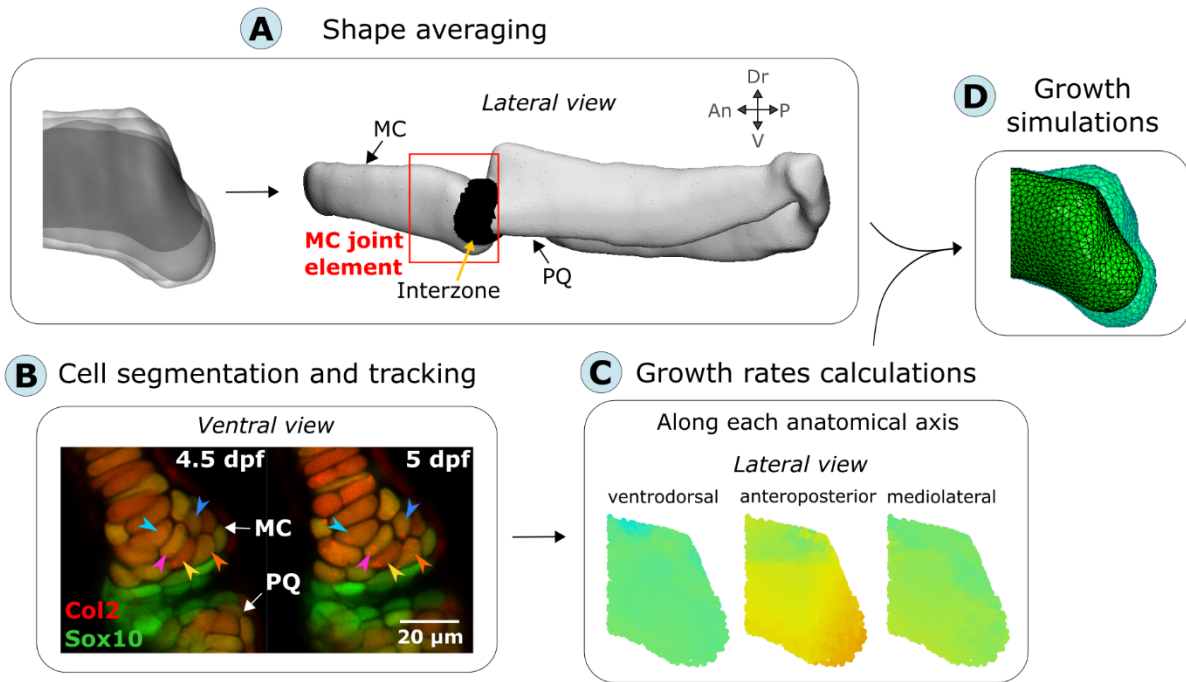


Figure 5.1: **Process of growth patterns characterisation.** A) Average shapes for free-to-move and immobilised larvae were generated at each time point (3.5, 4, 4.5, 5 and 5.5 dpf). B) Cells were segmented and tracked between each time point. C) Growth rates and orientations were calculated based on the tracked cell-level data. D) Growth simulations were performed from the average shapes and the growth maps. An: anterior, Dr: dorsal, MC: Meckel's Cartilage, P: posterior, PQ: palatoquadrate, V: ventral.

5.2.1.1. Zebrafish husbandry/Zebrafish lines and anaesthetisation

Fish were maintained as described in Aleström (2020) at University of Bristol. All experiments were approved by the local ethics committee (Bristol AWERB) and performed under a UK Home Office Project Licence. Transgenic lines *Tg(col2a1aBAC:mCherry)* (Mitchell et al. 2013) and *Tg(-4.9sox10:eGFP)* (Carney et al. 2006) have been previously described. In the immobilised group, wild type larvae were anaesthetised in 0.1 mg.ml⁻¹ tricaine methanesulphonate (MS222) in Danieau's buffer from 3 dpf prior to the start of recorded jaw movements (Kimmel et al. 1995). The solution was refreshed twice daily.

5.2.1.2. Characterising growth from cell-level data

Growth maps were calculated for twelve-hour interval time windows (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf) for immobilised specimens following the methodology described in Chapter 3. Briefly, confocal image stacks centred on the jaw joint line were obtained at twelve-hour intervals from 3.5 to 5.5 dpf for double transgenic *Tg(col2a1aBAC:mCherry; -4.9sox10:eGFP)* anaesthetised larvae. Imaging was performed by Dr. Elizabeth A. Lawrence and Mengdi Wang. Cells were segmented in Fiji and the 3D

cell centroid's coordinates in the Meckel's cartilage (MC) joint element were extracted at each time point. MC joint cells were manually tracked between images from two consecutive timepoints using manual labelling in MATLAB (R2018a, The MathWorks, Inc., Natick, Massachusetts, United States). The position of cell centroids with respect to each other over time was used to calculate the growth tensors $\dot{V}$ (units s^{-1}) in cubic regions of interests (ROIs) using equation 3.2 as detailed in Chapter 3 (Graner et al. 2008). The cubic ROIs were of size length 15 μm and mapped the MC joint element. After calculations, the local growth tensors $\dot{V}$ in each ROI can be represented by an ellipsoid whose axes represent the three directions for growth and their radius the rate of growth along these directions. Growth maps displaying the local deformation rates and directions were generated from the local growth ellipsoids for each time window. Interpolation between ROIs centres was performed in Abaqus CAE (Dassault Systemes, 2019) by importing the growth maps as analytical mapped fields. In this Chapter, ventrodorsal (VD)/anteroposterior (AP)/mediolateral (ML) growth is defined as the growth rates of the growth ellipsoid axis whose angle from the anatomical VD/AP/ML axis is the smallest amongst the three ellipsoid axes. All growth maps will be analysed following this terminology. The growth ellipsoid axes are displayed in Appendix 3.1, along with the angles between the growth ellipsoid axes and the anatomical VD/AP/ML axes. Sample numbers per time window for both free-to-move (acquired in Chapter 3) and immobilised specimens are listed in Table 5.1. The p-value for growth rates mean differences along each direction for growth between free-to-move and immobilised groups for each time window were obtained by running Shapiro-wilk test of normality followed by Mann-Whitney U-test with Bonferroni adjustments for multiple comparisons.

	3.5–4 dpf	4–4.5 dpf	4.5–5 dpf	5–5.5 dpf
Free-to-move	5	7	7	7
Immobilised	7	8	6	7

Table 5.1: Sample number per time window for growth rates analyses in the anterior MC joint element of free-to-move and immobilised larvae.

5.2.1.3. Average shape generation

Average shapes were generated at 3.5, 4, 4.5, 5 and 5.5 dpf for immobilised larvae following the methodology described in Chapter 3. Briefly, confocal image stacks of four to five larval zebrafish jaws (encapsulating the Meckel's cartilage (MC), the palatoquadrate (PQ) and the ceratohyal (C)) from the transgenic line *Tg(col2a1aBAC:mCherry)* were taken with a Leica SP8 confocal microscope at each time point in the ventral plane. Imaging was performed by Dr. Elizabeth A. Lawrence and Mengdi Wang. A 3D Gaussian grey filter was applied to the image stacks in Fiji. Image stacks were imported in Mimics

(Materialise NV, Leuven, Belgium) to be segmented. Only half-jaws (separated at the level of the midsagittal plane) were segmented. Each segmented half-jaw was divided into slices in the transversal plane. For each slice, an average shape outline was generated in MATLAB from the shape vertices of each segmented half-jaw. Averaged shape outlines were saved as image stacks and imported into Mimics where the resultant average half-jaw shape was generated. In the figures, the outlines of the average MC joint element were consistently cropped based on the increases over time of the distances between the tracked cells and the joint line.

5.2.1.4. Simulating zebrafish jaw growth

Growth in free-to-move and immobilised zebrafish jaws was simulated for each twelve-hour interval time window (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf) following the methodology described in Chapter 3. A non-manifold assembly combining the average half-jaw and the interzone (added as a volume filling the gap between the two joint elements using Boolean operations) was generated in Mimics and meshed in 3-matic (Materialise NV, Leuven, Belgium) with ten node tetrahedral elements. In Abaqus CAE (Dassault Systemes, 2019), a model for each twelve-hour time window was created. Cartilaginous regions were assigned homogeneous isotropic elastic material properties with Poisson's ratio 0.3 and Young's Modulus (YM) based on the nano-indentation measurements displayed in Table 5.2. Since no difference was observed between hypertrophic and immature cartilage material properties, all cartilaginous elements were assigned the same material properties with no distinction between regions (see Chapter 4). Simulations of jaw movement using viscoelastic instead of linear elastic material properties resulted in minor changes in load patterns, justifying the use of linear elastic material properties in this Chapter (see Appendix 3.2). The interzone was assigned isotropic elastic material properties with Poisson's ratio 0.3 and YM set at 0.025% of the cartilaginous YM based on Lawrence et al. (2021) and physiological boundary conditions were applied as detailed in Chapter 3. For each 12-hour period, growth strains derived from the growth maps were imported into Abaqus CAE as three distinct analytical mapped fields (one for each axis of the growth ellipsoids) and applied to the model. The Abaqus user subroutine UEXPAN was used to apply spatially varying expansion based on the strain fields to provide a prediction of growth and shape for each time-window. Outlines of the

	3.5–4 dpf	4–4.5 dpf	4.5–5 dpf	5–5.5 dpf
Free-to-move	85.04	142.01	142.01	142.01
Immobilised	72.75	82.91	117.44	151.96

Table 5.2: Zebrafish jaw cartilage Young's Moduli [kPa] in free-to-move and immobilised larvae based on nano-indentation measurements.

simulated growth shapes were obtained for the anterior MC joint element and cropped based on Abaqus results.

5.2.2. Investigating zebrafish jaw joint mechanoregulation

Joint growth mechanoregulation was investigated for two time-windows: 4 to 4.5 dpf and 4.5 to 5 dpf. Before 4 dpf, jaw movements are still erratic in some larvae hence 3.5–4 dpf analyses were not performed. Analyses were not conducted from 5 to 5.5 dpf due to a discrepancy in the free-to-move 5.5 dpf average shape, in which the depth was smaller than that of the 5 dpf average shape, which made shape comparisons difficult to interpretate. Loading fields were therefore extracted from jaw movement simulations at 4 and 4.5 dpf and integrated into 4–4.5 and 4.5–5 growth simulations respectively.

5.2.2.1. Simulating zebrafish jaw movements with finite element models

Jaw movement simulations were performed on free-to-move 4 and 4.5 dpf finite element (FE) models in Abaqus CAE. Muscle contractions engaged during opening/closure as shown in Figure 5.2 were applied to the models based on (Brunt et al. 2016a). Muscle attachment points and directions were estimated from confocal scans of double transgenic *Tg(col2a1aBAC:mCherry;smyhc1:EGFP)* larvae and are shown in Figure 5.2. Muscle forces enabling physiological jaw displacement (jaw opening of 37.2 μm based on the average jaw displacement for 5 dpf larvae (Brunt et al. 2015)) were used and are listed in Table 5.3. Jaw closure and opening were simulated in subsequent steps with each step decomposed into five increments. Muscle directions were not updated with jaw motion, resulting at peak opening and peak closure in minor errors to the direction provided in Appendix 3.3. Hydrostatic stress and strain fields were extracted into MATLAB for each time increment from peak closure to peak opening. As muscles were modelled as point loads, non-physiologic stress concentrations were obtained at the loaded nodes which were therefore removed from the extracted stress and strain fields.

	4 dpf	4.5 dpf
adductor mandibularis (am)	2.84	4.35
intermandibularis anterior (ima)	1.25	2.90
intermandibularis posterior (imp)	1.50	3.47
interhyal (ih)	1.50	3.47

Table 5.3: Muscle forces [nN] in the lower jaw enabling physiological movement in FE simulations.

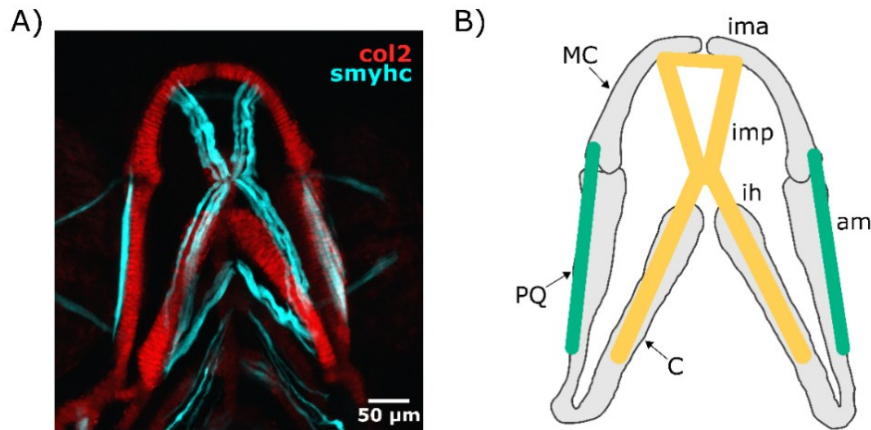


Figure 5.2: **Lower jaw muscles.** A) Maximum projection of ventral confocal image stacks expressing *Col2a1aBAC:mcherry* (red) and *smyhc1:EGFP* (cyan) of a 4 dpf larva. B) Scheme of the muscles engaged during lower jaw opening (yellow) and closure (green) in the ventral plane. C: ceratohyal, MC: Meckel's cartilage, PQ: palatoquadrate, am: adductor mandibularis, ih: interhyal, ima: intermandibularis anterior, imp: intermandibularis posterior.

5.2.2.2. Integrating mechanical stimuli arising from movements

Growth simulations combining biological and mechanobiological contributions were obtained by integrating the loading fields arising from jaw movement simulations to the growth maps obtained from cell-level data. The baseline levels of biological contributions to growth were assumed to be the growth tensors (V matrices) calculated from immobilised larvae but applied to the free-to-move shapes which from now on are called G_{imm} . Loading fields were used to alter G_{imm} and obtain new mechanobiological growth maps which were applied in growth simulations. Two different methods to calculate the mechanobiological growth maps were implemented to test different hypotheses. Detailed are provided below. Since hydrostatic stress and strain patterns were similar (Appendix 3.4), only the hydrostatic stress fields were used in the calculations for simplicity and uniformity with previous studies (Shefelbine and Carter 2004b, Giorgi et al. 2014). Mesh convergence study was conducted showing that the chosen mesh was appropriate when modelling jaw movements using the methodology described in this Chapter (Appendix 3.5).

Calculation of mechanobiological growth map: Method 1:

Hypothesis: Average loads across loading history direct jaw joint morphogenesis

The average hydrostatic stress field across loading history S was calculated from the hydrostatic stress fields of all step increments in MATLAB. At each node of the model mesh the average hydrostatic stress writes as follow:

$$S = \frac{\sum_{i=1}^N \sigma_{hi}}{N} \quad (5.1)$$

where S is the average hydrostatic stress at the considered node, σ_{hi} is the hydrostatic stress at the considered node in one of the step increments and N the number of step increments from peak opening to peak closure.

To test the hypothesis that tension promotes growth (Vanderploeg et al. 2008), a new growth map $G_{tension}$ was calculated (Figure 5.3A and B). At each node, we set:

$$G_{tension} = G_{imm} + aS \cdot I_3 \quad (5.2)$$

where G_{imm} is the growth tensor obtained from immobilised larvae at the considered node, a is a mechanoregulatory growth modulating variable which influences the impact of the average hydrostatic stress S on the mechanobiological growth map (units [$m^2N^{-1}s^{-1}$]) and I_3 is the identity matrix of order 3×3 .

From 4 to 4.5 dpf, a was incrementally increased from $1e9 \text{ m}^2N^{-1}s^{-1}$, a value with which it had minor influence on the mechanobiological growth map, to $4e9 \text{ m}^2N^{-1}s^{-1}$ where joint overgrowth was obvious as shown in Appendix 3.6. The modulating variable value which predicted physiological MC depth growth was chosen ($a = 2e9 \text{ m}^2N^{-1}s^{-1}$). The same value was used from 4.5 to 5 dpf.

Similarly, to test the hypothesis that compression promotes growth (Kisiday et al. 2004), another growth map $G_{compression}$ was calculated based on the following equation applied to each node of the mesh model (Figure 5.3A and B):

$$G_{compression} = G_{imm} - bS \cdot I_3 \quad (5.3)$$

where G_{imm} is the growth tensor obtained from immobilised larvae at the considered node, b is a mechanoregulatory growth modulating variable which influences the impact of the average hydrostatic stress S on the mechanobiological growth map (units [$m^2N^{-1}s^{-1}$]) and I_3 is the identity matrix of order 3×3 .

After incrementally increasing the modulating variable value, $b = 3e9 \text{ m}^2N^{-1}s^{-1}$ was chosen because it predicted physiological MC depth growth from 4 to 4.5 dpf (see Appendix 3.6). The same value was used from 4.5 to 5 dpf. The mechanoregulatory growth modulating variables a and b encapsulate the number of jaw openings occurring over twelve hours (approximately 57 thousand openings between 4 and 4.5 dpf according to Brunt et al. (2015)). For both $G_{tension}$ and $G_{compression}$, the contribution of the average hydrostatic stress field to growth was isotropic: the same hydrostatic stress field and modulating variable were applied along all directions. The obtained mechanobiological growth maps $G_{tension}$ and $G_{compression}$ were qualitatively compared to the growth map calculated from free-to-move larvae G_{free} . Growth simulations were run in Abaqus CAE using $G_{tension}$ and $G_{compression}$ based

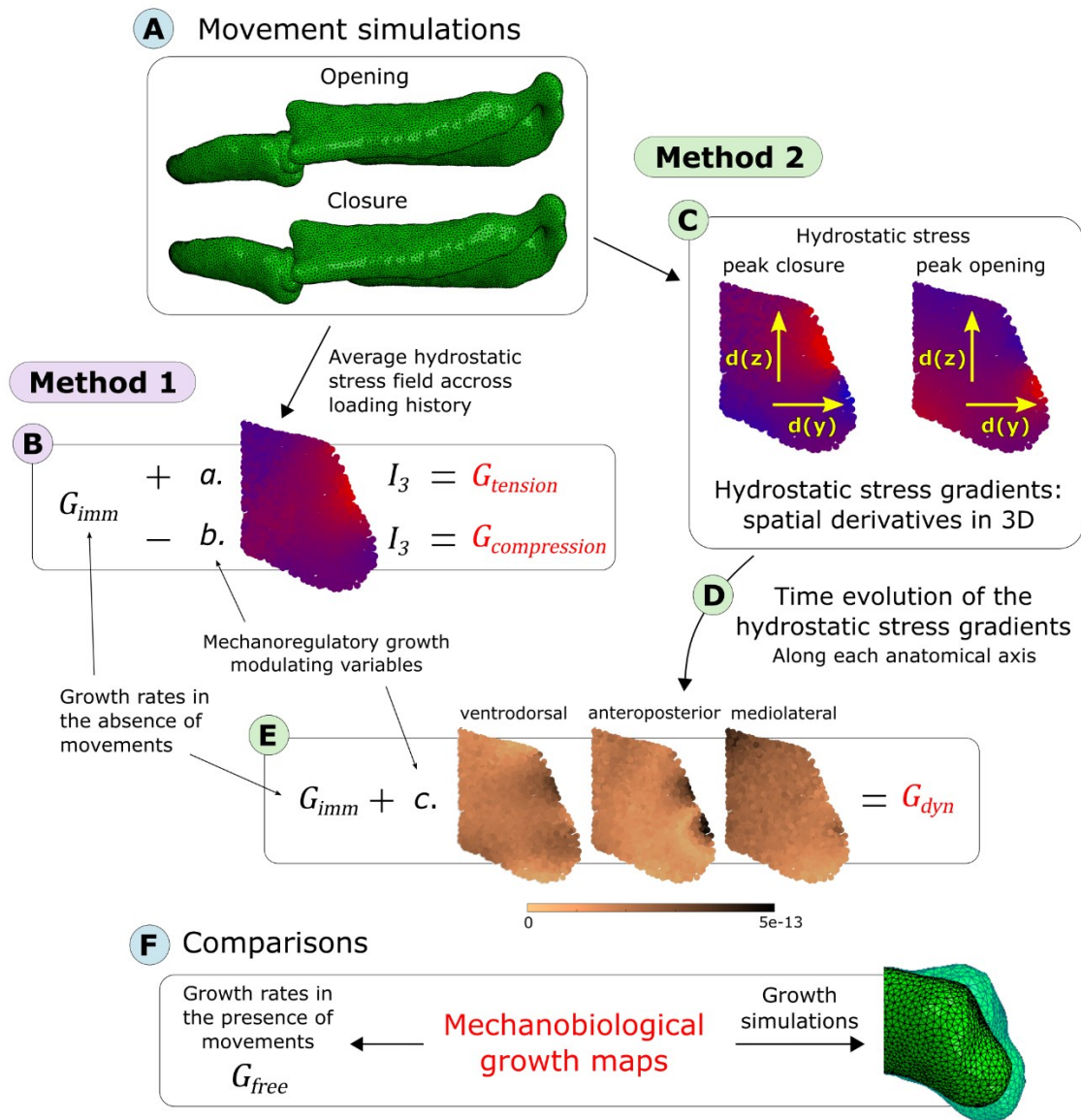


Figure 5.3: **Process of integration of mechanical stimuli arising from movements into mechanobiological growth maps of zebrafish jaw joint growth.** A) Jaw opening and closure were simulated. B) Method 1: Average hydrostatic stress fields across the loading history was extracted and subtracted or added to the growth rates in the absence of movements G_{imm} after being weighted by a mechanoregulatory growth modulating variable a or b respectively. The newly obtained mechanobiological growth maps were called $G_{tension}$ and $G_{compression}$ respectively. C) Method 2: The time evolution of the hydrostatic stress gradients was used. Hydrostatic stress gradients at peak closure and peak opening were calculated along each anatomical axis. D) The time evolution of the hydrostatic stress gradients between peak opening to peak closure along each anatomical axis was calculated E) and was added to G_{imm} after being weighted by a mechanoregulatory growth modulating variable c . The newly obtained mechanobiological growth map was called G_{dyn} . F) The obtained mechanobiological growth maps $G_{tension}$, $G_{compression}$ and G_{dyn} were compared to the growth map in the presence of movements G_{free} . Growth simulations were run using $G_{tension}$, $G_{compression}$ and G_{dyn} and compared to growth simulations using G_{free} .

on the same methodology than explained in Section 5.2.1.4 and qualitatively compared to growth simulations using G_{free} (Figure 5.3D).

Calculation of mechanobiological growth map: Method 2:

Hypothesis: Dynamic changes in load spatial patterns drive jaw joint growth

The dynamic changes in hydrostatic stress patterns over jaw motion were used to calculate a new growth map G_{dyn} . For this, for each node of the mesh model, the hydrostatic stress gradients $\nabla\sigma_h$ along each anatomical axes x , y and z were calculated in MATLAB from the hydrostatic stress vectors at peak closure and peak opening (Xing 2015) (Figure 5.3C.1). Here, these hydrostatic stress gradients were expressed in the form of diagonal matrices:

$$\nabla\sigma_h = \begin{pmatrix} \frac{\partial\sigma_h}{\partial x} & 0 & 0 \\ 0 & \frac{\partial\sigma_h}{\partial y} & 0 \\ 0 & 0 & \frac{\partial\sigma_h}{\partial z} \end{pmatrix} \quad (5.4)$$

where σ_h is the hydrostatic stress at the considered node and (x, y, z) are the anatomical axes.

The dynamic changes in hydrostatic stress patterns over jaw motion were computed as the absolute value of the difference between hydrostatic stress gradients at peak opening and at peak closure (Figure 5.3C.2). A new growth map G_{dyn} was calculated at each node of the mesh model based on the following equation (see Figure 5.3C.3):

$$G_{dyn} = G_{imm} + c \cdot |\nabla\sigma_{h \text{ peak opening}} - \nabla\sigma_{h \text{ peak closure}}| \quad (5.5)$$

where G_{imm} is the growth tensor obtained from immobilised larvae at the considered node, c is a mechanoregulatory growth modulating variable which influences the impact of the hydrostatic stress gradients on the mechanobiological growth map (units $[m^3N^{-1}s^{-1}]$) and $\nabla\sigma_{h \text{ peak opening}}$ and $\nabla\sigma_{h \text{ peak closure}}$ are the hydrostatic stress gradients at peak opening and peak closure respectively.

From 4 to 4.5 dpf, c was incrementally increased from $1e10 \text{ m}^3N^{-1}s^{-1}$, a value with which it had minor influence on the mechanobiological growth map, to $1.5e11 \text{ m}^3N^{-1}s^{-1}$ where joint overgrowth was obvious as shown in Appendix 3.6. The modulating variable value which predicted physiological MC depth growth was chosen ($c = 5e10 \text{ m}^3N^{-1}s^{-1}$). The same value of c was used from 4.5 to 5 dpf. The number of jaw openings which occur over twelve hours is once again encapsulated in the modulating variable. The contribution of mechanical fields to growth was anisotropic: the same modulating variable value c was used along all anatomical axes, but the stress gradients varied between axes. The newly obtained mechanobiological growth map G_{dyn} was qualitatively compared to the growth map

calculated from free-to-move larvae G_{free} . Growth simulations were run in Abaqus CAE using G_{dyn} and qualitatively compared to growth simulations using G_{free} (Figure 5.3D). Terminologies and methods for calculation of the mechanobiological growth maps are summarised in Table 5.4.

Growth map	Description	Contribution of mechanical loads	Hypothesis tested	Mechanoregulatory growth modulating variable
G_{free}	Growth rates obtained from free-to-move cell-level data	Normal baseline	-	-
G_{imm}	Growth rates obtained from immobilised cell-level data	None	-	-
$G_{tension}$	Mechanobiological growth map	Average hydrostatic field across load history	Tensions promote growth	$a = 2e9 \text{ m}^2\text{N}^{-1}\text{s}^{-1}$
$G_{compression}$			Compressions promote growth	$b = 3e9 \text{ m}^2\text{N}^{-1}\text{s}^{-1}$
G_{dyn}		Time evolution of the hydrostatic stress gradients	Dynamic load patterns promote growth	$c = 5e10 \text{ m}^3.\text{N}^{-1}\text{s}^{-1}$

Table 5.4: Overview of growth maps terminologies and methods. a , b and c are mechanoregulatory growth modulating variables which influence the impact of the hydrostatic stress fields on the mechanobiological growth map.

5.3. Results

5.3.1. In the absence of movement, growth is differentially altered along anatomical axes

Qualitative observations were made on average free-to-move and immobilised Meckel's cartilage (MC) joint elements from 3.5 to 5.5 dpf (MC joint element is highlighted in Figure 5.4A). Free-to-move MC length increases over the whole timeframe were distinctly higher than in immobilised larvae as seen in Figure 5.4Bb, d. Immobilised MC depth remained constant from 3.5 to 5.5 dpf whereas free-to-move MC depth noticeably increased (Figure 5.4Bb, d). The decrease of free-to-move MC depth from 5 dpf (Figure 5.4Bb, green outline) to 5.5 dpf (cyan outline) is not physiological and comes from the use of different clutches of fish between ages. Free-to-move MC width at the level of the joint increased slightly over the investigated timeframe whereas immobilised MC width remained unchanged over

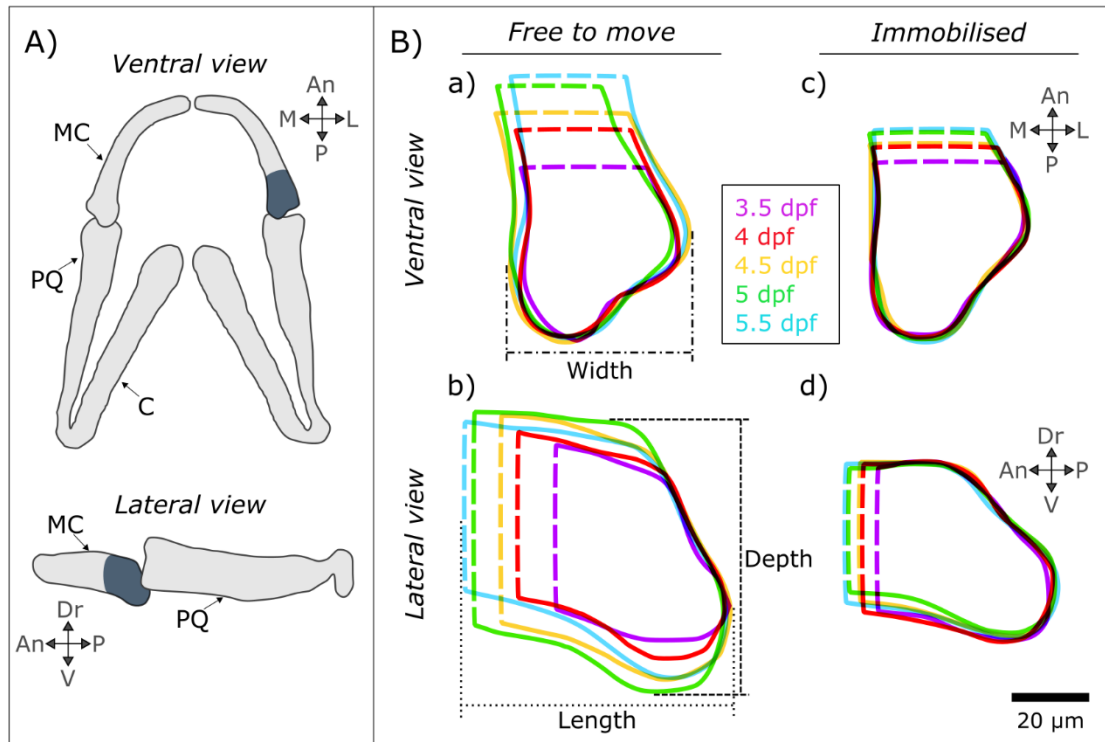


Figure 5.4: **In the absence of movement, growth is the most compromised along the ventrodorsal and anteroposterior axes.** A) Sketches of the jaw in the ventral and lateral planes showing in dark the joint element for which growth is characterised (anterior Meckel's cartilage (MC) element). B) Shape outlines of average MC joint element from 3.5 to 5.5 dpf for both free-to-move (a, b) and immobilised (c, d) larvae in the ventral (a, c) and lateral (b, d) planes. An: Anterior, C: Ceratohyal, Dr: Dorsal, L: Lateral, M: Medial, MC: Meckel's cartilage, P: Posterior, PQ: Palatoquadrate, V: Ventral.

time (Figure 5.4Ba, c). In conclusion, in the absence of movement, growth was differentially altered along anatomical axes, with depth and length increases being the most compromised.

To quantify growth pattern alterations in the absence of movement, growth rates and orientations were quantified in both free-to-move and immobilised larvae based on tracked cell-level data. Ventrodorsal (VD) growth rates (along the depth) in immobilised larvae were significantly altered in all four time-windows compared to free-to-move larvae as shown in Figure 5.5A, B. From 4 dpf, when jaw movements are well established in free-to-move larvae, there was a drop of about 0.02 h^{-1} in the average VD growth rate in the immobilised compared to the free-to-move groups in all three time-windows (4–4.5, 4.5–5 and 5–5.5 dpf) as shown in Figure 5.5B. Inconsistently with the average shape outlines shown in Figure 5.4, the VD growth rates from 3.5 to 4 dpf in free-to-move larvae were found to be negative (meaning tissue contraction). Mismatch in the order of magnitude of 3.5–4 dpf growth rates may have arisen from difficulties to image 3.5 dpf larvae due to their small size. Anteroposterior (AP) growth rates (along the length) in immobilised larvae were only significantly different from free-to-move larvae in one time window, from 5 to 5.5 dpf, as shown in Figure 5.5C, D. Surprisingly, in this

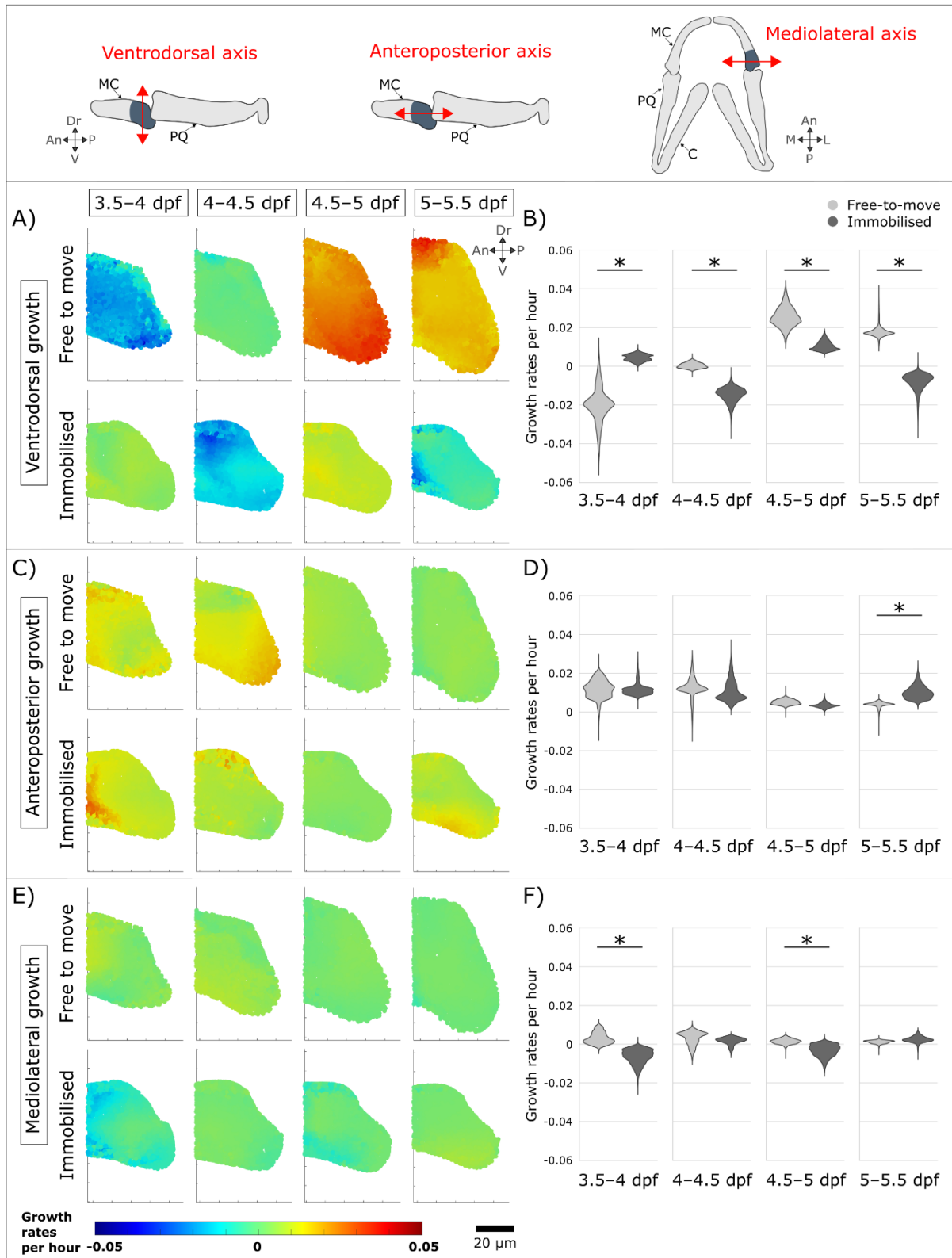


Figure 5.5: **Immobilisation leads to altered growth rates, primarily along the ventrodorsal axis.** (A, C, E) Ventrodorsal (VD)/anteroposterior (AP)/mediolateral (ML) growth rates for each time window (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf) in the MC joint element for both free-to-move and immobilised larvae. Results are displayed in one section in the mid-lateral plane. (B, E, F) Quantitative comparison of VD/AP/ML growth rates between free-to-move and immobilised groups. * indicates significant difference between

free-to-move and immobilised means ($p < 0.05$). An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, MC: Meckel's Cartilage, P: Posterior, PQ: Palatoquadrate, V: Ventral.

time window, immobilised AP growth rates were higher than free-to-move growth rates. The difference between immobilised and free-to-move AP growth rates from 5 to 5.5 dpf was about four times smaller than for VD growth rates over the same time window. Mediolateral (ML) growth rates were significantly lower in immobilised compared to free-to-move larvae from 3.5 to 4 dpf and from 4.5 to 5 dpf as shown in Figure 5.5E, F. The drop in the average ML growth rate in the immobilised compared to the free-to-move group from 4.5 to 5 dpf was three times smaller than for VD growth rates over the same time window. In conclusion, decreases in the growth rates when jaw movements were absent compared to the free-to-move groups were stronger along the ventrodorsal axis which explains the shape changes observed between immobilised and free-to-move larvae, especially regarding MC depth.

As previously performed for the free-to-move fish (described in Chapter 3), growth was simulated in free-to-move and immobilised larvae using FE methods in order to confirm that the calculated growth rates drive the observed shape changes. In growth simulations, free-to-move length increases (Figure 5.6A-ii) were higher than in immobilised larvae (Figure 5.6B-ii) as seen in real shape averages from 3.5 to 5.5 dpf. Consistent with the real shape averages, no depth increase was simulated in immobilised larvae (Figure 5.6B-ii) contrary to free-to-move larvae (Figure 5.6A-ii). No depth increase was predicted in free-to-move growth simulations from 3.5 to 4 dpf (pale red outline in Figure 5.6A-ii) which confirms the inconsistency observed with the low 3.5–4 growth rates along the ventrodorsal axis (Figure 5.5A). Nonetheless, the global patterns of free-to-move and immobilised growth were correctly simulated using the growth rates obtained from cell-level data (depth increases only in free-to-move larvae, length increases higher in free-to-move larvae). In conclusion, MC growth was differentially reduced along different anatomical axes by the absence of movements, with growth rates being most compromised along the ventrodorsal axis (depth).

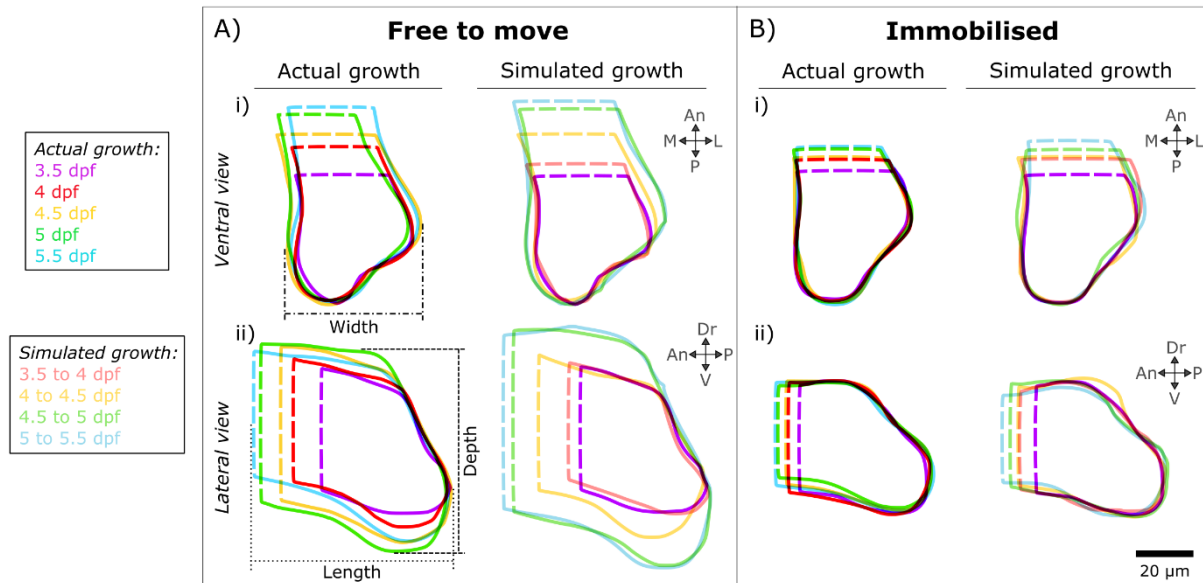


Figure 5.6: **Growth simulations correctly predict growth patterns in free-to-move and immobilised MC joint elements.** (A, B) Average shape outlines at 3.5, 4, 4.5, 5 and 5.5 dpf (left: actual growth) along with simulated growth outlines (right: simulated growth) for each twelve-hour time window (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf) for (A) free-to-move and (B) immobilised larvae in the (i) ventral and (ii) lateral planes. In each time window, the average shape at the starting point was used as the initial shape when simulating growth (e.g. 4 dpf average shape is the initial shape of 4 to 4.5 dpf growth simulation). The decrease of free-to-move MC depth in 5.5 dpf (A-ii cyan outline) compared to 5 dpf (green outline) is not physiological and makes the comparison between 5.5 actual growth and 5 to 5.5 dpf simulated growth (dark and pale cyan outlines) harder. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

5.3.2. Average hydrostatic stresses over joint motion do not directly promote jaw joint mechanoregulated growth

How do mechanical stimuli arising from jaw movements regulate jaw joint growth? From studies on cartilage explants we know that cyclic loading promotes chondrogenesis (Anderson and Johnstone 2017, Salinas et al. 2018) but which and how mechanical stimuli drives joint growth is still unclear. In this study I first test the hypothesis that the average hydrostatic stresses over joint motion promote growth which was the approach taken in previous mechanobiological models of joint morphogenesis (Shefelbine and Carter 2004b, Giorgi et al. 2014, Dixit et al. 2020). I focussed my analyses after 4 dpf, when jaw movements are well established.

Immobilised growth rates were used as the baseline of biological contributions to joint growth and were applied to the free-to-move shapes from 4 to 4.5 and 4.5 to 5 dpf (G_{imm} in Figures 5.7A-i and 5.8A-i). The simulated shapes (red outlines in Figures 5.7A-iii and 5.8A-iii) did not grow in depth, contrary to when free-to-move growth rates (G_{free} in Figures 5.7A-ii and 5.8A-ii) were used (green outlines in Figures 5.7A-iii and 5.8A-iii). Length increases from the initial shape (blue outlines) were

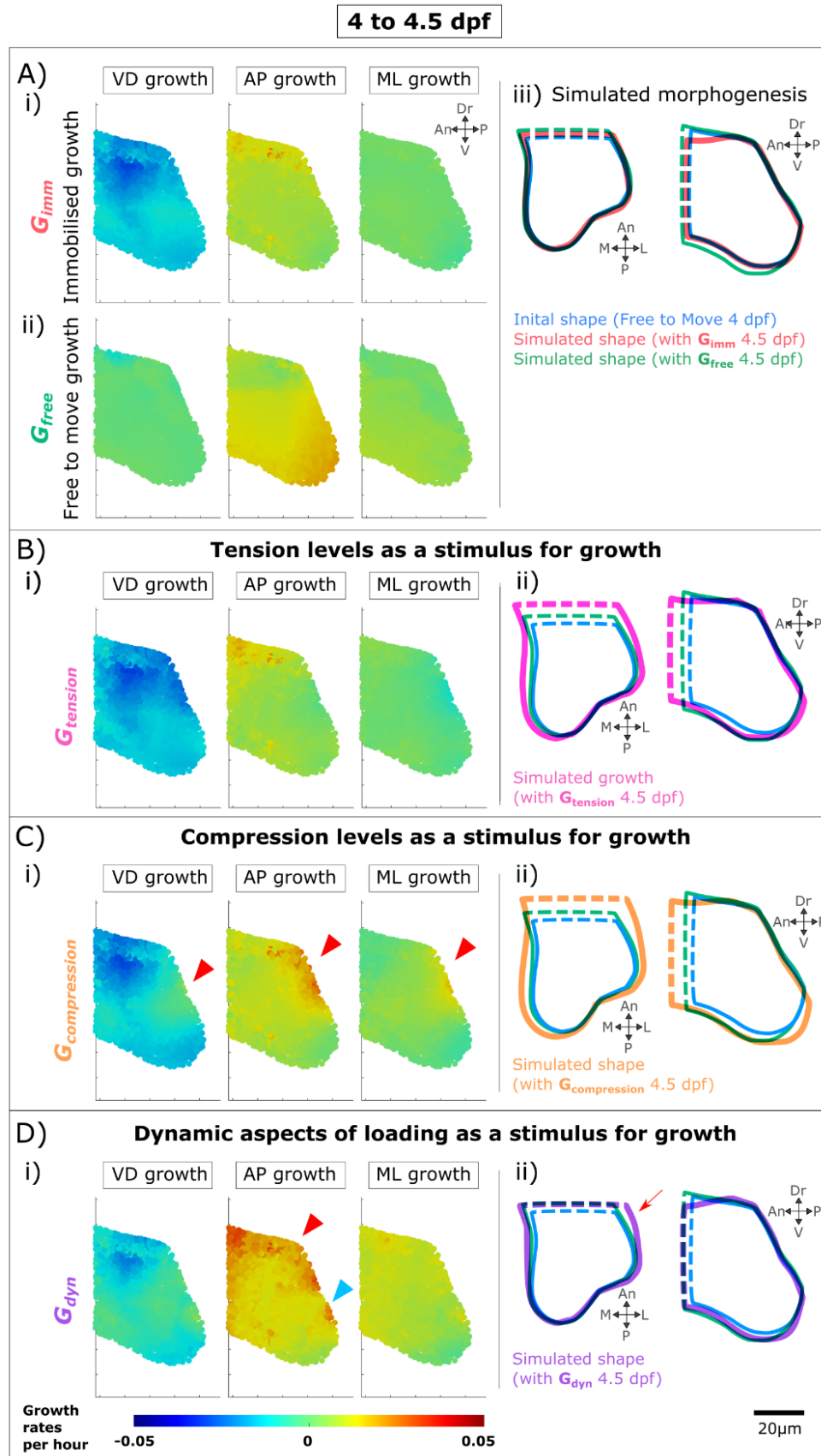


Figure 5.7: **Biological and mechanobiological contributions to jaw joint morphogenesis from 4 to 4.5 dpf.** A) Biological contributions to morphogenesis in the absence of movements lead to undergrowth of

MC depth and length compared to free-to-move. A-i) Immobilised ventrodorsal (VD), anteroposterior (AP) & mediolateral (ML) growth rates applied to free-to-move 4 dpf shape. A-ii) Free-to-move growth rates. A-iii) Outlines of simulated morphogenesis with immobilised or free-to-move growth rates promoting growth. B–C) Average hydrostatic stress levels when used as the mechanoregulatory factor fail to simulate physiological jaw joint morphogenesis. B-i) Mechanobiological growth rates with tension promoting growth. B-ii) Outlines of simulated morphogenesis with tension promoting growth. C-i) Mechanobiological growth rates with compression promoting growth. Red arrowheads point to local areas of elevated growth rates which are not physiological. C-ii) Outlines of simulated morphogenesis with compression promoting growth. D) Dynamic patterns of mechanical stimuli (from compression to tension) lead to a more physiological growth pattern than average hydrostatic stress levels. D-i) Mechanobiological growth rates integrating the dynamic patterns of hydrostatic stress gradients. Red/blue arrowheads point local areas of elevated/reduced growth rates which are not physiological. D-ii) Outlines of simulated morphogenesis. Red arrow shows MC width overgrowth. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

more pronounced using G_{free} (green outlines) than G_{imm} (red outlines) as shown in Figures 5.7A-iii and 5.8A-iii.

To include mechanical stimuli arising from movements to growth simulations, jaw movements were simulated using FE methods. Average hydrostatic stress distributions through the full joint motion were obtained at 4 dpf and at 4.5 dpf and are shown in Figures 5.9A. Average hydrostatic stresses were mostly spread in compression rather than in tension (Figure 5.9A), and a peak of compression was observed at the level of the jaw joint at both 4 and 4.5 dpf (Figure 5.9A, red arrowheads). I first tested the hypothesis that average tensions over the loading cycle promote growth. Mechanobiological growth maps $G_{tension}$ were generated from both 4 to 4.5 and 4.5 to 5 dpf by linearly combining the biological baseline G_{imm} and the average hydrostatic stress distribution at 4 dpf and 4.5 dpf respectively. For both time windows, growth maps differences between the mechanobiological growth map $G_{tension}$ and the biological baseline G_{imm} were very subtle as shown in Figures 5.7B-i and 5.8B-i. Mechanobiological growth simulations using $G_{tension}$ showed a physiological increase in MC depth, but overgrowth of both MC length and MC width (Figure 5.7B-ii). From 4.5 to 5 dpf, there was no increase of MC depth and MC length even decreased (blue asterisk in Figure 5.8B-ii) compared to growth simulations using just the immobilised growth rates G_{imm} (red outline in Figure 5.8A-iii). Therefore, from both 4 to 4.5 dpf and 4.5 to 5 dpf, jaw joint mechanoregulated morphogenesis could not be accurately simulated using averaged tension levels as the mechanobiological stimulus.

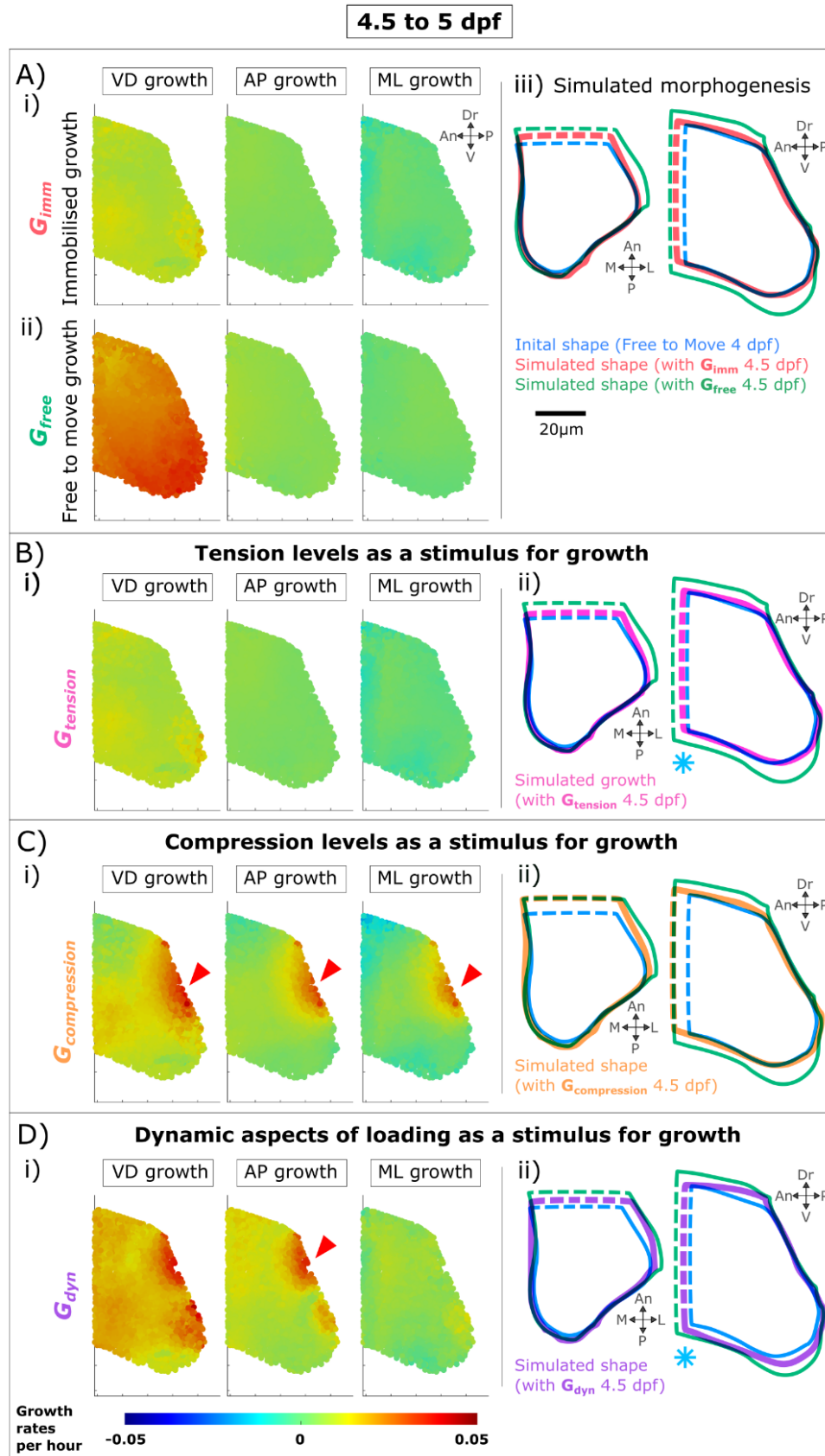


Figure 5.8: **Biological and mechanobiological contributions to jaw joint morphogenesis from 4.5 to 5 dpf.** A) Biological contributions to morphogenesis in the presence or absence of movements. A-i)

Immobilised ventrodorsal (VD)/anteroposterior (AP)/mediolateral (ML) growth rates applied to free-to-move 4 dpf shape. A-ii) Free-to-move growth rates. A-iii) Outlines of simulated morphogenesis. B) Average tension and compression levels are unlikely mechanoregulatory stimuli for jaw joint morphogenesis. B-i) Mechanobiological growth rates with tension promoting growth. B-ii) Outlines of simulated morphogenesis. C-i) Mechanobiological growth rates with compression promoting growth. Red arrowheads point local areas of elevated growth rates which are not physiological. C-ii) Outlines of simulated morphogenesis. D) Dynamic patterns of mechanical stimuli likely contribute to the mechanoregulation of jaw joint morphogenesis. D-i) Mechanobiological growth rates integrating the dynamic patterns of hydrostatic stress gradients. D-ii) Outlines of simulated morphogenesis. Blue asterisk indicates MC length shorting of the mechanobiological growth simulation compared to when Gimm is used. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

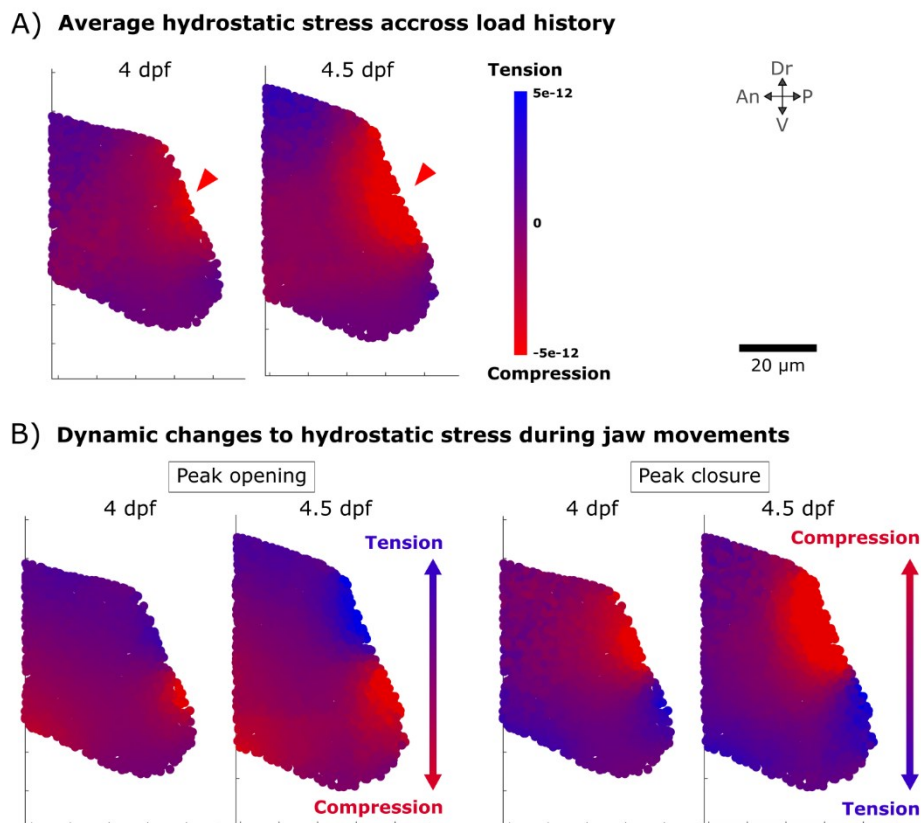


Figure 5.9: **Compression and tension levels arising from jaw movements.** A) Average hydrostatic stress distribution experienced over one cycle of mouth opening and closure at 4 and 4.5 dpf. Red arrowheads indicate a peak of compression at the level of the jaw joint. B) Dynamic changes to hydrostatic stresses from peak opening to peak closure showing a shift of hydrostatic stress gradients (from compression to tension) along the ventrodorsal axis. An: Anterior, Dr: Dorsal, P: Posterior, V: Ventral.

I then tested the hypothesis that average compressions over the loading cycle promote growth. New mechanobiological growth maps $G_{compression}$ were generated from 4 to 4.5 and 4.5 to 5 dpf. For both time windows, the mechanobiological growth maps $G_{compression}$ exhibited spots of locally increased growth rates at the level of the joint line which are not seen in the free-to-move growth maps G_{free} (Figure 5.7C-i and 5.8C-i red arrowheads). From 4 to 4.5 dpf, mechanobiological growth simulations using $G_{compression}$ showed a rescue of MD depth, but overgrowth of both MC length and MC width (Figure 5.7C-ii). From 4.5 to 5 dpf, there was a rescue of MC length but no MC depth nor width increases using $G_{compression}$ (Figure 5.8C-ii). Therefore, jaw joint mechanoregulated growth could not be accurately simulated by direct integration of average compressions over joint motion.

5.3.3. Dynamic changes in load patterns are promising candidates of jaw joint mechanoregulation

Since the direct integration of average hydrostatic stress distributions over the full joint motion failed to simulate jaw joint growth mechanoregulation, I investigated the dynamic changes in hydrostatic stress patterns during jaw motion at both 4–4.5 and 4.5–5 dpf. At peak opening, the dorsal aspect of the rudiment experiences tension whereas the ventral aspect is in compression, creating a stress gradient from tension to compression along the ventrodorsal axis (Figures 5.9B). At peak closure however, the dorsal aspect is in compression whereas the ventral aspect is in tension, creating a reversed gradient along the ventrodorsal axis compared to peak opening (Figures 5.9B). I hypothesised that the switch in the ventrodorsal hydrostatic stress gradient from peak opening to peak closure is promoting growth along the rudiment's depth. The time evolutions of hydrostatic stress gradients along the three anatomical axes were therefore calculated and combined with G_{imm} to generate a new mechanobiological growth map called G_{dyn} .

From 4 to 4.5 dpf, simulating growth using the mechanobiological growth map G_{dyn} resulted in a shape (Figure 5.7 D-ii, purple outline) which resembled the shape obtained using the free-to-move growth rates G_{free} (Figure 5.7 D-ii green outline). MC depth and length increases almost matched between G_{dyn} and G_{free} simulated shapes. MC width away from the joint line was slightly bigger in G_{dyn} simulated shape than using G_{free} (red arrow in Figure 5.7 D-ii). In terms of growth rates, the mechanobiological growth map G_{dyn} was improved compared to the immobilised growth map G_{imm} with ventrodorsal (VD) growth rates having increased overall (Figure 5.7 A-i and D-i). When compared to the free-to-move growth rates G_{free} , anteroposterior (AP) growth rates were higher at the dorsal aspect in G_{dyn} contrary to G_{free} where higher growth rates were observed at the ventral aspect (Figure 5.7 A-ii and D-i, red and blue arrowheads). G_{dyn} mediolateral (ML) growth rates were overall slightly higher compared to G_{free} explaining the slight MC width overgrowth (Figure 5.9 A-ii and D-i).

Overall, from 4 to 4.5 dpf, despite small discrepancies, the mechanobiological growth simulations using G_{dyn} were the most satisfying. It shows that the dynamic patterns of mechanical stimuli rather than direct integration of average compression or tension levels take part in jaw joint mechanoregulation.

Results from 4.5 to 5 dpf corroborated my hypothesis that dynamic patterns of mechanical stimuli promote jaw joint morphogenesis. Simulating growth using the mechanobiological growth map G_{dyn} resulted in a shape (Figure 5.8 D-ii, purple outline) much like the shape obtained using the free-to-move growth rates G_{free} (Figure 5.8 D-ii, green outline), albeit slightly smaller. The mechanobiological growth map G_{dyn} (Figure 5.8 D-i) exhibited similarities with the free-to-move growth map G_{free} (Figure 5.8 A-ii) with notably the G_{dyn} VD growth rates having increased compared to that of G_{imm} (Figure 5.8 A-i). However, a spot of higher AP growth rates was seen at the level of the joint line in G_{dyn} (Figure 5.8 D-i) which was not observed in the free-to-move growth rates G_{free} (Figure 5.8 D-ii). Overall, from both 4 to 4.5 and 4.5 to 5 dpf, integrating the dynamic hydrostatic stress patterns across jaw joint motion resulted in more faithful growth simulations than when the average tensions and compressions are directly used. Dynamic switches from tension to compression are a promising candidate of jaw joint mechanoregulation.

5.4. Discussion

In this research, growth patterns of zebrafish jaw joint morphogenesis were analysed and simulated in the presence or absence of movement. Results show that growth rates in the absence of movement are differentially reduced along different anatomical axes compared to free-to-move specimens, with ventrodorsal growth being the most compromised. Synthesising cell-level data into mechanobiological models of jaw joint growth enabled me to test hypotheses regarding to how mechanical stimuli experienced by joint elements over motion promote growth. The simulation results highlighted the role of dynamic changes to loading in influencing growth dynamics.

Experimental studies have previously shown how movements influence joint shapes and reported a loss of interlocking shape features and a general lack of growth when these movements are disrupted (Sotiriou et al. 2019, Bridglal et al. 2020). In addition to shape comparisons at the macro-scale, I directly quantified growth patterns in the presence or absence of movement revealing that mechanical stimuli from embryonic movements influence growth anisotropy in the zebrafish jaw. It is known that, in the absence of muscle contraction, oriented cell behaviours which may impact growth anisotropy are disrupted. A notable change in cell behaviour is the abnormal intercalation of hypertrophic chondrocytes into disorganised stacks which is observed in the pharyngeal cartilage of paralysed larval zebrafish (Shwartz et al. 2012, Brunt et al. 2016b). Cell morphology is also altered in the absence of

muscle contraction with cells from immobilised larvae being rounder and smaller compared to free-to-move zebrafish (Shwartz et al. 2012, Brunt et al. 2016b). Alterations of both cell intercalation and cell morphology were also observed in embryonic growth plates of muscleless-limb mice, suggesting that growth anisotropy is important in multiple aspects of skeletal development (Shwartz et al. 2012).

Despite evidence at the macro- and cell-levels, the process of mechano-regulated growth in joints is still unclear. The current literature lacks understanding of which mechanical stimuli influence transient cartilage growth. In articular cartilage tissue engineering, studies showed that compressive dynamic loading has a positive impact on chondrocytes with increased gene expression and extracellular matrix production (Mauck et al. 2000, Kisiday et al. 2004), whereas static loads tend to downregulate chondrogenesis (Davisson et al. 2002, Sharma et al. 2007). Dynamic hydrostatic pressure loading has been shown to promote growth in articular cartilage tissue engineering bioreactors (Elder and Athanasiou 2009, Correia et al. 2012). To assess how such mechanical stimuli may influence transient cartilage growth and especially joint shaping, mechanobiological models of joint growth have been used. Incorporation of average hydrostatic compressions arising over joint motion into mechanobiological simulations enabled prediction of the emergence of shape features seen under normal or altered loading conditions (Giorgi et al. 2015, Dixit et al. 2020). In this research however, the mechanobiological simulations in which compressive or tensile hydrostatic stress distributions, averaged over joint motion, were used as promoters of mechanoregulated growth did not accurately predict growth and morphogenesis of the zebrafish jaw joint. Rather, I found that the dynamic changes from tension to compression are the most likely stimuli influencing growth. This demonstrates that joint mechanoregulated growth is not directly and solely linked to the magnitude of mechanical stimuli experienced over motion. This discovery fits with data from cartilage tissue engineering in which static loading does not have a positive influence on chondrogenesis contrary to dynamic loading (Grodzinsky et al. 2000, Sharma et al. 2007). In the embryo, static loading—through rigid paralysis (continuous activation of muscle contraction)—disturbs joint morphology in larval zebrafish jaws and embryonic chick limbs (Osborne et al. 2002, Brunt et al. 2016b). Another mechanobiological model of axolotl salamander joint growth which used poroelastic material properties suggested that the pressure patterns arising from static loading did not correlate with tissue growth (Comellas et al. 2022). It is believed that static loads change the extracellular environment of the chondrocytes through increased ion concentrations and osmolarity through fluid expression, which then has a degenerative effect on chondrocyte metabolism (Milner et al. 2012). Chondrocyte metabolism is indeed mediated by activation of ion channels. A number of these channels, such as the calcium permeable transient reception potential vanilloid type 4 (TRPV4) channel, are gated by osmolarity and mechanical stimuli (Phan et al. 2009, Lewis et al. 2011, Khatib et al. 2022). TRPV4 is a major channel which is

downregulated in high osmotic (hypertonic) environments and which has recently been shown to be crucial for joint growth mechano-regulation (Phan et al. 2009, Khatib et al. 2022). Consistently, Zhou et al. (2016) showed that increased osmolarity reduces calcium signalling in bovine juvenile and adult articular cartilage. Activation of ion channels during joint morphogenesis is therefore a key element of the underlying mechanisms of joint mechano-regulated growth.

A strength of this research is the direct synthesis of tracked cell-level data in my mechanobiological models when previous mechanobiological simulations of joint growth used extrapolated cell data and hypothesised how they impact growth rates. Previous computational simulations assumed that the biological contributions to joint growth were proportional to chondrocyte density (D'Costa and Shepherd 2009, Giorgi et al. 2015, Dixit et al. 2020). In this research, zebrafish jaw joint growth at the macro-scale was directly quantified from tissue geometry changes at the cellular level. This enabled precise and specific characterisation of the cell-level growth and therefore less uncertainty when testing different hypotheses regarding to how mechanical stimuli influence growth.

Though the zebrafish jaw joint has many similarities with mammalian synovial joints, a major difference lies in the delayed cavitation compared to when most of the morphological changes take place (Askary et al. 2016). However, this research investigates a critical time of joint morphogenesis, right after movements are established, and provide valuable insights to deepen our understanding of the processes driving joint morphogenesis. Another difference between zebrafish and larger mammal models is the small size of the larvae and thus the small number of chondrocytes constituting the jaw. It is therefore possible that individual cell behaviours have a great impact on tissue shape and thus that oriented behaviours trigger pronounced growth anisotropy in the zebrafish. Growth anisotropy may be less noticeable in larger animal models.

A discrepancy was observed within the 5.5 dpf average shape with MC depth being smaller than 5 dpf MC depth. This decrease in depth was not physiological and comes from the use of different clutches of fish at 5 dpf and at 5.5 dpf. It has limited my ability to perform the same mechanobiological analyses as from 4 to 4.5 and 4.5 to 5 dpf. However, it is unlikely that new insights would have been obtained from the analysis of a third time window given that my results were consistent between the two analysed windows. Other limitations in this work arose from modelling muscles as point loads in fixed directions which resulted in minor errors to the direction during jaw motion and in non-physiologic stress concentrations at the muscle insertion sites. In future work, the cross section of a tendon could rather be represented by a small contact area and muscle' force and direction as a combination of axial and shear stresses. Another limitation lies in the determination of the mechanoregulatory growth modulating variables through incremental testing. Since no formal optimisation was pursued, slight

variations in the most optimised mechanoregulatory growth modulating variables are expected. Similarly, quantification of simulation performances was not conducted and conclusions were drawn from visual inspection only.

In conclusion, insights on the mechanoregulation of joint morphogenesis were obtained from the synthesis of biological cell-level data into novel mechanobiological models of joint growth. Marked changes in growth anisotropy between free-to-move and immobilised zebrafish larvae were correlated with dynamic patterns of mechanical stimuli. Overall, this research offers avenues for improvement in simulations of joint development and potentially other organs. It provides advance in our understanding of mechanoregulation in the developing joint and increases our understanding of the origins of conditions such as hip dysplasia and arthrogryposis.

Chapter 6

6. Mechanoregulated cell dynamics underlying murine joint morphogenesis

6.1. Introduction

Despite advances made in understanding the drivers of joint morphogenesis, the cellular dynamics underlying it are still poorly characterised. As detailed in Section 2.2.1, experimental studies on the early processes of joint morphogenesis have shown that asymmetric expression of several active cell processes between and within the two opposing skeletal elements in the longitudinal and ventrodorsal axes, such as influx of progenitors, cell proliferation and cell differentiation, may mould joint surfaces into interlocking and reciprocal shapes ([Pacifci et al. 2005](#), [Kahn et al. 2009](#), [Shwartz et al. 2016](#), [Zhang et al. 2020](#)). Cell morphometric observations in the murine elbow joint and the zebrafish jaw joint have suggested that cell size, orientation and arrangements are other processes that contribute to joint morphogenesis ([Shwartz et al. 2012](#), [Brunt et al. 2015](#)). Other prenatal model systems such as limb buds or growth plates have given insights on the role that cellular dynamics may have on growth. Computational models of the murine limb bud has shown that spatially heterogeneous rates of cell proliferation alone are not sufficient to predict correct bud elongation ([Boehm et al. 2010](#), [Marcon et al. 2011](#)). Rather, growth simulations showed that murine limb bud elongation is driven by anisotropic behaviours correlated with cell orientation and oriented cell division ([Boehm et al. 2010](#), [Marcon et al. 2011](#)). Morphometric analyses of large number of cells in prenatal murine tibial growth plates have shown that chondrocyte volume, orientation and density are spatially differentially distributed along the longitudinal axis giving an insight of how each zone of the growth plate contributes to its elongation ([Rubin et al. 2021](#)). Recently, in our group, similar analyses were performed in the murine distal humerus at several developmental stages and showed that increases in cell size and number are important drivers of joint morphogenesis ([Huang 2022](#)). Cell size and number were also disturbed in the absence of skeletal muscles ([Huang 2022](#)), reinforcing that the cellular dynamics involved in joint

shaping adapt in response to mechanical loading with consequences for joint morphology as detailed in Section 2.2.2.2 (Roddy et al. 2011a, Roddy et al. 2011b, Schwartz et al. 2012, Roddy et al. 2017). Such analyses have prompted questions on the contribution of these cellular dynamics to tissue changes at the macro-scale level. Translating co-ordinated cell dynamics into tissue growth by the means of computational techniques would greatly deepen our understanding of the processes involved in joint morphogenesis.

In Chapters 3 and 5, modes of growth were explored in the zebrafish jaw joint giving new insights on synovial joint morphogenesis. The very small number of cells in the zebrafish has however limited my ability to generalise results because morphological changes of individual cells had important consequences on joint shape. Global cell behaviours were therefore hard to identify and separating out relative contributions unfeasible. The mouse overcomes these disadvantages. As a mammal, it is also more applicable to human joints with joint shapes being more similar and the timing of cavitation analogous. In this Chapter, the methodology used in the first Chapters of this thesis was expanded to the murine distal humerus. Growth rates between several developmental stages were quantified and the contribution of several key cellular dynamics—cell proliferation, ECM deposition and cell volume expansion—were decomposed. This was done for both normal mouse embryos and Splotch-delayed ($Pax3^{Sp-d/Sp-d}$) muscleless-limb embryos to quantify the extent to which cell activities involved in joint shaping are impacted by skeletal muscles.

6.2. Methods

6.2.1. Data collection

In this Chapter, numerical data of segmented cells in the murine distal humeri of control and Splotch-delayed ($Pax3^{Sp-d/Sp-d}$) muscleless-limb embryos at several developmental stages were processed. The numerical cell data sets encapsulate the chondrocytes' position, shape and size, and were provided by Dr. Huang, which were obtained as follows. Humeri of control and Splotch-delayed ($Pax3^{Sp-d/Sp-d}$) muscleless-limb embryos at several developmental stages from Theiler stage TS23 (corresponding to embryonic day e14.5) to TS27 (e18.5, close to birth) were imaged at high resolution using inline phase contrast 3D microtomography at Diamond Light Source Ltd. in Oxfordshire, UK. Beamline I13-2. Raw scans (Figure 6.1A) were filtered in Fiji to isolate distal humeri's chondrocytes (light grey dots in Figure 6.1B) from other tissues and from the extracellular matrix (Schindelin et al. 2012). In a previous study, analyses showed that embryonic chondrocytes from murine distal humeri of control and Splotch-delayed ($Pax3^{Sp-d/Sp-d}$) embryos at TS24 and TS27 imaged by phase contrast enhanced synchrotron tomography had similar size and shape as when they were stained for histology (Pierantoni et al. 2021)

as shown in Figure 6.2. Chondrocytes in the distal humeri of the filtered image stacks were segmented in Fiji using the 3D spot segmentation plugin from the 3D ImageJ Suite ([Ollion et al. 2013](#)). Examples of labelled cells are shown in Figure 6.1C. Cells were then approximated as ellipsoids and measurements including their centroid positions, cell radii, unit vectors for cell axes and cell volume were returned. Details are provided in Dr. Huang's thesis ([2022](#)).

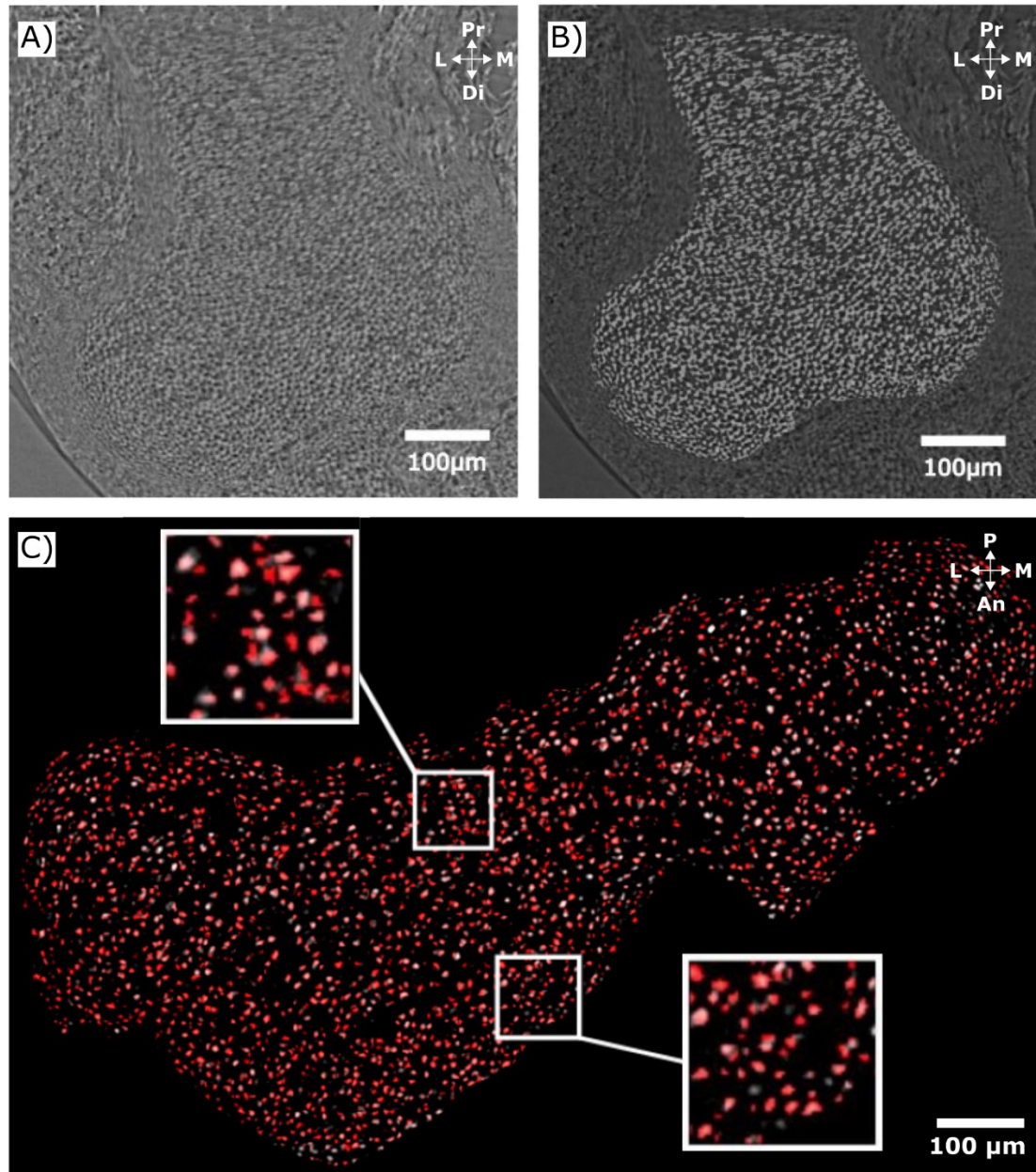


Figure 6.1: **Process of cell segmentation from synchrotron image stacks.** (A) Raw synchrotron image stack in the coronal plane. (B) Segmentation and thresholding of the distal humerus highlighting cells (shown in the coronal plane). (C) Cell segmentation in the axial plane. Labelled cells show up in red while cells which were not segmented appear in grey. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal. From ([Huang 2022](#)).

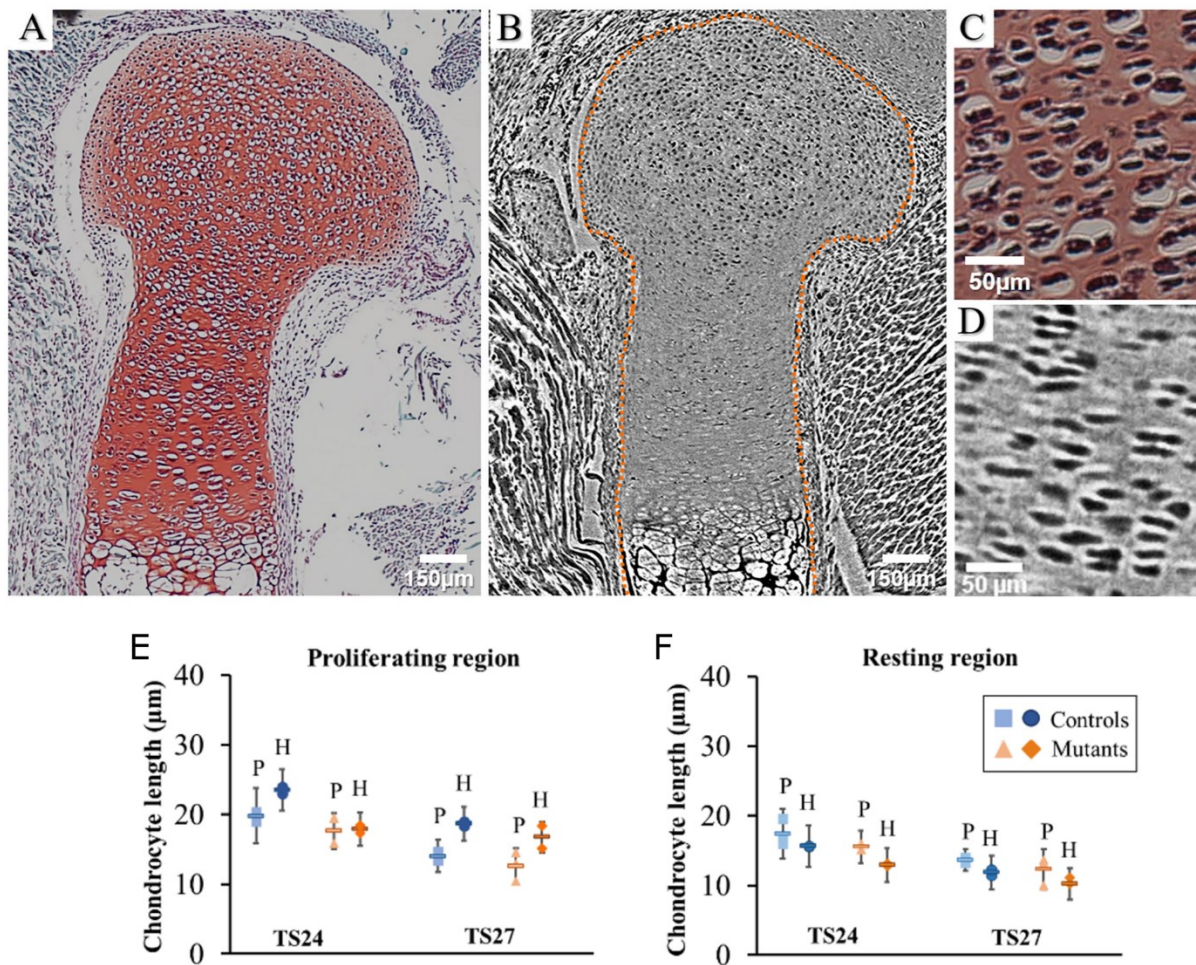


Figure 6.2: There was a good correlation between chondrocytes' characteristics imaged using phase contrast enhanced tomography and histological methods. (A) Safranin O - fast green stained histological slices and (B) tomographic longitudinal view for a TS27 control. To ease comparison, the contrast was inverted in the phase contrast images (compared to Figure 6.1) so chondrocytes appear darker, similarly to the histology staining. The edges of the limb are highlighted by a line. (C) and (D) Higher magnification of proliferating chondrocytes imaged from a histological slice and by phase contrast synchrotron tomography, respectively. (E) Chondrocytes sizes in the proliferating and (F) resting regions. In the graphs: H= Manually measured values from histological slicing (of two controls and two mutants at both TS24 and TS27 based on 25 to 30 chondrocytes for each region) and P= values obtained with the automated 3D analysis of the tomographic volumes. Mean values and standard deviation are also indicated. Figure and legend from (Pierantoni et al. 2021).

6.2.2. Morphometric analyses

From the isolated distal humerus cell data, I generated a volume part for all samples in Mimics (Materialise NV, Leuven, Belgium) and exported them in 3-matic (Materialise NV, Leuven, Belgium) where they were cropped proximally where the medial and lateral condyles converge into the humeral diaphysis. The remaining epiphysis volume was calculated for each sample. The distal humeri from all

developmental stages and groups were manually aligned in 3-matic at mid condyle inflection and transformation matrices describing this alignment exported. Rudiments' width and condylar depth were measured. To test for significant differences between developmental stages of controls and muscleless-limbs, Kruskal-Wallis tests were performed ($p < 0.05$), followed by Conover-Iman post-hoc tests for pairwise comparisons with adjustment for multiple testing using the False Discovery Rate (FDR). Mann-Whitney U tests ($p < 0.05$) were performed to assess the differences between controls and muscleless-limbs during individual developmental stages.

6.2.3. Characterising tissue growth from cell-level data

From the provided spreadsheets of cell/ellipsoid measurements, I performed growth analyses following the workflow described below and illustrated in Figure 6.3. One muscleless-limb sample at TS23 was removed from the analyses due to a complete absence of the medial condyle which, if included, would create a discrepancy between the number of samples taken into account in each humeral region (e.g. condyles and humerus shaft) which was detrimental for the numerical calculations. Experimental groups and n numbers are listed in Table 6.1.

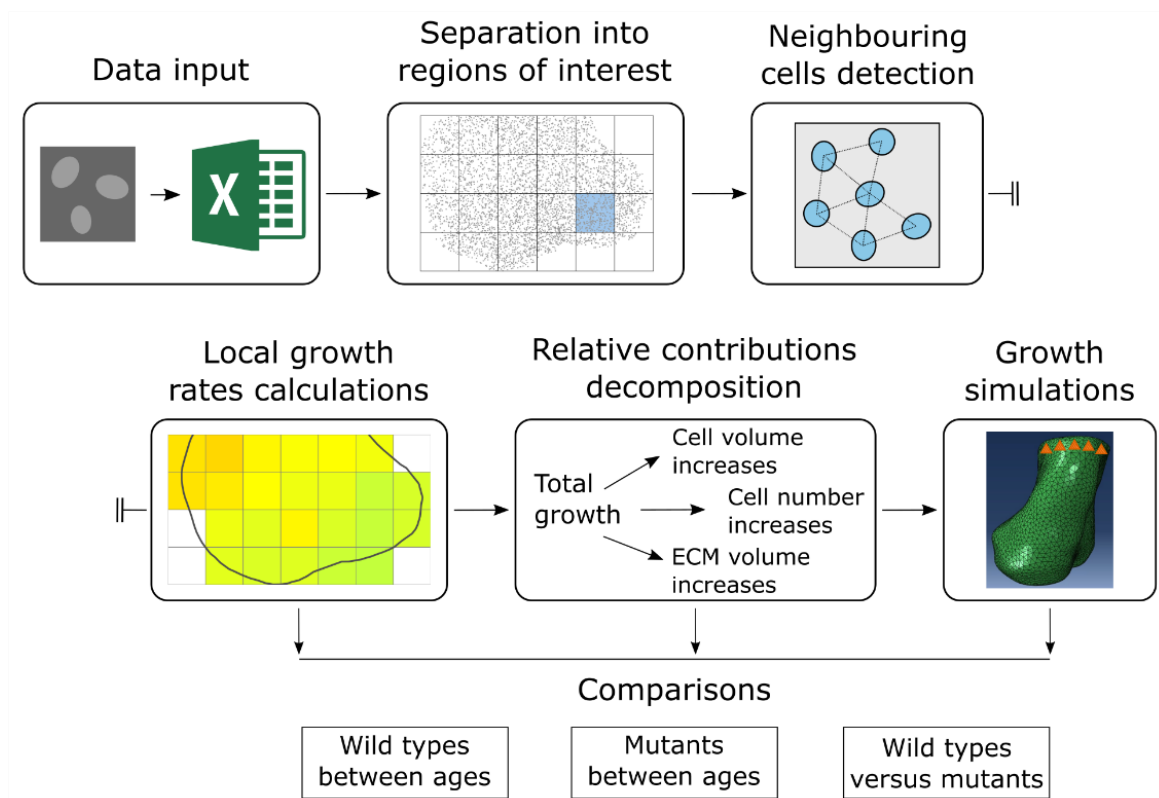


Figure 6.3: **Growth analyses workflow.**

Type	Theiler stages	n number
Control	TS23	4
	TS24	4
	TS25	4
	TS27	4
Muscleless-limb	TS23	3
	TS24	4
	TS27	4

Table 6.1: Experimental groups and n number.

6.2.3.1. Separation into regions of interests

I imported the provided cell/ellipsoid measurements into MATLAB (R2018a, The MathWorks, Inc., Natick, Massachusetts, United States). Transformation matrices exported from 3-matic and which describe sample alignments (see section 6.1.2) were used to accordingly align the cell centroids of each sample in MATLAB. To perform subsequent growth calculations in local regions, all specimens were separated out into cubic regions of interest (ROIs). To build the ROIs, a cubic grid of side length 100 μm was superimposed on a representative control rudiment at TS23 (specimen number 4) in MATLAB. For the rest of the specimens, to ensure that ROIs consistently represented the same anatomical area across samples and over time, e.g. the medial condyle, a ratio (r) between the width of the specimen and the width of the representative specimen was calculated, then a cubic grid of side length $r \times 100 \mu\text{m}$ was superimposed on the specimen as illustrated in Figure 6.4A. Specimen widths, ratios and grid sizes are reported in Appendix 4.1.

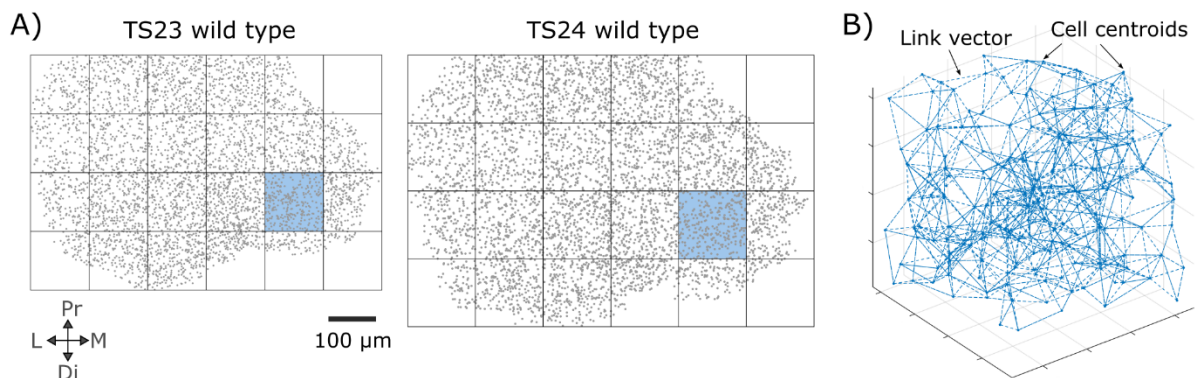


Figure 6.4: **ROI definition and neighbouring cells detection.** (A) ROIs of two representative specimens at TS23 and TS24 controls in the frontal view. ROIs represent the same anatomical area over time as illustrated with the blue box. (B) Representative 3D motif obtained when neighbouring cell centroids within a ROI are connected by link vectors, zoomed in region.

6.2.3.2. Neighbouring cells detection

In order to quantify local tissue geometries—formed by cell arrangements—I devised a way to calculate the connectivity of the cells. For each ROI, cells within the ROI's limits were detected and their neighbouring cells searched within a limit radius, called the neighbour search limit distance, specific to each specimen. For later growth calculation, the method employed for detecting neighbouring cells is not a decisive factor as long as it is consistently applied across all samples. Some parameters were therefore arbitrarily chosen. The neighbour search limit distance was estimated from the distances between each cell and its twelve closest cells in each ROI. A gaussian curve was fitted to the distribution of these distances. The z-score corresponding to 75% of the set was obtained for each ROI and then averaged across all ROIs. The obtained average was set as the specimen's neighbour search limit distance. The neighbour search limit distances for each specimen are reported in Appendix 4.1. Visual verification was performed by ensuring that the motif obtained when vectors linking neighbouring cell centroids were plotted such as shown in Figure 6.4B was alike across specimens in term of density of connections.

6.2.3.3. Local growth rates calculations

Next, growth rates between two timepoints (t and $t+\Delta t$) were calculated in each ROI based on equations from Graner et al. (2008) as done previously with the zebrafish. These equations however had to be adapted because of the impossibility of tracking cells over time. According to Graner et al, and like for the zebrafish, the relative position of cell centroids in an ROI, at a timepoint t and in a specimen s , forms a pattern which can be described by a texture matrix $M_{t,s}$ calculated from link vectors l connecting neighbouring cell centroids (Figure 6.5, t panel). Such texture matrices M_s^t were calculated in each ROI for each specimen.

$$M_{t,s} = \langle l \otimes l \rangle \quad (6.1)$$

where $\langle \dots \rangle$ denotes averaging taking all links within an ROI into consideration, $\otimes$ is the outer (or tensor) product, l is a link vector connecting two neighbouring cells, $M_{t,s}$ is the texture matrix within an ROI at time point t , in specimen s . $M_{t,s}$ has the dimension of $[m^2]$ and, in 3D, is a 3x3 matrix.

Then, in each ROI, and unlike with the zebrafish, the average texture matrix across samples was calculated for each time point t (e.g. TS23) and was called M_t . M_t has the dimension of $[m^2]$. Over time the link vectors l may change in length and direction, changing the pattern's overall geometry. As a reminder, the “statistical symmetrised velocity gradient” defined in Chapter 3 (matrix V equation 3.2) (Graner et al. 2008) is a tool to describe at which rate and in which direction the pattern of the tissue

deforms between timepoints t and $t+\Delta t$. A second equation was derived for the growth tensor V by Graner et al (2008):

$$V = \frac{BM^{-1}}{2} \quad (6.2)$$

where B describes the change in tissue patterns exclusively from link vectors l which have not undergone topological rearrangement, that is which have not appeared nor disappeared as a result to cell division or death between successive timepoints.

$$B = \left\langle \frac{d(l \otimes l)}{dt} \right\rangle \quad (6.3)$$

where l is a link vector connecting two neighbouring cells present in both successive timepoints, $\langle \dots \rangle$ denotes averaging taking all links present in both successive timepoints within an ROI into consideration, $\otimes$ is the outer (or tensor) product.

Because cell tracking was not possible, I set the assumption that new links created between t and $t+\Delta t$ (due to cell division) form a similar geometry than the one when all the links are taken into account. It implies that the creation of new links between t and $t+\Delta t$ is homogeneous within a ROI with no specific direction. Under this assumption B is equivalent to the variation of the texture matrix M between t and $t+\Delta t$.

$$B \sim \frac{\Delta M}{\Delta t} \quad (6.4)$$

where ΔM is the variation of the texture matrices between t and $t+\Delta t$ in the ROI ($\Delta M = M_{t+\Delta t} - M_t$ where M_t and $M_{t+\Delta t}$ are the texture matrices of the ROI at time points t and $t+\Delta t$ respectively).

Equation 6.2 therefore writes:

$$V \sim \frac{\Delta M}{\Delta t} M^{-1} / 2 \quad (6.5)$$

where V is the statistical velocity gradient matrix between time points t and $t+\Delta t$ within an ROI, Δt is the time interval between time points t and $t+\Delta t$, ΔM is the variation of the texture matrices between t and $t+\Delta t$ in the ROI ($\Delta M = M_{t+\Delta t} - M_t$ where M_t and $M_{t+\Delta t}$ are the texture matrices of the ROI at time points t and $t+\Delta t$ respectively), M^{-1} is the inverse matrix of M , where M is the mean between M^t and $M^{t+\Delta t}$, which was a requirement for deriving equation 6.2 (Graner et al. 2008).

The growth tensor V describes local tissue expansions or contractions due to the increase or decrease of the distance between cell centroids (resulting from cell volume expansion/contraction and/or ECM volume increase/decrease) as shown in Figure 6.5, case 1. As discussed in Chapter 3, V is a statistical equivalent of a strain rate based on discrete objects.

To account for the pronounced increase in the number of cells seen in the mouse model over gestation, a second term was considered, the “statistical topological rearrangement rate” (growth tensor P of dimension 3×3) which characterises the effect of topological changes on the cell pattern (Graner et al. 2008). It quantifies the impact that the creation or destruction of link vectors l between t and $t+\Delta t$ (due to cell division or death) has on tissue growth as illustrated in Figure 6.5, case 2. Similarly than for V , P is a statistical description based on discrete objects equivalent to the plastic strain rate. It is the continuous counterpart of individual topological changes T when a new link is created, that is, when cells have divided (the destruction of links was neglected because the number of cells increased overtime in the datasets). P writes as:

$$P = \frac{TM^{-1}}{2} \quad (6.6)$$

$$T = \dot{n}_a M_a \quad (6.7)$$

where P is the statistical topological rearrangement rate matrix between time points t and $t+\Delta t$ within an ROI, $\dot{n}_a$ is the rate of link appearance per unit time, M_a is the cell pattern (texture matrix) formed by link vectors which have appeared between t and $t+\Delta t$, M^{-1} is the inverse matrix of M , where M is the mean between M^t and $M^{t+\Delta t}$ for consistency with equation 6.5.

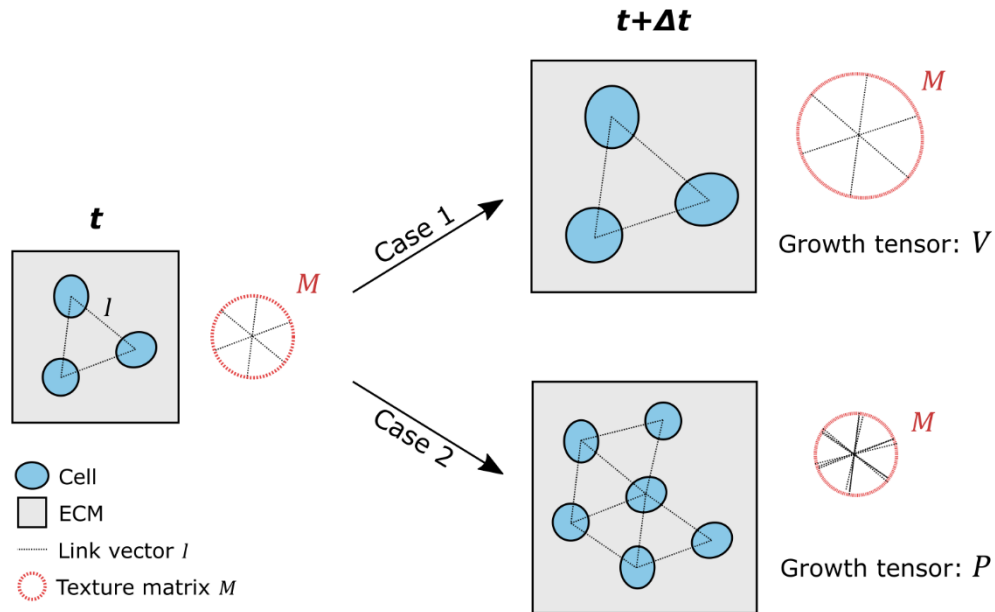


Figure 6.5: **Local growth rates calculations.** In an ROI, link vectors l connecting neighbouring cells are used to calculate the texture matrix M which describes the local tissue geometry and is here represented by a sphere. Between t and $t+1$, the distance between neighbouring cells may increase due to cell volume expansion and/or ECM deposition (case 1). Tissue growth in this case is described by the growth tensor V . Alternatively, tissue growth may be due to an increase in the number of cells (case 2) and is described by the tensor P . In a biological tissue, growth occurs as a combination of cases 1 and 2.

The rate of link appearance per unit time $\dot{n}_a$ is set by Graner et al. (2008) as:

$$\dot{n}_a = \frac{1}{\Delta t} \frac{\Delta N_a}{N_{tot}} \quad (6.8)$$

where ΔN_a is the number of appeared links (that exist at $t+\Delta t$ but not yet at t), Δt is the time interval between time points t and $t+\Delta t$, N_{tot} is the total number of links.

Here I approximate the number of links at a time point t as proportional to the average number of cells across samples at this time point called nbC_t , and the number of appeared links as proportional to the variation between t and $t+\Delta t$ of the average number of cells $\Delta nbC = nbC_{t+\Delta t} - nbC_t$. M_a , the cell pattern or texture matrix formed by link vectors which have appeared between t and $t+\Delta t$, is here considered to be $M_{t+\Delta t}$, the texture matrix at time point $t+\Delta t$ when all the links are taken into account. This approximation holds under the previously made assumption that new links created between t and $t+\Delta t$ (due to cell division) form a similar geometry than the one when all the links are taken into account. Once again, it implies that the creation of new links between t and $t+\Delta t$ is homogeneous within a ROI with no specific direction. It resulted that:

$$P \sim \frac{1}{\Delta t} \frac{\Delta nbC}{nbC_{t+\Delta t}} M_{t+\Delta t} M^{-1}/2 \quad (6.9)$$

where P is the statistical topological rearrangement rate matrix between time points t and $t+\Delta t$ within an ROI, ΔnbC is the variation between t and $t+\Delta t$ of the average number of cells, Δt is the time interval between t and $t+\Delta t$, $nbC_{t+\Delta t}$ is the average number of cells at $t+\Delta t$, $M_{t+\Delta t}$ is the texture matrix at time point $t+\Delta t$, M^{-1} is the inverse matrix of M , where M is the mean between M^t and $M^{t+\Delta t}$ for consistency with equation 6.5.

Taken together, V and P completely characterise the rate of changes in tissue strain. V describes the rate of tissue expansions due to the increase or decrease of the distance between cell centroids (Figure 6.5, case 1), and P describes the rate of tissue changes due to the appearance of new cells (Figure 6.5, case 2). The total growth tensor is set as $G = V + P$ (6.10). It is a second order tensor of dimension 3x3 and as the dimension of a strain rate [s^{-1}]. Equations 6.5, 6.9 and 6.10 give:

$$G \sim \frac{\Delta M}{\Delta t} M^{-1}/2 + \frac{1}{\Delta t} \frac{\Delta nbC}{nbC_{t+\Delta t}} M_{t+\Delta t} M^{-1}/2 \quad (6.11)$$

where Δt is the time interval between time points t and $t+\Delta t$; ΔM is the variation of the texture matrices between t and $t+\Delta t$ in the ROI ($\Delta M = M_{t+\Delta t} - M_t$ where M_t and $M_{t+\Delta t}$ are the texture matrices of the ROI at time points t and $t+\Delta t$ respectively); M^{-1} is the inverse matrix of M , where M is the mean between M^t and $M^{t+\Delta t}$ for consistency with equation 6.5; ΔnbC is the variation between t and $t+\Delta t$ of the average number of cells; $nbC_{t+\Delta t}$ is the average number of cells at $t+\Delta t$, $M_{t+\Delta t}$ is the texture matrix at time point $t+\Delta t$

Because cells could not be tracked over time, the direction of link deformation could not be assessed unlike with the zebrafish. Consequently, the obtained matrices G at each ROI can be represented by a sphere whose radius correspond to the local tissue growth rate. Maps of local growth rates were generated from the growth tensors G for each of the time windows listed in Figure 6.6.

6.2.4. Quantifying the relative contribution of cell processes to growth

The impacts of three drivers for growth—cell volume, ECM deposition and cell number changes—were explored by successively simulating growth when each of these drivers was kept constant or was the only active one. This step could not be performed for zebrafish due to the small number of cells and the fact that they were closely packed. New tissue material created between t and $t+\Delta t$, was schematically separated into cell and ECM material deposition. A simplified representation was adopted to further decompose the former into volume increases of “existing cells at time t ” and “newly formed cells between t and $t+\Delta t$ ”. Mathematically, I therefore decomposed the local growth tensor G , which represents total growth, into the followings growth tensors:

- $G_{cell\ volume}$ due to a volume increase of “existing cells at time t ”
- $G_{new\ cell\ volume}$ due to the appearance of “newly formed cells between t and $t+\Delta t$ ”
- G_{ecm} solely due to an increase in ECM volume

Figure 6.7 provides a scheme of this representation and how each three contributions to overall tissue growth was mathematically assessed is detailed below. For each vector link l , the distance covered by the cell cytoplasm, l_{cell} , and the one covered by the ECM, l_{ecm} (see Figure 6.8), were calculated using the cell ellipsoid radii and the direction of its axes. Therefore:

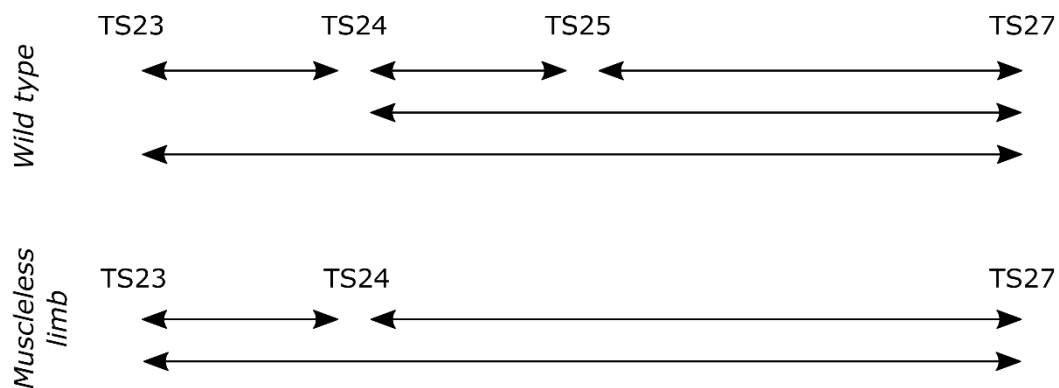


Figure 6.6: **List of time windows for which growth rate calculations were performed.** These time windows enable comparison between experimental groups and between ages.

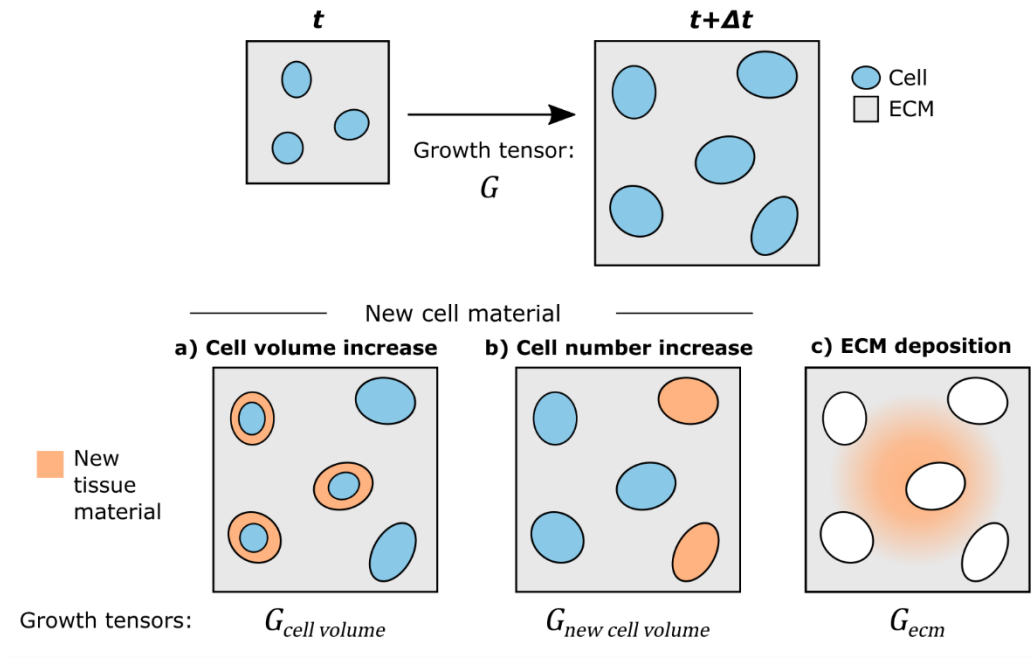


Figure 6.7: New tissue material arises from increases of cell volume (a), cell number (b) and ECM volume (c).

$$l = l_{cell} + l_{ecm} \quad (6.6)$$

and

$$M = M_{cell} + M_{ecm} + M_{c.e} + M_{e.c} \quad (6.7)$$

where

$$M_{cell} = \langle l_{cell} \otimes l_{cell} \rangle \quad (6.8)$$

$$M_{ecm} = \langle l_{ecm} \otimes l_{ecm} \rangle \quad (6.9)$$

$$M_{c.e} = \langle l_{cell} \otimes l_{ecm} \rangle \quad (6.10)$$

$$M_{e.c} = \langle l_{ecm} \otimes l_{cell} \rangle \quad (6.11)$$

where l_{cell} is the distance covered by the cells' cytoplasm and l_{ecm} the distance covered by ECM between two neighbouring cells, $\langle \dots \rangle$ denotes averaging taking all links within an ROI into consideration, $\otimes$ is the outer (or tensor) product, M_{cell} , M_{ecm} , $M_{c.e}$ and $M_{e.c}$ are texture matrices.

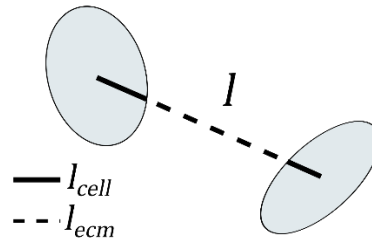


Figure 6.8: Link vectors l connecting two neighbouring cells are decomposed into the distance covered by the cell cytoplasm, l_{cell} , and the distance covered by the ECM, l_{ecm} .

We set:

$$V_{cell} = \frac{\Delta M_{cell}}{\Delta t} M^{-1}/2 \quad (6.12)$$

$$P_{cell} = -\frac{1}{\Delta t} \frac{\Delta nbC}{nbC} M_{cell}^{t+1} M^{-1}/2 \quad (6.13)$$

$$V_{ecm} = \frac{\Delta M_{ecm}}{\Delta t} M^{-1}/2 \quad (6.14)$$

$$P_{ecm} = -\frac{1}{\Delta t} \frac{\Delta nbC}{nbC} M_{ecm}^{t+1} M^{-1}/2 \quad (6.15)$$

$$V_{c.e} = \frac{\Delta M_{c.e}}{\Delta t} M^{-1}/2 \quad (6.16)$$

$$P_{c.e} = -\frac{1}{\Delta t} \frac{\Delta nbC}{nbC} M_{c.e}^{t+1} M^{-1}/2 \quad (6.17)$$

$$V_{e.c} = \frac{\Delta M_{e.c}}{\Delta t} M^{-1}/2 \quad (6.18)$$

$$P_{e.c} = -\frac{1}{\Delta t} \frac{\Delta nbC}{nbC} M_{e.c}^{t+1} M^{-1}/2 \quad (6.19)$$

where Δt is the time interval between time points t and $t+\Delta t$; ΔM_{cell} , ΔM_{ecm} , $\Delta M_{c.e}$ and $\Delta M_{e.c}$ are the variations of the texture matrices M_{cell} , M_{ecm} , $M_{c.e}$ and $M_{e.c}$ between t and $t+\Delta t$ in the ROI (e.g. $\Delta M_{cell} = M_{cell}^{t+\Delta t} - M_{cell}^t$ where M_{cell}^t and $M_{cell}^{t+\Delta t}$ are the texture matrices M_{cell} of the ROI at time points t and $t+\Delta t$ respectively); M^{-1} is the inverse matrix of M , where M is the mean between M^t and $M^{t+\Delta t}$; ΔnbC is the variation between t and $t+\Delta t$ of the average number of cells, nbC is the mean between the average number of cells across samples at t and $t+\Delta t$ (nbC^t and $nbC^{t+\Delta t}$ respectively).

Therefore:

$$V = V_{cell} + V_{ecm} + V_{c.e} + V_{e.c} \quad (6.20)$$

$$P = P_{cell} + P_{ecm} + P_{c.e} + P_{e.c} \quad (6.21)$$

The contribution of cell volume change to the overall tissue growth shown in Figure 6.7a and called $G_{cell\ volume}$, refers to the change of tissue geometry solely due to an increase of l_{cell} over time (as a reminder, l_{cell} is the distance covered by the cell cytoplasm between two cell centroids when l is the total distance as shown in Figure 6.7). It is described by the sum of V_{cell} and $V_{c.e} + V_{e.c}$ when l_{ecm} (the distance covered by the ECM) is assumed constant over time and was here estimated as:

$$G_{cell\ volume} \sim V_{cell} + (V_{c.e} + V_{e.c}) \cdot \frac{V_{cell}}{V_{cell} + V_{ecm}} \quad (6.22)$$

V_{cell} is not impacted by increases in the distance covered by the ECM (increases in l_{ecm}) but $V_{c.e} + V_{e.c}$ is. $(V_{c.e} + V_{e.c}) \cdot \frac{V_{cell}}{V_{cell} + V_{ecm}}$ is therefore an estimation of which fraction of $(V_{c.e} + V_{e.c})$ is due to an increase of l_{cell} solely (when l_{ecm} is assumed constant over time). Tests were performed to estimate this fraction as detailed in Appendix 4.2.

The contribution of newly formed cells between t and $t+1$ to tissue growth, $G_{new\ cell\ volume}$, is estimated from the change in cell number between t and $t+1$ as shown in Figure 6.7b. It is mathematically described by the quantities P_{cell} and $P_{c.e} + P_{e.c}$ when l_{ecm} is assumed constant over time. To estimate which fraction of $P_{c.e} + P_{e.c}$ was due to an increase of l_{cell} solely, the same tests

than for $(V_{c.e} + V_{e.c})$ detailed in Appendix 4.2 were performed and the same conclusions were drawn. Therefore, the impact of new cell volume was estimated as:

$$G_{new\ cell\ volume} \sim -P_{cell} - (P_{c.e} + P_{e.c}) \cdot \frac{P_{cell}}{P_{cell} + P_{ecm}} \quad (6.23)$$

The impact of ECM deposition to tissue growth G_{ecm} (Figure 6.7c), corresponds to the change of tissue geometry due to an increase of l_{ecm} and is encapsulated in both quantities V and P when l_{cell} is assumed constant over time. The total contribution of ECM deposition to tissue growth was approximated as:

$$G_{ecm} \sim V_{ecm} + (V_{c.e} + V_{e.c}) \cdot \frac{V_{ecm}}{V_{cell} + V_{ecm}} - P_{ecm} - (P_{c.e} + P_{e.c}) \cdot \frac{P_{ecm}}{P_{cell} + P_{ecm}} \quad (6.24)$$

6.2.5. Simulating embryonic murine humerus growth with a finite element model

6.2.5.1. Initial shape generation

In order to simulate growth using finite element (FE) methods, initial shapes were generated as an average of the original shapes listed in Appendix 4.1. In MATLAB, aligned rudiments were divided into slices of 10 μm depth in the transversal plane, an example is shown in Figure 6.9A. For each slice, a shape outline was obtained from the cell centroids for each specimen and an average outline was generated at each timepoint as shown in Figure 6.9B, C. Averaged shape outlines were saved as image stacks and imported into Mimics where resultant average shapes were generated at each timepoint.

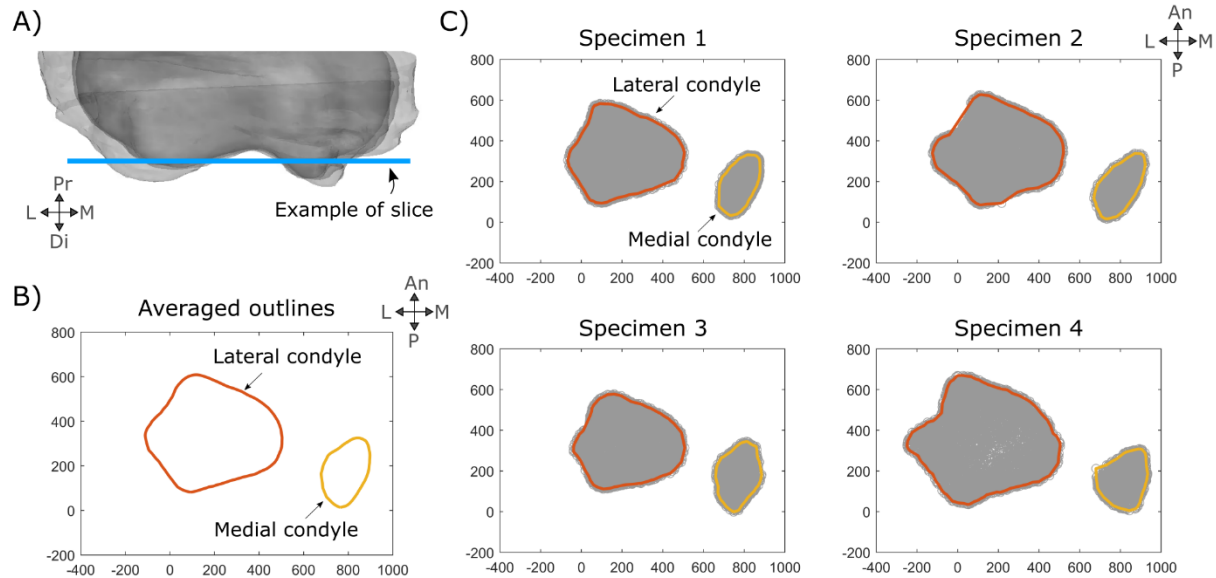


Figure 6.9: Shape averaging. (A) For each experimental group, distal humeri are aligned and sliced transversally. (B, C) For each slice, average outlines (B) are obtained from the shape outlines of each specimen (C). The slice marked in blue in (A) is shown as an example in (B, C). Units are μm . An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

In 3-matic, the average shapes were meshed with ten node tetrahedral elements and exported to Abaqus CAE (Dassault Systemes, 2019) where the models listed in Figure 6.6 were created.

6.2.5.2. Material properties and boundary conditions

In Abaqus, all models were assigned homogeneous isotropic elastic material properties with Poisson's ratio 0.3 and Young's Modulus 1.11 MPa based on bending measurements taken on unmineralized embryonic mouse ribs (Tanck et al. 2004). The proximal end of the humerus was fixed along the proximodistal direction as shown in Figure 6.10.

6.2.5.3. Growth maps integration

The growth maps deriving from cell-level data were incorporated into the models to induce growth. For each model, strains derived from the associated growth maps (growth rates multiplied by the considered the time-interval) were imported into Abaqus CAE as an analytical mapped field and applied to the model. The coordinates of the ROI centres were assigned the calculated strains and interpolation was performed between ROI centres to assign strains to each element. Isotropic growth equal to the assigned strains was applied for each time-window. The same process was repeated for 'full', constant cell size, constant ECM volume and constant cell number simulations (refer to Table 6.2).

6.2.5.4. Quantitative analysis of "full" simulation accuracy

"Full" simulations were compared to the average shape at the older age using the part comparison tool in 3-matic. For each time window, the simulated shape and the average shape at the older age were manually aligned and the simulated shape was cropped to match the height of the average shape. Part comparison, an automated tool which calculates the distance between surface nodes of two parts, was performed. The outcome map was superposed to the simulated shape. Median, and 1st and 3rd quartile distances were extracted.

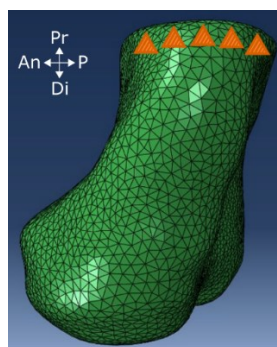


Figure 6.10: **Abaqus model showing boundary conditions.** The proximal end was fixed along the proximodistal axis as marked by orange triangles. An: anterior; Di: distal; P: posterior; Pr: proximal.

Simulations	Drivers for growth taken into account			Growth tensors
	<i>Cell size increase</i>	<i>ECM volume increase</i>	<i>Increase in cell number</i>	
'Full' simulation	x	x	x	G
Constant cell size		x	x	$G - G_{cell\ volume}$
Constant ECM volume	x		x	$G - G_{ecm}$
Constant cell number	x	x		$G - G_{new\ cell\ volume}$

Table 6.2: Simulations of embryonic murine humerus growth, performed for controls and muscleless-limbs and for all time windows listed in Figure 6.6.

6.2.5.5. Estimation of the percentage of growth in “partial” growth simulations

For each time window, the mean growth rates for the four growth simulations (“full” simulation, constant cell size, constant ECM volume and constant cell number simulations) were calculated. The percentage of growth in a simulation was approximated as the ratio of the mean growth rate of the considered simulation over the mean growth rate of the “full” simulation.

6.3. Results

6.3.1. Dynamics of growth in developing joint are spatially and temporally heterogeneous

The murine distal humerus undergoes pronounced growth and shape changes from TS23 (embryonic day e14.5) to TS27 (e18.5) as shown in Figure 6.11A&D, with significant increases in volume (Figure 6.11E), epiphyseal width (Figure 6.11F) and condylar depths (Figure 6.11G) over the entire period studied (TS23–TS27). Growth did not progress linearly over the three time windows studied, with the most pronounced increase in epiphysis volume evident at TS25 (Figure 6.11E). Significant differences ($p < 0.05$) between timeframes were only found between TS23 and TS25, between TS23 and TS27, and between TS24 and TS27, indicating that changes in volume and shape between TS23 & TS24 and between TS25 & TS27 were subtle (Figure 6.11). Particularly evident in the shape changes over the entire timeframe was outgrowth of the lateral condyles on the anterior aspect, as highlighted with arrows in Figure 6.11B–D.

A 3D growth map was obtained from numerical data of cells' positions in the prenatal murine distal humerus from TS23 to TS27. The growth map gives a quantitative and absolute measure of the local growth which occurs throughout the considered time window and is expressed in volume increase per unit volume (strain). From TS23 to TS27, the posterior side of the humerus exhibited local areas of elevated growth (red nodes in Figure 6.12), whereas local areas of very low growth were seen on the anterior side, proximally to the lateral condyle (blue nodes in Figure 6.12), creating an anterior-

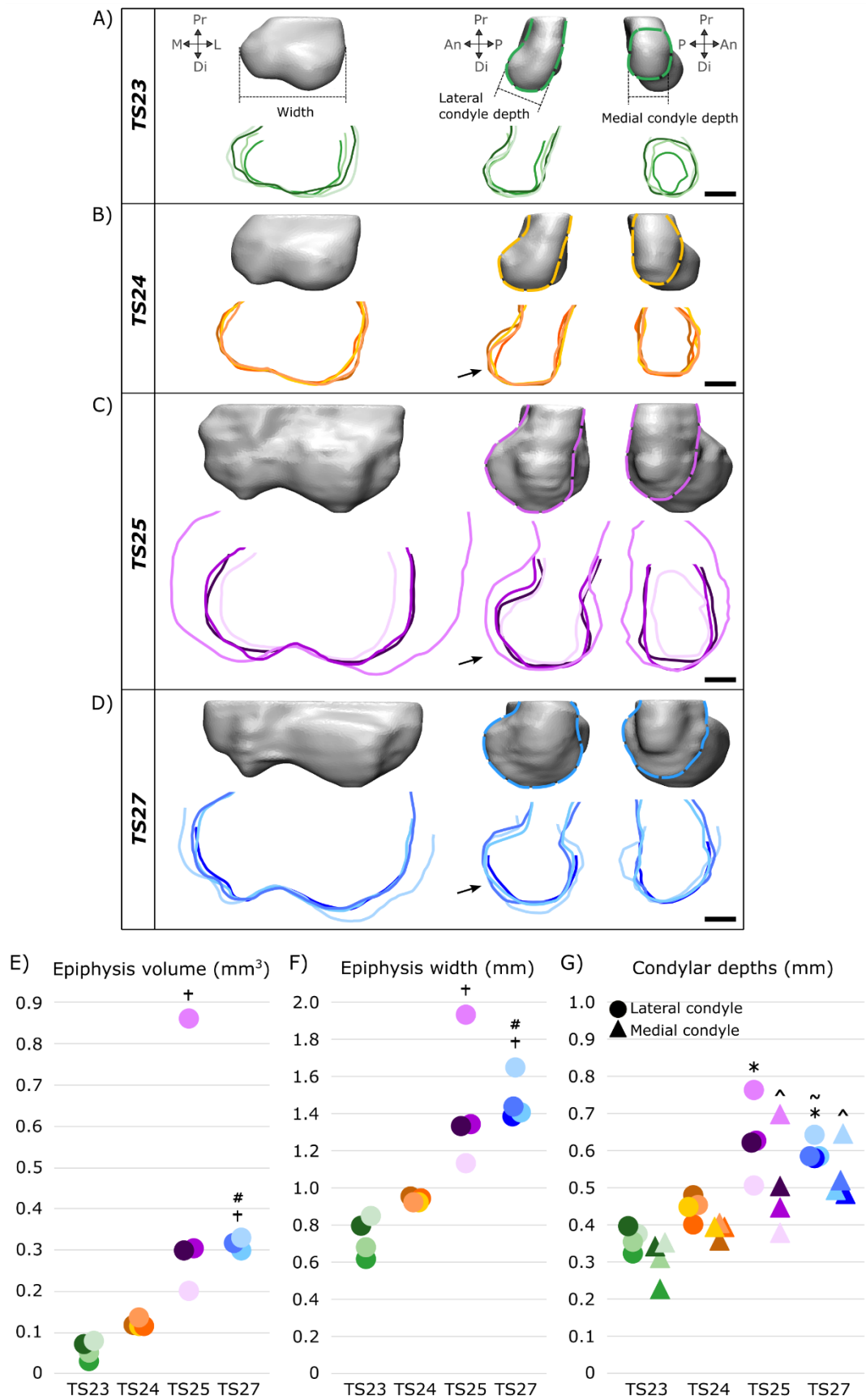


Figure 6.11: Temporal changes in distal humerus shape and size between TS23 and TS27 show that growth does not progress linearly. (A–D) 3D views of average control distal humeri along with original

shape outlines in the frontal, lateral and medial planes at TS23 (A), TS24 (B), TS25 (C) and TS27 (D) (n=4 at each stage). In the 3D views, outlines of the lateral (middle) and medial (right) condyles are shown with dashed lines. Arrows show anterior outgrowth of the lateral condyle. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal. Scale bars are 200µm. (E) Epiphysis volume for each sample (n=4 at each stage except for TS25 where n=3). (F) Epiphysis widths, as annotated in (A) (n=4 at each stage). (G) Lateral and medial condyle depths as annotated in (A) (n=4 at each stage). † indicates significant difference ($p<0.05$) with TS23 group. # indicates significant difference ($p<0.05$) with TS24 group. * indicates significant difference ($p<0.05$) with TS23 group for the lateral condyle. ~ indicates significant difference ($p<0.05$) with TS24 group for the lateral condyle. ^ indicates significant difference ($p<0.05$) with TS23 group for the medial condyle.

posterior bias symbolised by an arrow in Figure 6.12B. I hypothesise that high local growth rates at the posterior aspect, combined with a form of restraint of the posterior aspect which interacts with the ulna, promote anterior outgrowth of the lateral condyle as shown in Figure 6.11B–D.

The full timeframe TS23–TS27 was decomposed into three time-windows: TS23–TS24, TS24–TS25 and TS25–TS27. Growth was most pronounced from TS24 to TS25 with an average growth across the rudiment reaching 67% of the average growth occurring over the full timeframe TS23–TS27 (Figure 6.13B) which is consistent with the growth patterns observed on the rudiment shapes in Figure 6.11. The average growth across the rudiment from TS23 to TS24 reached only 23% of the full timeframe growth (Figure 6.13A) and only 6 % from TS25 to TS27 (Figure 6.13C).

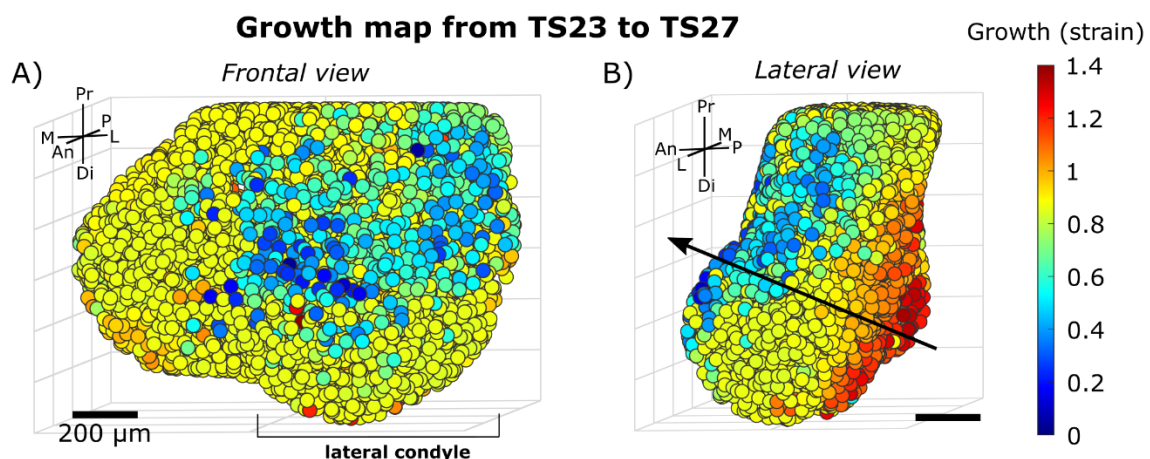


Figure 6.12: **Growth in the TS23–TS27 control humerus is higher posteriorly than anteriorly.** (A, B) External frontal (B) and lateral (C) views of the 3D TS23–TS27 growth map calculated from cell-level data of control samples. Arrow indicates a bias towards high growth in the posterior (red nodes) compared to anterior (blue nodes) aspects. Dots represent nodes from the meshed volume which was used for morphogenesis simulations. Scale bars are 200µm. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

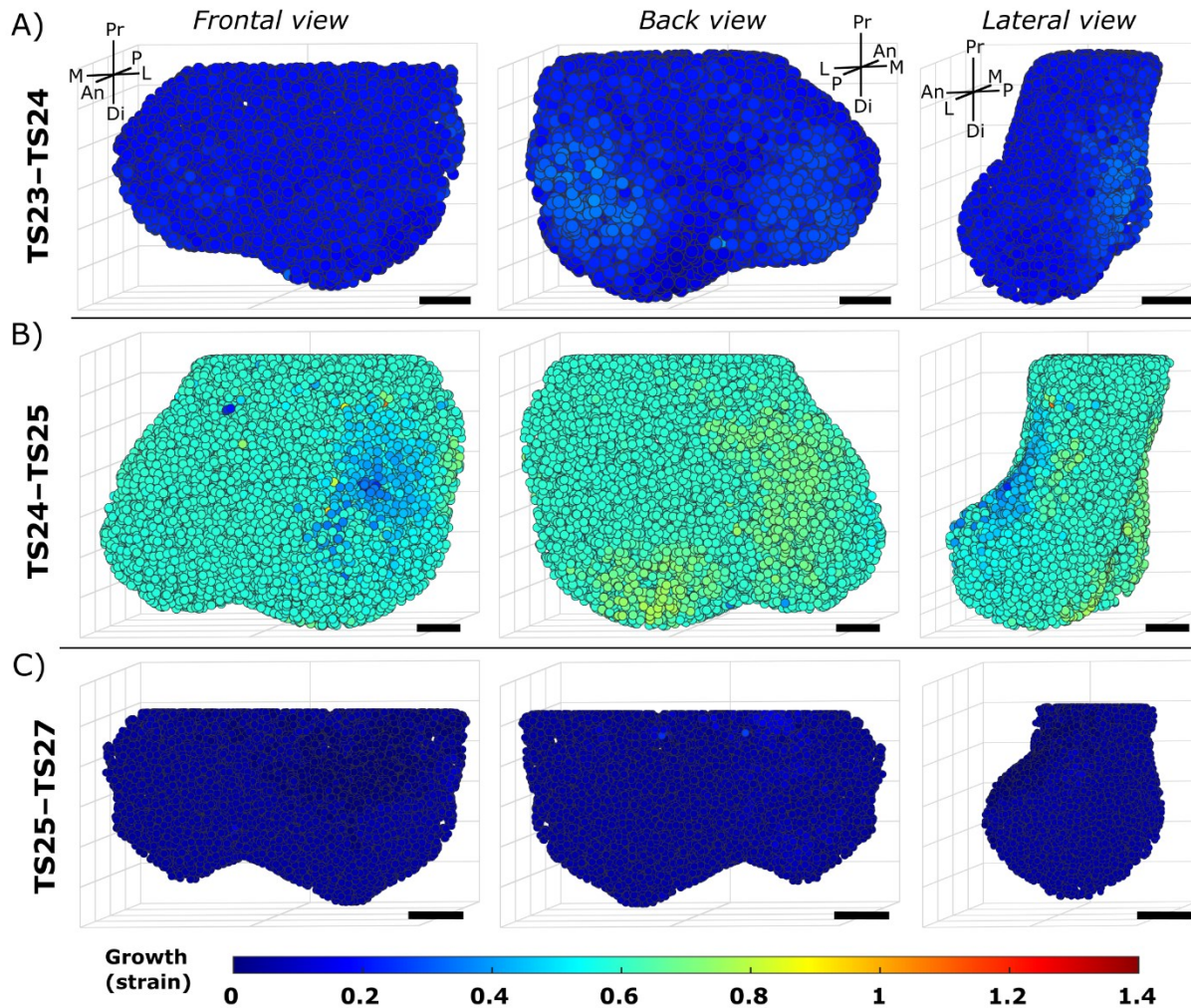


Figure 6.13: **Growth in prenatal murine distal humerus is the most pronounced from TS24 to TS25.** (A–C) External views of 3D growth maps control humeri from TS23 to TS24 (A), TS24 to TS25 (B) and TS25 to TS27 (C) calculated from cell-level data. Dots represent nodes from the meshed volume which was used for morphogenesis simulations. Scale bars are 200 μm . An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

Growth in the distal humerus was simulated over the entire timeframe using FE methods. From TS23–TS27, the simulated shape visually resembled the original shapes at TS27, with the rudiments being of similar size (Figure 6.14 B–C) and outgrowth of the lateral condyle being correctly replicated (Figure 6.14 B–C, hollow arrowheads), proving that the necessary drivers for shape determination were captured. Part comparison analyses (Chargo et al. 2019) were performed to quantitatively assess the distances between surface nodes of the simulated shapes and of the stage-matched average real shapes. Overall, surface nodes of the simulated shapes were very close to those of the average TS27 shape (Figure 6.14D). 75% of the simulated surface nodes were at a maximum distance of 47 μm from the average TS27 shape, which corresponds to about 3% of the average rudiment width at TS27 (3rd quartile in Figure 6.14D).

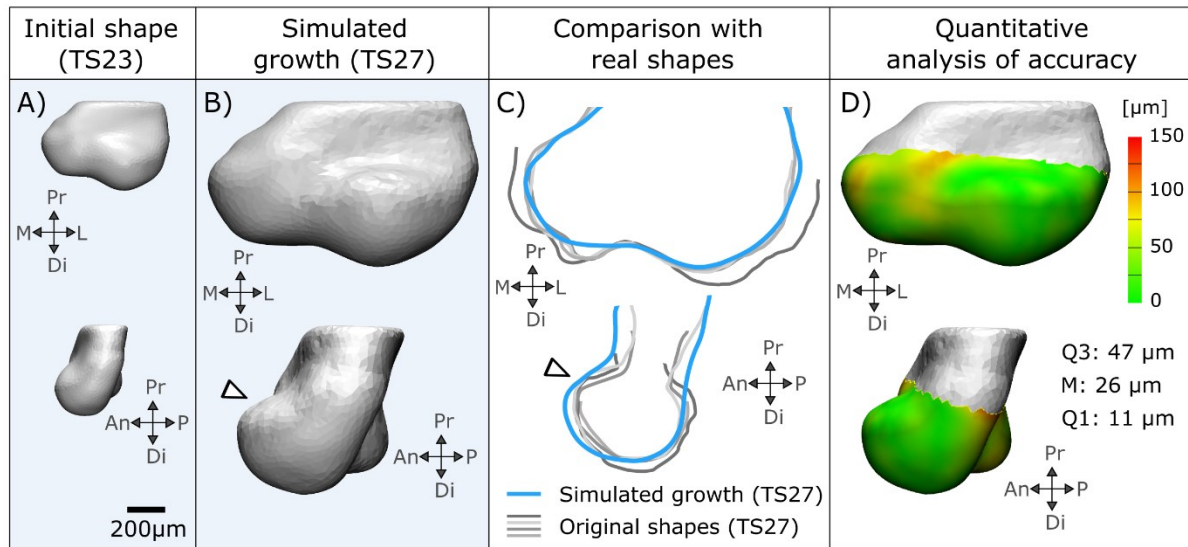
Growth simulations from TS23 to TS27

Figure 6.14: **Accurate 3D growth simulation from TS23 to TS27.** (A, B) The TS23 to TS27 growth map (Figure 6.12) was applied to the average TS23 shape (A) and growth was simulated (B). (C) The resultant shape was visually similar to the original shapes at TS27 and exhibits anterior outgrowth of the lateral condyle (hollow arrowhead). (D) Part comparison analysis shows strong resemblance between the simulated shape and the average TS27 shape. The simulated shape has been cropped to match the height of the average TS27 shape. 1st and 3rd quartiles of distance from surface nodes along with median distance are shown. Scale bars are 200 µm. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

Similarly, computational simulations of growth for each of the individual time-windows accurately simulated size and shape changes. For all time windows, surface nodes of the simulated shapes were very close to those of the stage-matched average real shapes with distances between surface nodes being very small compared to the width of the humerus (Figure 6.15). In all simulated shapes, at least 75% of the surface nodes were at a distance of less than 3.7% of the width of the stage-matched average real shapes (Figure 6.15A–D). The high levels of accuracy of the simulations compared to the actual shapes over growth indicate that cell number, cell volume and matrix volume are sufficient to simulate growth and morphogenesis of the prenatal joint over the timescale assessed, and therefore indicating that growth anisotropy (which could not be characterised) is not a critical driver to murine joint morphogenesis over the studied period. To sum up, growth in the distal humerus varies over time, with growth being most pronounced from TS24 to TS25, and anterior outgrowth of the lateral condyle seems to be driven by spatially heterogeneous growth dynamics.

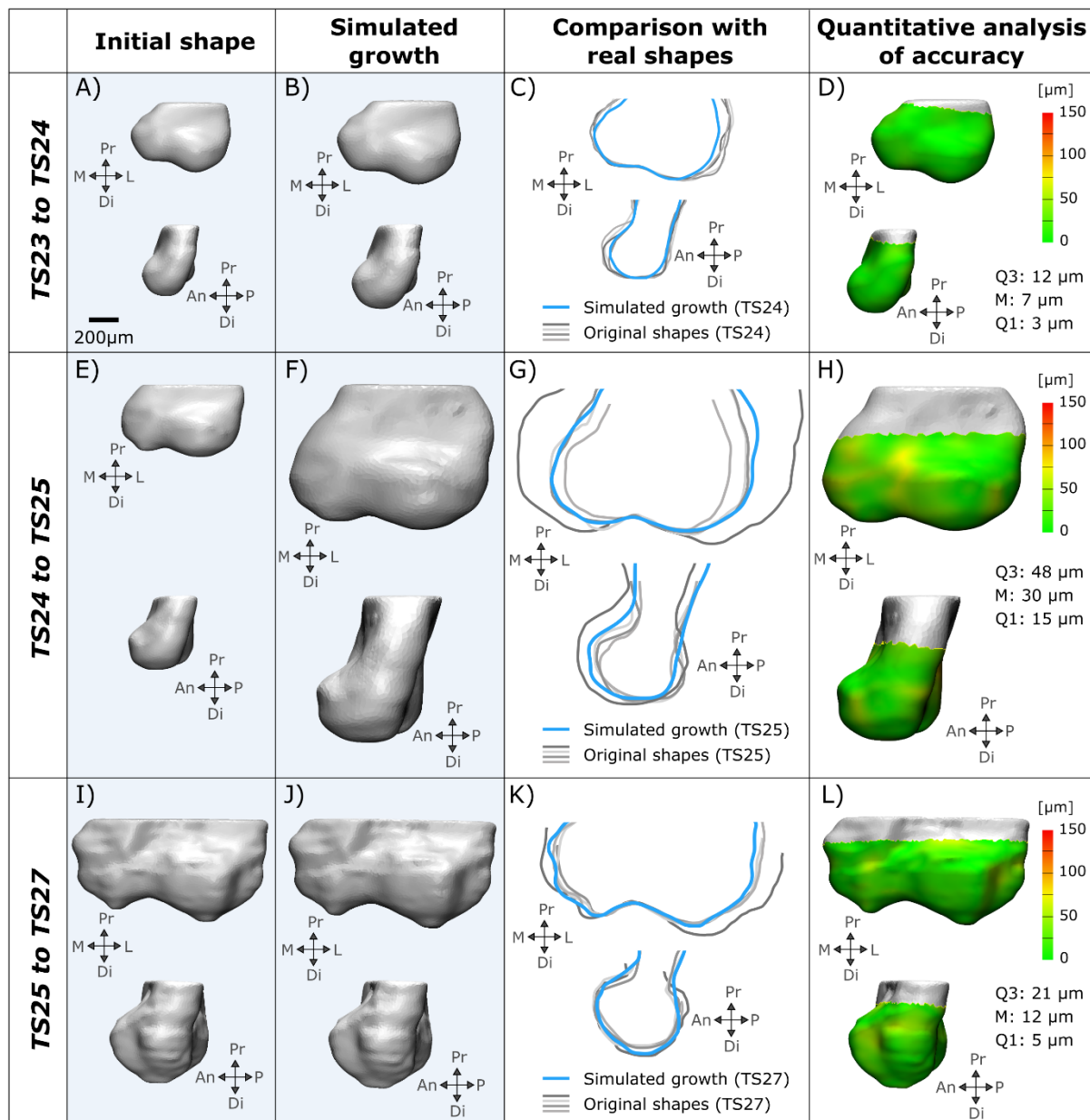


Figure 6.15: **Simulated growth was decomposed into several time windows.** (A–L) 3D growth simulations from TS23 to TS24 (A–D), TS24 to TS25 (E–H) and TS25 to TS27 (I–L). (A, E, I) Initial shapes are the shape averages at TS23 (A), TS24 (E) and TS25 (I). (C, G, K) Simulated growth shapes are compared to the original shapes at TS24 (C), TS25 (G) and TS27 (K). (D, H, I) Quantitative analysis of accuracy was performed by obtaining the distance from surface nodes between the simulated shapes and the average shapes. The simulated shapes have been cropped to match the height of the average shapes. 1st and 3rd quartiles of distance from surface nodes along with median distance are shown. Scale bar is 200 µm. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

6.3.2. The importance of growth contributors varies over development

I next sought to quantify the relative importance of increases in cell volume, cell number and ECM volume to growth over the entire timeframe, and in each of the composite time-windows. The

contributions from cell volume, cell number and ECM volume increases were selectively suppressed within the equations used to calculate the growth rates. Growth simulations were performed with each of these parameters suppressed and the resultant shapes compared to the full simulations.

Suppression of ECM volume increases had the most impact on growth over the entire timeframe compared to suppression of cell number or cell volume increases. When ECM volume increases were suppressed in the TS23–TS27 growth simulations, the simulated volume increase reached only 47% of the volume increase of the shape grown in the full simulation, as shown in Figure 6.16A&B (green • outline). When cell number or cell volume increases were suppressed, 72% (Figure 6.16A&B, black ♦ outline) and 79% (Figure 6.16A&B, red + outline) of the total growth was simulated respectively. Therefore, ECM deposition was the main driver for joint growth over the full timeframe, contributing almost half of the increase in epiphysis volume.

The importance of ECM deposition was even more pronounced from TS23 to TS24 than over the full timeframe. When ECM deposition was suppressed from TS23–24, no visible growth was simulated (Figure 6.16C, green • outline), while suppression of cell volume expansion and cell proliferation did not visibly alter the shape predictions compared to the full simulation (Figure 6.16A, red + and black ♦ outlines). From TS24 to TS25, in contrast to both the full duration studied, and to TS23–24, cell volume expansion was the main driver of cartilage growth, with cell number and ECM deposition contributing almost equally. When cell volume increases were suppressed, the simulated shape reached only 59% of the volume increase of the shape from the full simulation (Figure 6.16D, red + outline), while suppressing cell number or ECM volume increases led to simulated shapes with respectively 67% or 74% of the volume grown in the full simulation (Figure 6.16D, black ♦ and green • outlines). From TS25 to TS27, ECM volume and cell number increases were dominant contributors to growth, with no visible growth simulated in the case of a constant ECM volume (Figure 6.16E, green • outline), and the simulated shape when a constant cell number was applied reaching only 9% of the volume increase of the full simulation (Figure 6.16E, black ♦ outline). The preponderance of these two drivers to growth is due to the minimal growth that occurs in that window, with only minor shape or size changes (Figure 6.11C, D). A constant cell volume resulted in overgrowth of the shape predictions (Figure 6.16E, red + outline), a consequence of the decrease in the average size of the cells from TS25 to TS27 as shown in Figure 6.17. In summary, the relative contributions of cell volume expansion, cell proliferation and ECM deposition for growth at the level of the joint varied between time-windows. ECM deposition was at first the main driver for growth from TS23 to TS24. Then, from TS24 to TS25, cell expansion was the main driver of cartilage growth, while cell number increases and ECM deposition were also important

contributors. From TS25 to TS27, only minor epiphyseal shape changes occurred, driven by ECM deposition and cell number increases.

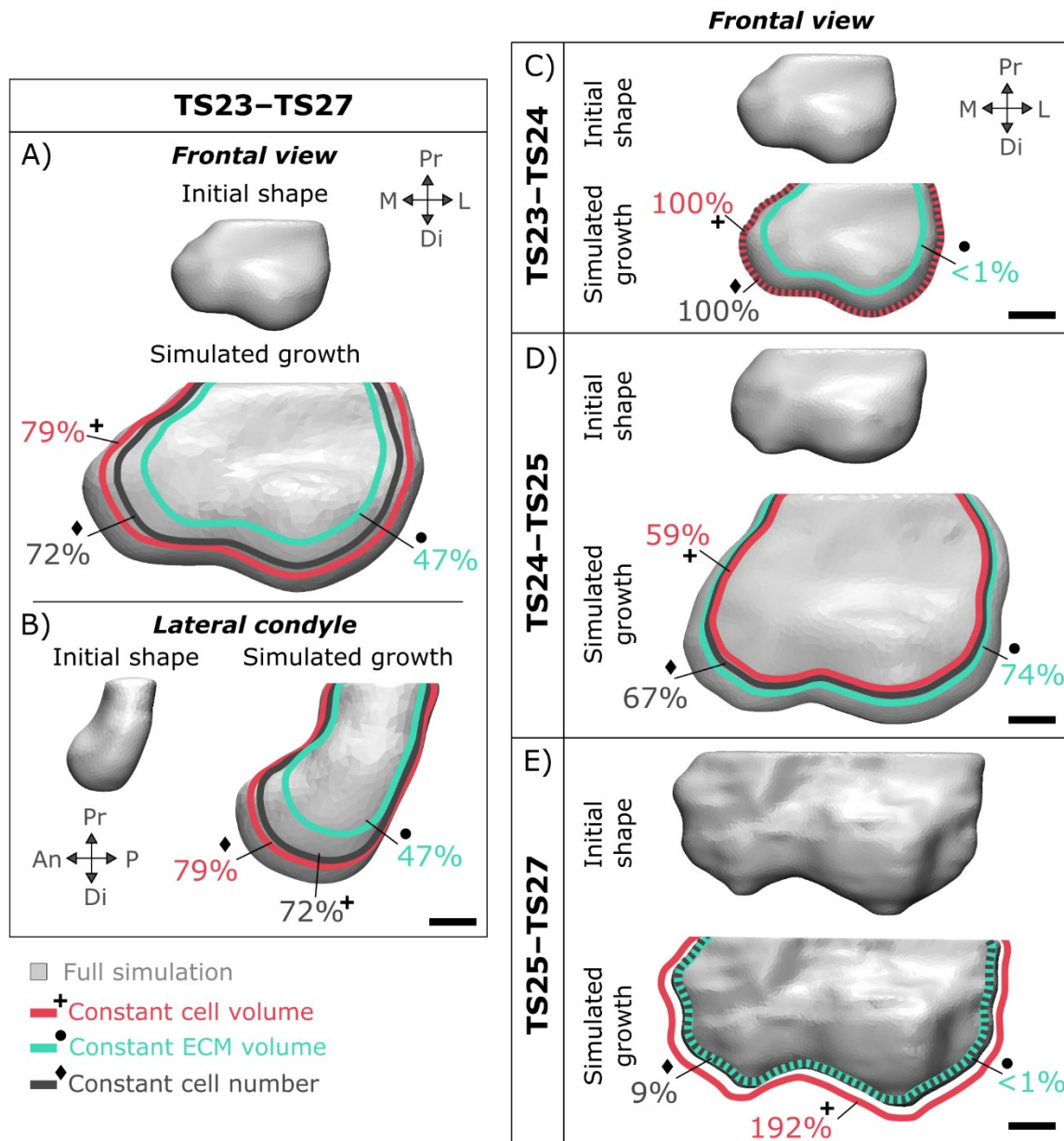


Figure 6.16: **The importance of growth contributors varies between time-windows with ECM volume increases being the main driver for growth between TS23 and TS27.** (A–E) Outlines of simulated shapes from TS23 to TS27 (A, B), TS23 to TS24 (C), TS24 to TS25 (D) and TS25 to TS27 (E) controls when ECM volume (green, ●), cell volume (red, +) and cell number (black, ♦) changes are successively suppressed are compared to the full simulation (grey 3D shape). (A, C–E) Frontal views. (B) Lateral view with outlines of the lateral condyle for TS23–TS27 simulations. A percentage of growth for each type of partial simulation relative to the full simulation is shown, where within each time window, the full simulation is assigned 100% and the initial shape 0%. Greater than 100% indicates overgrowth. Scale bars are 200 μm. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

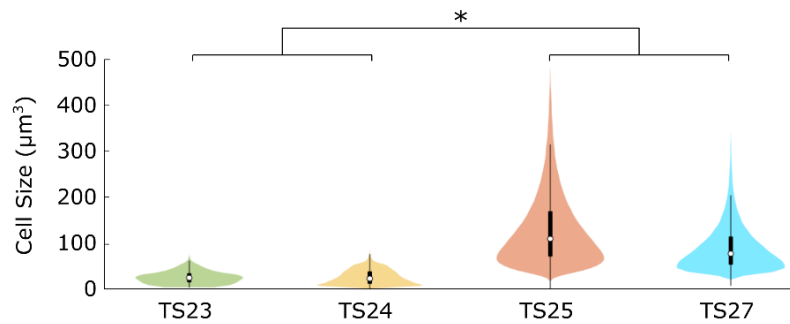


Figure 6.17: **Cell size changes from TS23 to TS27 in controls.** Segmented cells from all four samples at each developmental stages are combined in one violin plot. * indicates significant difference between TS23/TS24 groups and TS25/TS27 groups ($p < 0.05$) (ANOVA test with Tukey's HSD post hoc and Bonferroni adjustments). Data from Dr Huang (Huang 2022).

6.3.3. Absence of skeletal muscle associated with shape deformities

In animal models of fetal akinesia, shape abnormalities resulting from the absence of skeletal muscles or muscle contractions have been described (Osborne et al. 2002, Sotiriou et al. 2019, Bridglal et al. 2020) but changes in cell characteristics in the developing joint epiphyses have not been characterised. To address the gap in understanding, I applied my workflow (Figure 6.3) to Splotch-delayed ($Pax3^{Sp-d/Sp-d}$) mouse embryos which lack limb skeletal muscles. High-resolution phase contrast synchrotron tomography was performed on “muscleless-limbs” at TS23, TS24 and TS27. Shape features of the distal humeri were quantified as previously.

Splotch-delayed limbs increased in size and changed in shape over the timeframe studied (Figure 6.18). Some abnormalities in shape were evident from the outlines, in particular delayed anterior outgrowth of the lateral condyle (Figure 6.18A&B, black arrowheads), and an abnormal protuberance of the medial condyle in the frontal plane (Figure 6.18A–C, black arrows). Shapes were more variable within stages in the muscleless-limbs compared to controls, evident both in the outlines and in the spread of the measurements (Figure 6.18). One sample at TS23 was missing the medial condyle completely (Figure 6.18, hollow arrow). Quantitative morphological analyses showed a significant ($p < 0.05$) increase in volume of the muscleless-limb epiphyses from TS23–TS27 (Figure 6.18D). Average epiphyseal volumes appeared slightly lower than the controls at TS27, but there were no significant differences for volume between the muscleless-limbs and controls (Figure 6.18D). Width of the muscleless-limb epiphyses significantly ($p < 0.05$) increased from TS23–TS27 (Figure 6.18E). Average epiphyseal width was significantly ($p < 0.05$) lower than the controls at TS27 (Figure 6.18D). Corroborating the abnormal outgrowth of the lateral condyle seen in the outlines, lateral condyle depth in muscleless-limbs was consistently significantly ($p < 0.05$) lower than that of controls (Figure 6.18F). There were no significant differences for medial condyle depth between muscleless and control

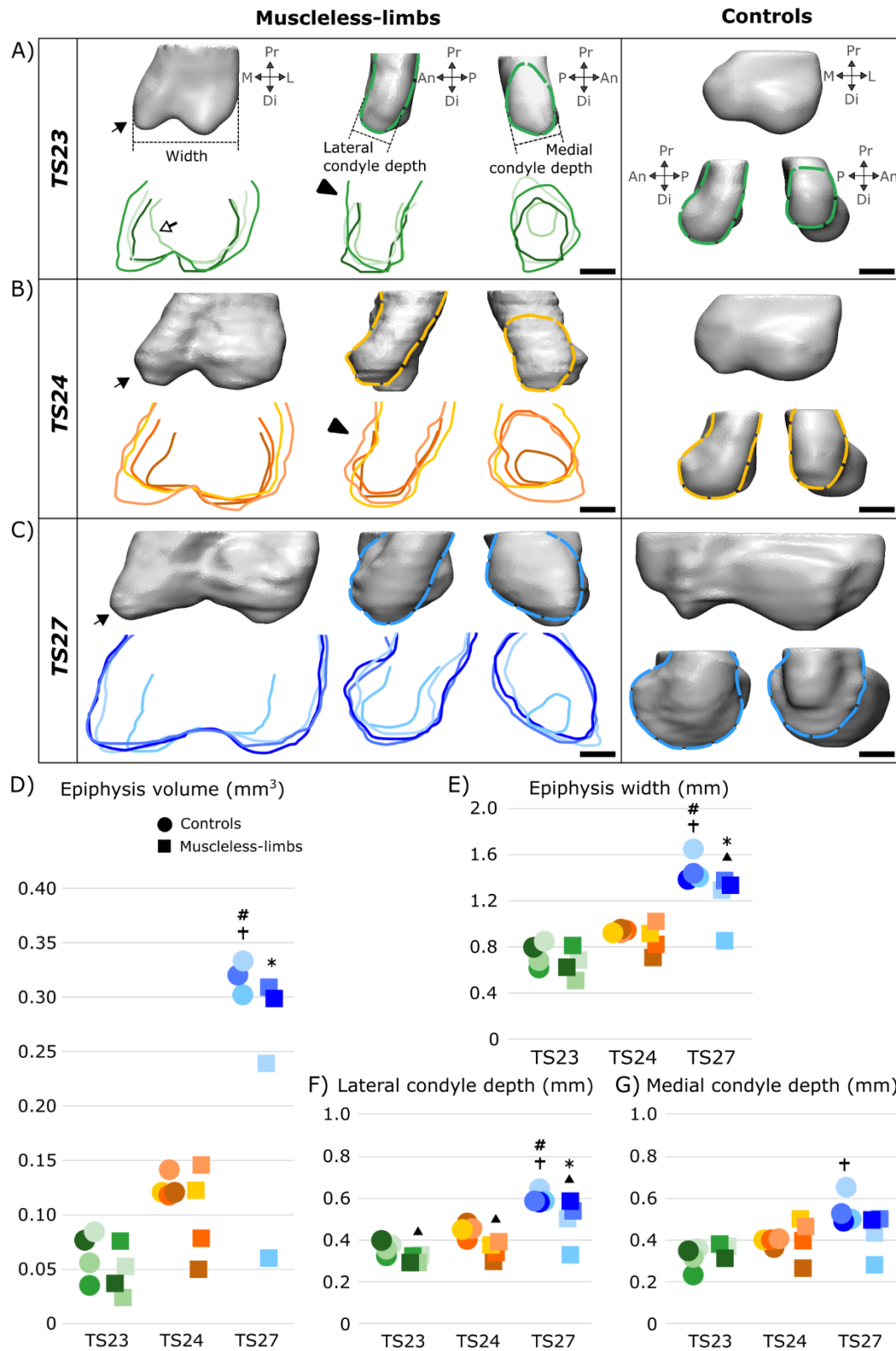


Figure 6.18: **Muscleless-limb distal humeri at TS23, TS24 and TS27 exhibit distinctive shape features compared to controls.** (A–C) 3D views of average Splotch-delayed ($Pax3^{Sp-d/Sp-d}$) muscleless-limb and control distal humeri along with original shape outlines (muscleless-limbs only) in the frontal, lateral and medial planes at TS23 (A), TS24 (B) and TS27 (C) ($n=4$ at each stage). In the 3D views, outlines of the lateral

(middle) and medial (right) condyles are shown with dashed lines. Arrows show outgrowth of the muscleless-limb medial condyle in the frontal plane. Hollow arrow indicates absent medial condyle in one muscleless-limb at TS23. Black arrowheads show delayed (compared to controls) anterior outgrowth of the muscleless-limb lateral condyle at TS23 and TS24. Scale bars are 200 μ m. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal. (D–G) Epiphysis volumes (D) (n=4 at each stage except for TS25 controls where n=3), epiphysis width (E) (n=4 at each stage), lateral condyle depth (F) (n=4 at each stage) and medial condyle depth (G) (n=4 at each stage except for TS23 muscleless-limbs where n=3) for controls (circle) and muscleless-limbs (squares). Epiphysis width and condylar depths are annotated in (A). † indicates significant difference ($p<0.05$) with TS23 controls group. # indicates significant difference ($p<0.05$) with TS24 controls group. * indicates significant difference ($p<0.05$) with TS23 muscleless-limbs group. ▲ indicates significant difference ($p<0.05$) between controls and muscleless-limbs at the corresponding developmental stage.

groups (Figure 6.18G). However, control, but not muscleless, limbs had a significantly increase in medial condyle depth between TS27 and TS23 (Figure 6.18G).

6.3.4. Absence of skeletal muscle affects growth dynamics in developing joint

3D growth maps were obtained for the full investigated timeframe TS23–TS27 and growth simulated using finite element analyses. Spatial heterogeneity was disrupted by the absence of skeletal muscle. Firstly, growth at the anterior aspect was slightly reduced in the muscleless-limbs compared to controls as shown by the predominance of green nodes in the muscleless-limbs (Figure 6.19D) rather than yellow nodes (Figure 6.19A), while the posterior aspect was less affected (yellow nodes in Figure 6.19B, E). Secondly, the location of areas of elevated (red nodes) or reduced growth (blue nodes) differed in the muscleless-limbs compared to controls. For example, in the lateral condyle, areas of reduced or elevated growth were missing at the anterior and posterior aspects in the muscleless-limbs (white arrowheads Figure 6.19A–B, D–E). In the muscleless-limbs, higher growth was seen at the posterior aspect, proximally to the lateral condyle (black double arrowheads in Figure 6.19B, E). Disrupted locations of high or low growth impacted the pronounced antero-posterior bias in growth which was seen in the controls and which, in the absence of muscle, is more proximal to the condyles as shown by arrows in Figure 6.19C, F. Disruption to the antero-posterior bias in growth is likely responsible for reduced anterior outgrowth of the lateral condyle in the muscleless-limbs (Figure 6.18A–B, arrowheads) as suggests the absence of shape differentiation of the lateral condyle from the diaphysis at the anterior aspect in muscleless-limb growth simulations in Figure 6.19G–J (red arrow). In the individual window TS23–TS24 subtle changes were observed between control and muscleless-limb growth maps (Figure 6.20A), and high accuracy was achieved when simulating growth (Figure 6.21A–D). From TS24–TS27, growth at the anterior aspect was markedly reduced in the muscleless-limbs

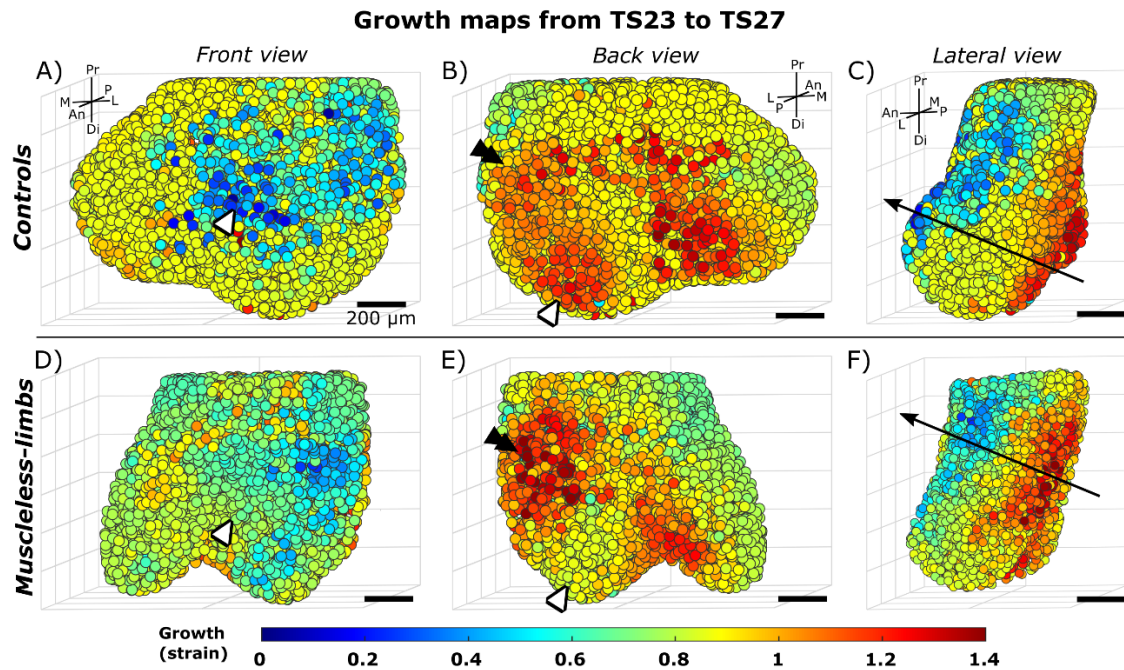


Figure 6.19: **In the absence of skeletal muscle, growth patterns are spatially disturbed with areas of disturbed growth co-localising with the emergence of abnormal shape features.** (A–F) TS23–TS27 3D growth maps for controls (A–C) and muscleless-limbs (D–F) calculated from cell-level data. Dots represent nodes from the meshed volume which was used for morphogenesis simulations. White arrowheads indicate areas of reduced (in A, D) or elevated (in B, C) growth seen in controls but not in muscleless-limbs. Black double arrowheads (in E) indicate an area in which growth was greater in muscleless-limbs compared to controls. Arrows (in C, F) indicate a bias towards high growth in the posterior (red nodes) compared to anterior (blue nodes) aspects in both controls and muscleless-limbs but this bias being more proximal in muscleless-limbs (F) compared to controls (C). (G–J) 3D growth simulations from TS23 to TS27 in muscleless-limbs. Initial shapes are the shape averages at TS23 (G). Simulated growth shape (H) is compared to the original shapes at TS27 (I). Red arrow indicates no shape differentiation of the lateral condyle from the diaphysis at the anterior aspect. (J) Quantitative analysis of accuracy was performed by

obtaining the distance from surface nodes between the simulated shapes and the average shapes. The simulated shapes have been cropped to match the height of the average shapes. 1st and 3rd quartiles of distance from surface nodes along with median distance are shown. Scale bars are 200 μm . An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

compared to controls as seen with the presence of blue nodes in Figure 6.20B. Muscleless-limbs also displayed an abnormal protuberance of the medial condyle, visible in the frontal plane (Figure 6.18A–C, black arrows). This abnormal outgrowth correlates with elevated growth detectable at the medial condyle in the individual time window TS24–TS27 (Figure 6.20B, red arrowhead). Outgrowth of the medial condyle was replicated in growth simulations as shown in Figure 6.21E–H, red arrow.

Overall, in the absence of skeletal muscle, changes to growth dynamics compared to controls are complex and corroborate the hypothesis that mechanical stimuli arising from the articulation between the skeletal elements regulates prenatal joint morphogenesis. Shape abnormalities likely lead to a non-physiological distribution of loads from interaction between the distal humerus and the radius and ulna which likely trigger modified concentrations of growth.

6.3.5. Absence of skeletal muscle disrupts balance between growth contributors

Finally, I quantified the effects of absent skeletal muscle on the relative contributions to joint growth of cell volume, cell number and ECM volume increases. Growth simulations were performed when each of these parameters were suppressed and compared to the full simulations and to control simulations for the full timeframe TS23–TS27, and for the composite time windows (TS23–TS24 and TS24–TS27). For the full examined timeframe, ECM deposition was the main driver for growth in the muscleless-limbs. When ECM deposition was suppressed, only 31% of the total growth was simulated (Figure 6.22A&B, green • outline). Therefore, ECM volume increases contributed more to growth in muscleless-limbs than in controls, in which 47% of the total growth was simulated in controls when ECM volume was set constant (Figure 6.22A&B, green • outlines). A major difference between muscleless-limbs and controls lies in the contribution of cell volume increases to growth. When cell volume increases were suppressed, 92% of growth was simulated in the muscleless-limbs, compared to 79% in the controls (Figure 6.22A&B, red + outline) meaning that cell volume increases contribution to growth was reduced in the absence of skeletal muscles. When the number of cells was kept constant, 77% of total growth was simulated in the muscleless-limbs and 72% in controls (Figure 6.22A&B, black ♦ outlines). Therefore, the contribution of cell number increases to growth in muscleless-limbs was slightly lower than that of controls.

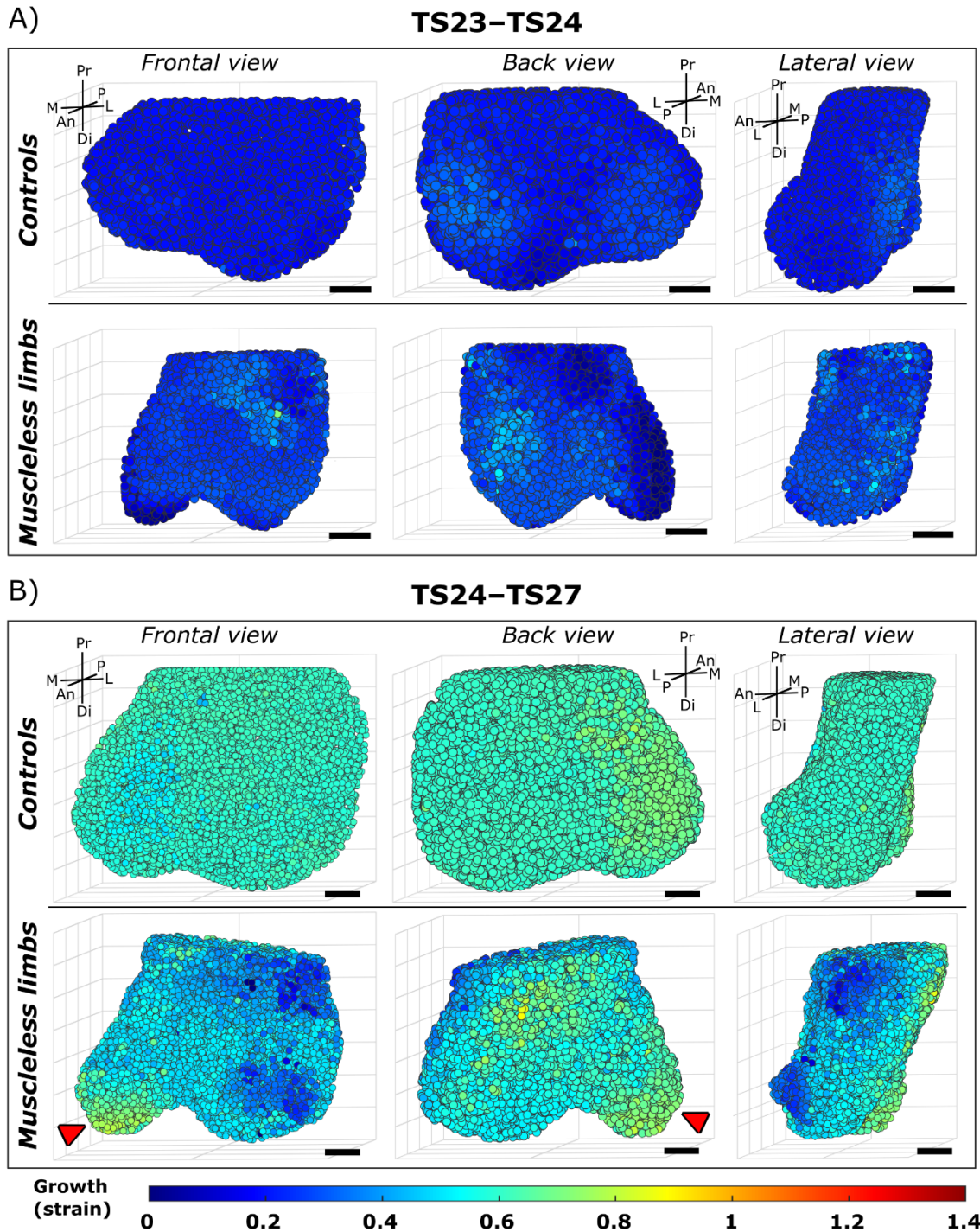


Figure 6.20: Growth patterns in the absence of skeletal muscles in the individual time windows TS23–TS24 and TS24–TS27. (A–B) 3D growth maps for controls and muscleless-limbs calculated from cell-level data in individual time-windows TS23–TS24 (A) and TS24–TS27 (B). Dots represent nodes from the meshed volume which was used for morphogenesis simulations. Red arrowheads indicate an area of elevated growth in the muscleless-limbs which coincides with outgrowth of the medial condyle in the absence of skeletal muscle. Scale bars are 200 μ m. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

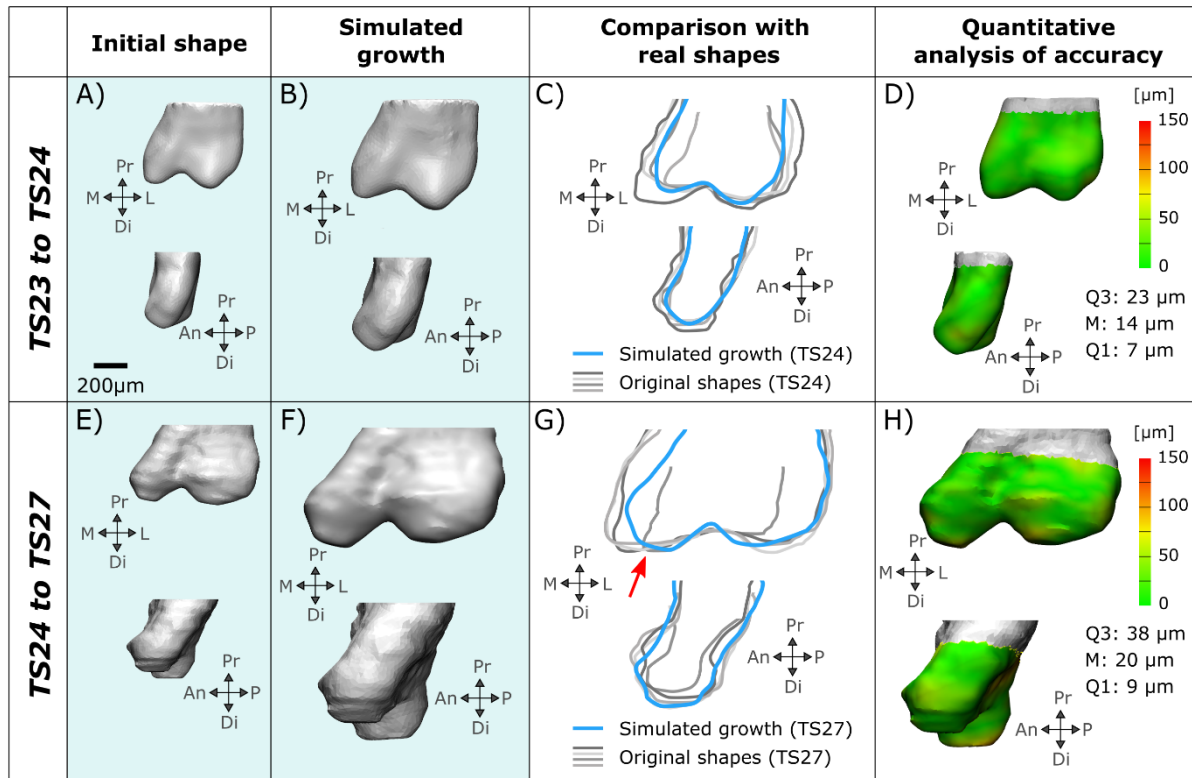


Figure 6.21: **Simulating growth in the muscleless-limb distal humerus in the individual time windows TS23–TS24 and TS24–TS27.** (A–H) 3D growth simulations from TS23 to TS24 (A–D) and TS24 to TS27 (E–H). (A, E) Initial shapes are the shape averages at TS23 (A) and TS24 (E). (B–C, F–G) Simulated growth shapes are compared to the original shapes at TS24 (C) and TS24 (G). Red arrow indicates simulated outgrowth of the medial condyle. (D, H) Quantitative analysis of accuracy was performed by obtaining the distance from surface nodes between the simulated shapes and the average shapes. The simulated shapes have been cropped to match the height of the average shapes. 1st and 3rd quartiles of distance from surface nodes along with median distance are shown. Scale bar is 200 µm and applies to all. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

From TS23 to TS24, growth was mostly driven by ECM deposition in both groups with only 4% of the total growth being simulated in the muscleless-limbs, and less than 1% in the controls when ECM volume was kept constant (Figure 6.22C, green • outline). In the absence of skeletal muscle, the average size of the cells decreased (Figure 6.23A), leading to overgrowth of the shape prediction when cell volume changes were suppressed (Figure 6.22C, red + outline), and the number of cells increased (Figure 6.23B), changes that were not observed in controls. From TS24 to TS27, ECM deposition was once again the main driver for growth in muscleless-limbs with 36% of growth simulated when ECM deposition was prevented. Cell volume expansions and cell number increases only minorly contributed to growth in muscleless-limbs, with 79% of growth simulated with cell volume kept constant and 85% of growth simulated when cell numbers were kept constant (Figure 6.22D). In contrast, in the controls during this window, each of the three drivers contributed almost equally to growth (Figure 6.22D). In

conclusion, in the absence of skeletal muscle, an imbalance between the growth drivers arose with, ECM volume being dominant over the other two processes over the entire time span and within the time windows studied.

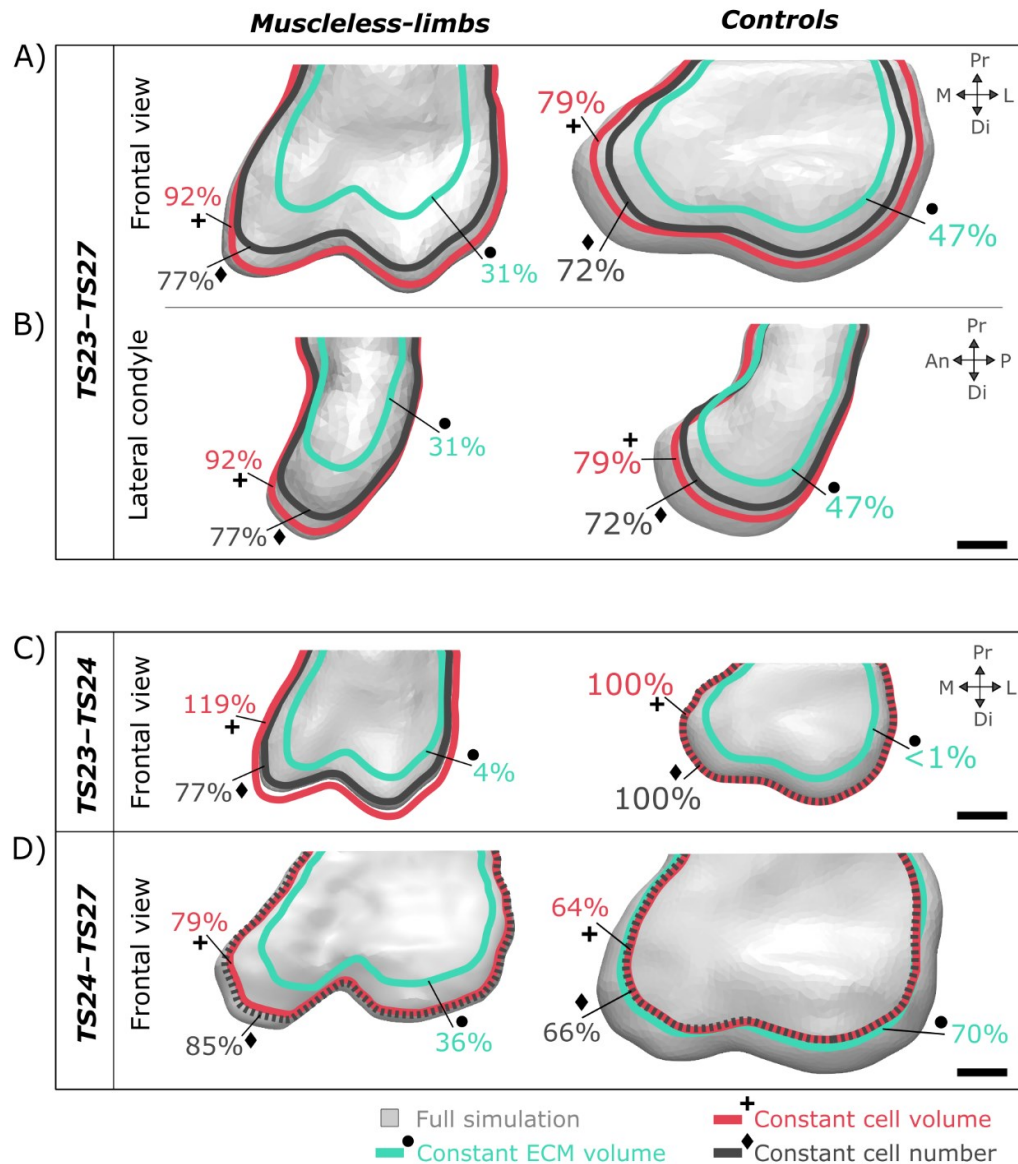


Figure 6.22: **In the absence of skeletal muscle, the contribution of cell volume increases to growth is reduced compared to controls.** (A–D) Muscleless-limb and control outlines of simulated shapes from TS23 to TS27 (A, B), TS23 to TS24 (C) and TS24 to TS27 (D) when either ECM volume (green, •), cell volume (red, +) and cell number (black, ◆) changes are successively suppressed. Grey 3D shapes are the full simulations. (A, C, D) Frontal views. (B) Lateral view with outlines of the lateral condyle for TS23–TS27 simulations. A percentage of growth for each type of partial simulation relative to the full simulation is shown, where within each time window, the full simulation is assigned 100% and the initial shape 0%. Greater than 100% indicates overgrowth. Scale bars are 200 μ m. An: anterior, Di: distal, L: lateral, M: medial, P: posterior, Pr: proximal.

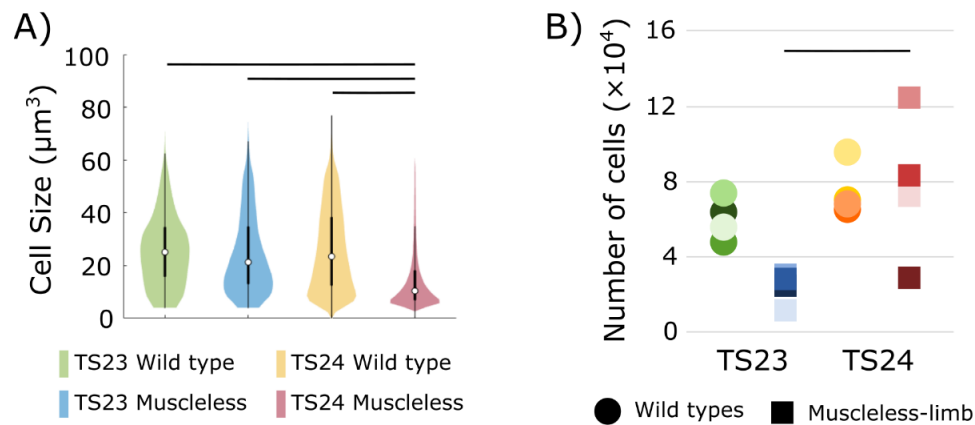


Figure 6.23: **Cell activities are disturbed in the absence of skeletal muscle.** (A) Cell size of controls and muscleless-limbs at TS23 and TS24. (B) Number of cells in controls (circles) and muscleless-limbs (squares) at TS23 and TS24. Solid bar indicates significant difference between groups ($p < 0.05$) (ANOVA test with Tukey's HSD post hoc and Bonferroni adjustments). Data from Dr Huang (Huang 2022).

6.4. Discussion

In this study, I aimed to discover what cell and tissue properties contribute to the growth and morphogenesis of the late prenatal joint, to provide insight into the biological and mechanobiological processes underlying this aspect of skeletal development, and to offer greater understanding of the aetiology of skeletal developmental disorders including hip dysplasia and arthrogryposis. The murine distal humerus were imaged using phase contrast synchrotron microscopy at multiple developmental stages and for both normal and akinesic conditions *in utero*. The epiphysis of the distal humerus increased over the period studied, and the key shape feature to become apparent over the period assessed was a pronounced anterior outgrowth of the lateral condyle. The impacts of cell number, cell volume expansion and increase in matrix volume, were assessed at the organ-level using biofidelic simulations of growth and morphogenesis. The growth simulations revealed that the anterior outgrowth of the lateral condyle occurred due to the pronounced growth concentrations at the posterior aspect of the distal humerus, at the site of interaction with the ulnar olecranon process (Figure 6.24). By manipulating the inputs to the computer simulation, I were able to identify which contributors were driving growth across different developmental stages. ECM volume is the dominant contributor to overall joint growth in the timeframe studied, with cell volume and cell number also important contributors from TS24–25, the time at which the biggest increase in epiphyseal volume occurred. In embryos devoid of limb skeletal muscle, growth in terms of epiphyseal volume was not significantly affected, but morphogenesis was abnormal, with the most obvious abnormality in shape

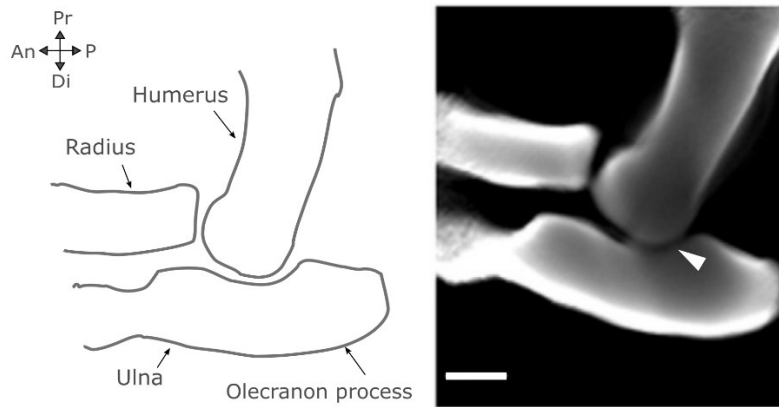


Figure 6.24: **The humerus interacts with the ulna at its posterior aspect.** Virtual section through the 3D reconstruction of an Alcian blue stained elbow joint of a control mouse at TS23. White arrowhead indicates the humerus-ulna interaction. Scale bar is 200 μ m. An: anterior, Di: distal, P: posterior, Pr: proximal. From (Nowlan et al. 2010b).

being the lack of anterior outgrowth of the lateral condyle. The growth simulations showed that in the muscleless limbs, the concentrations of growth rates at the posterior aspect of the rudiment were disrupted, corroborating the hypothesis that the mechanical forces from the articulation between the posterior distal humerus and the olecranon process of the ulna promote anterior outgrowth of the condyles over the period studied. Interaction of deformed shapes in the absence of muscles leads to a non-physiological distribution of loads which likely trigger modified concentrations of growth rates. The growth simulations also revealed that a lack of skeletal muscle is correlated with a disrupted balance in the contributors of growth, with cell number and cell volume contributions being decreased in the muscleless limbs. Therefore, the growth and morphogenesis of epiphyseal joints is a mechanoregulated process which depends on a delicate balance between cell number, cell volume and ECM volume, with specific shape features controlled by regional concentrations in growth rates, modulated by mechanical loading.

My-data demonstrates that mechanoregulated spatial heterogeneity underlies key aspects of prenatal joint morphogenesis. Spatial heterogeneity in cell activities has previously been indicated as a mechanism underlying aspects of joint morphogenesis (Roddy et al. 2011a, Shwartz et al. 2016, Zhang et al. 2020). (Roddy et al. 2011a) For example, prior to TS23, antero-posterior asymmetry of cell influx was observed in the mouse elbow with more cells being recruited at the anterior aspect of the humerus and which was thought to contribute to shaping the epiphyseal end (Zhang et al. 2020). In the chick embryo, medio-lateral asymmetry in cell proliferation and in biophysical stimuli due to fetal movements were hypothesised to correlate accelerated growth of the medial condyle compared to the lateral condyle (Roddy et al. 2011a). In the present study, the lateral condyle was bigger than the

medial condyle at all examined timepoints in the controls, while the growth maps revealed no obvious growth rate asymmetry between the condyles. The size difference between the condyles is therefore likely to have been established by asymmetric growth mechanisms acting prior to TS23.

Little has been published on the cell-level processes underlying joint morphogenesis. The work described in this study is the first to quantify their relative contribution to growth at the organ level in the murine joint. Under normal mechanical environment, growth is firstly driven by ECM deposition, then, cells expand and their number increases. I hypothesise that the delayed increases in cell volume and number compared to the initial increase in matrix volume is related to the response of chondrocytes to hypo-osmotic stimuli. Following exposure to hypotonic solutions, chondrocytes maximum volume tends to increase (Phan et al. 2009, Lewis et al. 2011). Initial ECM deposition from TS23 to TS24 could therefore have created a favourable environment for cell swelling. The response to hypo-osmotic stimuli is mediated by activation of ion channels amongst which some are mechano-regulated (Phan et al. 2009, Lewis et al. 2011). The transient reception potential vanilloid type 4 (TRPV4) is a Ca^{2+} permeable channel present in chondrocytes in mature and developing cartilage and is gated by tonicity and mechanical stimuli (Phan et al. 2009, Lewis et al. 2011, Khatib et al. 2022). It has recently been shown that TRPV4 activity is crucial for joint cartilage growth mechano-regulation partially through the control of cell proliferation (Khatib et al. 2022). Consistently, my results show that in the absence of skeletal muscle cell volume and cell number increases are reduced compared to controls. I showed that ECM volume still increases even in the absence of skeletal muscle, however, it is very unlikely that matrix synthesis proceeds normally. Altered GAG contents in embryonic cartilage of immobilised mice knees and zebrafish jaw joints have been reported (Mikic et al. 2004, Lawrence et al. 2021) as well as impaired collagen network formation in muscleless-limb mice elbows (Ahmed et al. 2022).

In this research, isotropic growth was used to simulate growth and led to accurate shape predictions. It suggests that orientation is less important to mouse joint growth than to zebrafish jaw joint growth as discussed in Chapter 3. One major difference between the mouse and the zebrafish as animal models is the number of cells. It is possible that in the zebrafish, because of the small number of cells, individual cell behaviours have a great impact on tissue shape. Therefore, oriented behaviours may trigger pronounced growth anisotropy in the zebrafish. The study of oriented cell processes such as migration and directed proliferation in the mouse by lineage tracking would enable us to quantify growth anisotropy and assess its importance in murine joint morphogenesis. It is also possible that the difference in the importance of growth orientation between the mouse and the zebrafish comes from the analysis of different timings of joint morphogenesis. Abnormal shape changes in the absence of skeletal muscles may also have been influenced by the disruption of oriented cell processes. Data from

our group showed a change in chondrocyte orientations in the distal humerus in the absence of skeletal muscle ([Huang 2022](#)). Alteration of cell orientation at the level of the joint line has also been reported in muscleless-limb mice elbows at e16.5 (about TS25) ([Kahn et al. 2009](#)).

A limitation of the work described in this Chapter is that growth analyses were only conducted on the distal humerus. The integration of the ulna and the radius would have provided insights on how interlocking shapes emerge, with especially the study of potential growth asymmetry between the opposing rudiments. The addition of the TS25 stage in the muscleless limb groups would have permitted a direct comparison of the temporal patterns of growth in the presence or absence of skeletal muscles. Still, the use of three developmental stages (TS23, TS24 and TS27) enabled multiple comparisons to be performed. In controls there was little growth from TS25 to TS27 so the gain from including this stage in the muscleless limbs would have been limited. Finally, since shape differences are already visible at TS23 between the two condyles in controls and between controls and muscleless limbs, investigating earlier developmental stages would have helped us to understand how these differences have arisen. It is possible that shape differences in the presence and absence of movements have arisen even prior to the supposed start of movements due to the absence of biological or mechanobiological cues from the muscle insertion points. Another limitation was the small number of samples in each experimental group (n=4), but the current data still enabled consistent, and statistically significant, quantification of growth patterns.

In conclusion, insights on the underlying biological and mechanobiological cell dynamics driving joint morphogenesis were obtained from the synthesis of biological cell-level data into simulations of murine prenatal joint morphogenesis. Spatial and temporal patterns of growth influence the formation of specific shape features which are impaired in the absence of skeletal muscles. The balance between the cell-level dynamics underlying joint morphogenesis is mechanically regulated with cell volume and number increases being compromised in the absence of skeletal muscle. In the next chapter, a summary of key findings, avenues for future research, and general conclusions are presented.

Chapter 7

7 General discussion

7.1 Outcomes and contributions

The research presented in this thesis investigated the process of joint growth and morphogenesis by synthesising cell-level data into a quantification of growth at the macro-scale. The underlying cell dynamics and the roles of mechanical forces were explored at multiple levels using novel analytical techniques. The advances brought about by this study's key achievements are discussed in this section.

Growth pattern characterisations in the zebrafish jaw joint and the distal mouse humerus brought new understandings specific to the strengths and limitations of each animal model. In the zebrafish, size and translucency enabled imaging of live specimens and, through cell tracking, quantification of growth anisotropy. Results showed that strong anisotropy underlies larval zebrafish jaw joint growth. In the mouse, growth anisotropy could not be quantified but it became clear that growth heterogeneity is a major factor for shape differentiation of the condyles. Growth orientations and heterogeneity dominate morphogenesis in several biological models, from limb bud expansion ([Boehm et al. 2010](#), [Morishita et al. 2015](#)) to folding of the drosophila wing ([Tozluoglu et al. 2019](#)), and this research revealed the importance of growth patterns to joint shaping.

To deepen our understanding of the cell dynamics underlying joint growth, formal quantification of the relative contributions of cell behaviours crucial to growth was undertaken in the mouse. It appeared that, in the investigated timeframe (TS23 to TS27) and in normally developing mice, growth is firstly driven by ECM volume increases then, after TS24, cell volume and cell number increases start contributing to growth to a similar level than ECM deposition. In previous studies, increased cell proliferation over time had been shown in the developing chick knee ([Roddy et al. 2011a](#)) but, to my knowledge, no quantification of the relative contribution of several cell processes had been undertaken.

In this research, the roles of mechanical loading on multiple aspects of joint development were explored. The cell-level dynamics, material properties and patterns of growth were compared between specimens raised under normal or altered mechanical conditions. In the zebrafish, growth orientations were disturbed when larvae were immobilised, further evidence for the importance of growth anisotropy in this animal model. Other studies have shown that oriented cell behaviours which might contribute to growth anisotropy, such as cell orientation and intercalation, are indeed compromised in immobilised larvae ([Shwartz et al. 2012](#), [Brunt et al. 2016b](#)). In the present study, delayed stiffening of the pharyngeal cartilage was also observed in the absence of movement with the viscoelastic properties of the tissue remaining abnormal in the investigated timeframe. This disruption of material properties is possibly an indication that the extracellular matrix content is abnormal in immobilised larvae. In the mouse, cell volume and number increases were reduced in the absence of skeletal muscle compared to wild types corroborating with decreased growth of the epiphysis. Alteration of growth heterogeneous patterns also coincided with shape changes at the macro-scale. Overall, the work presented in this thesis provides a comprehensive view of the impact of muscle forces on joint growth and morphogenesis by bringing together cell dynamics, patterns of growth and joint shape changes through the use of analytical techniques and growth simulations. Going a step further, hypotheses regarding to how mechanical stimuli promote growth in the joint was tested using mechanobiological models of joint growth in the zebrafish jaw. Simulations revealed that, rather than average tensions or compressions experienced during joint motion, the dynamic mechanical changes are the most likely stimuli influencing growth.

7.2 Future perspectives

The work conducted throughout this thesis opens new perspectives for future research. One possible avenue is the study of mutations which present joint malformations. For example, severe skeletal malformations are associated with mutations in the genes encoding type XI collagen. In the zebrafish, *col11a2* mutants present altered jaw morphologies with loss of joint space and joint hypermobility and is a model for the Skickler syndrome ([Lawrence et al. 2018](#)). Investigating growth patterns in mutants and correlating them to altered mechanical stimuli arising from movements will provide fundamental information on the onset of congenital conditions.

Further advances on joint morphogenesis mechanoregulation can be gained by simulating mouse elbow motion using FE methods. Patterns of mechanical stimuli during motion could be correlated with the growth patterns observed in this research. For example, the hypothesis that the humerus-

ulna interaction during joint motion promotes growth could be challenged. Going further, mechanical factors could be integrated to mouse joint growth simulations in an analogous way than for the zebrafish. Hypotheses regarding how the mechanical stimuli arising from movement influence growth could therefore be tested in an animal model that is closer to humans.

In the scope of this thesis, for both the zebrafish jaw joint and the mouse elbow, investigations were mainly performed on one joint element. In the zebrafish, cell segmentation and tracking in the posterior joint element of immobilised larvae were not reliable due to a weak expression of cartilage markers and scan resolution. Future improved cell segmentation methods could enable the analyses of both joint elements. In the mouse, only cell-level data of the distal humerus were available. Synchrotron scans though contain all three joint elements—distal humerus, proximal radius and proximal ulna—and it would be interesting to conduct similar analyses in all three joint elements. The mechanisms underlying the emergence of interlocking shape features could be revealed by notably the discovery of potential asymmetric growth patterns.

The research presented in this thesis on the zebrafish jaw joint suggested that growth anisotropy is important to joint morphogenesis. The methods used in this research did not enable quantification of growth anisotropy in the mouse elbow, but the accuracy of growth simulations suggested that cell orientation was less important for this animal model. Characterisation of oriented cell behaviours in the mouse, such as orientation, oriented division and migration could confirm this finding.

In the present study, the number of mouse samples is a limitation. More synchrotron scans from which the cell-level data were extracted were unfortunately not available. Data collection predated this doctorate and imaging could not be repeated. Despite low number of samples, especially for TS23 muscleless-limbs ($n=3$), the current data enabled robust and consistent quantification of growth patterns. The addition of the TS25 stage in the muscleless-limb groups would have permitted a direct comparison of the temporal patterns of growth in the presence or absence of skeletal muscles. Still, the use of three developmental stages (TS23, TS24 and TS27) enabled multiple comparisons to be performed. Finally, since shape differences are already visible at TS23 between the two condyles in wild types and between wild types and muscleless-limbs, investigating earlier developmental stages would have helped us to understand how these differences have arisen.

In this research, quantification of larval zebrafish cartilage material properties was experimentally performed showing that jaw movements influence cartilage stiffening and viscoelastic properties. However, no investigation of the cartilage content was performed and correlated to the biomechanical performances of the tissue. Indentation testing of the embryonic mouse knee or elbow cartilage would also expand our knowledge on embryonic cartilage material properties. As far as I am aware, in the

mouse, bending tests were performed on ribs ([Tanck et al. 2004](#)) but no investigation was conducted on the joint cartilage nor on the impact of muscle contraction.

7.3 Conclusions

The research presented in this thesis provides new and important insights into the factors driving joint morphogenesis. Cell dynamics were correlated with growth patterns and joint shape changes using computational models of joint growth. The impact of movements for the emergence of interlocking and physiological joint shapes was explored at multiple levels. Deepening our knowledge of the mechanisms of joint morphogenesis is important for our understanding of the onset of congenital musculoskeletal conditions. Such understanding could contribute to developing strategies for the treatment of musculoskeletal conditions through notably targeted physiotherapy.

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Appendix I: Chapter 3 supplementary material

Appendix I.1. Statistical analyses of jaw joint growth rates

Mean $\pm$ SD	Time window			
	3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Main direction	-2.3 $\pm$ 5.5 E-02	1.2 $\pm$ 3.0 E-02	2.8 $\pm$ 2.1 E-02	2.1 $\pm$ 1.8 E-02
Second direction	1.3 $\pm$ 2.9 E-02	0.2 $\pm$ 1.5 E-02	0.3 $\pm$ 1.1 E-02	-0.007 $\pm$ 1.2 E-02
Third direction	0.3 $\pm$ 0.7 E-02	0.1 $\pm$ 0.6 E-032	0.6 $\pm$ 4.9 E-03	0.08 $\pm$ 4.4 E-03

Table S1.1: List of means and standard deviations of growth rates for each group in the anterior joint element.

p-value	Time window			
	3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Main direction	< 0.001	< 0.001	< 0.001	< 0.001
Second direction	< 0.001	< 0.001	< 0.001	< 0.001
Third direction	< 0.001	0.017	0.011	< 0.001

Table S1.2: Lists of p-values obtained with Kolmogorov-Smirnov test of normality showing that groups were not normally distributed in the anterior joint element.

Comparison groups		Time window			
		3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Main direction	Second direction	< 0.001	0.126	< 0.001	< 0.001
Main direction	Third direction	< 0.001	< 0.001	< 0.001	< 0.001
Second direction	Third direction	< 0.001	> 1	0.001	> 1

Table S1.3: Lists of p-values obtained with Friedman test followed with Wilcoxon signed ranked test with Bonferroni adjustments to compare growth rates datasets in different directions for growth in each time-window in the anterior joint element. Bold indicates significant difference between comparison groups ($p < 0.05$).

Comparison groups		Direction for growth		
		Main	Second	Third
3.5–4 dpf	4–4.5 dpf	< 0.001	0.022	< 0.001
3.5–4 dpf	4.5–5 dpf	< 0.001	< 0.001	< 0.001
3.5–4 dpf	5–5.5 dpf	< 0.001	< 0.001	< 0.001
4–4.5 dpf	4.5–5 dpf	< 0.001	> 1	> 1
4–4.5 dpf	5–5.5 dpf	0.014	> 1	> 1
4.5–5 dpf	5–5.5 dpf	< 0.001	< 0.001	> 1

Table S1.4: Lists of p-values obtained with Friedman test followed with Wilcoxon signed ranked test with Bonferroni adjustments to compare growth rates datasets in different time-windows in each direction for growth in the anterior joint element. Bold indicates significant difference between comparison groups ($p < 0.05$).

Mean $\pm$ SD	Time window			
	3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Main direction	-0.08 $\pm$ 7.55 E-02	1.3 $\pm$ 5.9 E-02	2.2 $\pm$ 4.4 E-02	1.8 $\pm$ 4.5 E-02
Second direction	1.21 $\pm$ 3.3 E-02	0.5 $\pm$ 3.2 E-02	-0.4 $\pm$ 2.5 E-02	-0.3 $\pm$ 2.6 E-02
Third direction	0.5 $\pm$ 1.0 E-02	0.78 $\pm$ 9.79 E-03	0.2 $\pm$ 0.8 E-02	0.1 $\pm$ 0.6 E-02

Table S1.5: List of means and standard deviations of growth rates for each group in the posterior joint element.

p-value	Time window			
	3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Main direction	< 0.001	< 0.001	< 0.001	< 0.001
Second direction	< 0.001	< 0.001	< 0.001	< 0.001
Third direction	< 0.001	< 0.001	< 0.001	< 0.001

Table S1.6: Lists of p-values obtained with Kolmogorov-Smirnov test of normality showing that groups were not normally distributed in the posterior joint element.

<i>Comparison groups</i>		<i>Time window</i>			
		3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Main direction	Second direction	> 1	> 1	< 0.001	< 0.001
Main direction	Third direction	> 1	0.606	< 0.001	< 0.001
Second direction	Third direction	0.092	0.079	> 1	0.388

Table S1.7: Lists of p-values obtained with Friedman test followed with Wilcoxon signed ranked test with Bonferroni adjustments to compare growth rates datasets in different directions for growth in each time-window in the posterior joint element. Bold indicates significant difference between comparison groups ($p < 0.05$).

<i>Comparison groups</i>		<i>Direction for growth</i>		
		Main	Second	Third
3.5–4 dpf	4–4.5 dpf	> 1	> 1	> 1
3.5–4 dpf	4.5–5 dpf	> 1	0.002	> 1
3.5–4 dpf	5–5.5 dpf	> 1	< 0.001	< 0.001
4–4.5 dpf	4.5–5 dpf	0.055	0.028	> 1
4–4.5 dpf	5–5.5 dpf	> 1	0.025	> 1
4.5–5 dpf	5–5.5 dpf	> 1	> 1	> 1

Table S1.8: Lists of p-values obtained with Friedman test followed with Wilcoxon signed ranked test with Bonferroni adjustments to compare growth rates datasets in different time-windows in each direction for growth in the posterior joint element. Bold indicates significant difference between comparison groups ($p < 0.05$).

Appendix I.2. Mesh convergence study

A mesh convergence study was performed on a representative time window with visible shape changes (4 to 4.5 dpf). Initial shape assembly was remeshed in 3-matic with twice as many elements as shown in Figure S1.1A. Growth simulations from 4 to 4.5 dpf were performed in Abaqus CAE following the methodology described in Chapter 3 using either the baseline mesh assembly used throughout this thesis or the x2 remeshed assembly. The resulting shapes were overlaid showing subtle shape differences as shown in Figure S1.1B. To quantify the difference, the distance between surface nodes of the two simulated shapes was calculated using the part comparison analysis tool in 3-matic. Part comparison analysis showed that surface nodes from the two simulated shapes were at a maximum distance of 2 μm from each other as shown in Figure S1.1C. Given that shape differences using a mesh with twice as many elements were minor compared to the baseline mesh, the latter was considered suitable when modelling growth using the methodology described in this thesis.

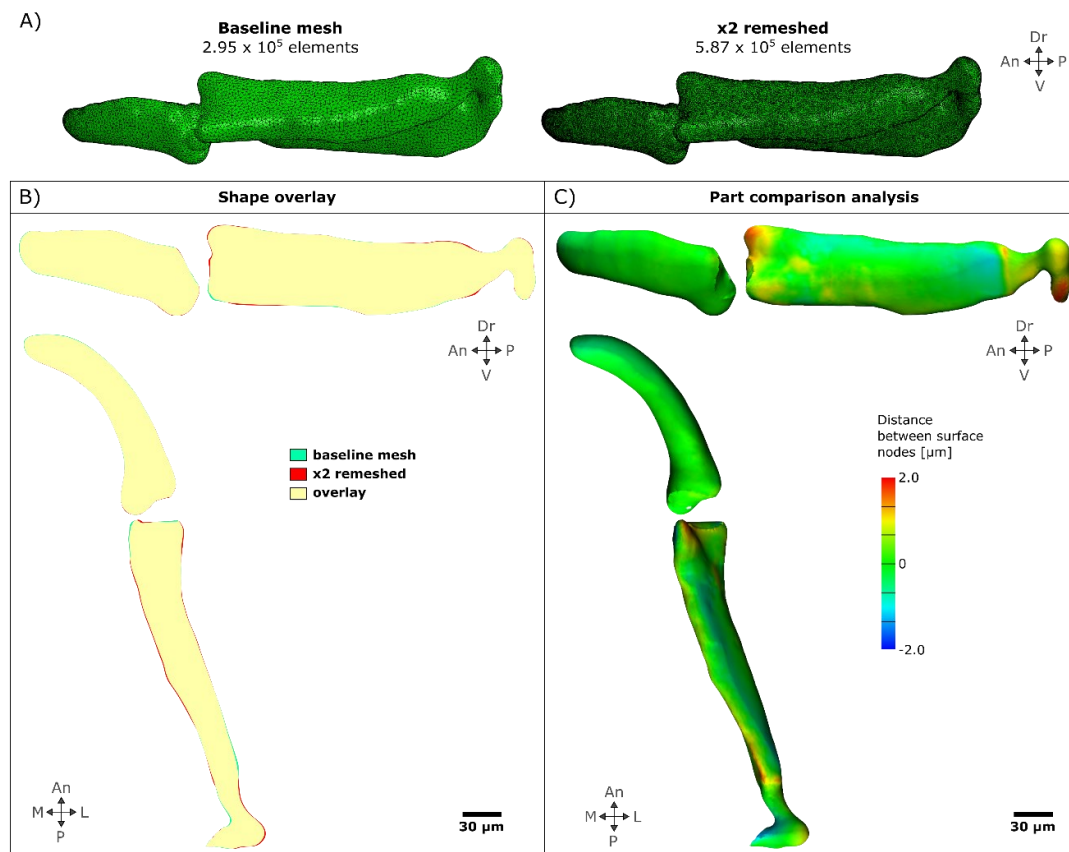


Figure S1.1: Mesh convergence study showing subtle shape differences when simulating growth from 4 to 4.5 dpf using twice as many mesh elements. A) Initial assembly at 4dpf with baseline mesh or meshed with twice as many elements (x2 mesh). B) Shape overlay of simulated shapes from 4–4.5 dpf using either the baseline mesh or the x2 mesh. C) Part comparison analysis between the two simulated shapes from 4–4.5 dpf showing subtle shape differences. An: anterior, Dr: dorsal, L: lateral, M: medial, P: posterior, V: ventral.

Appendix I.3. Median and minor axes of growth ellipsoids

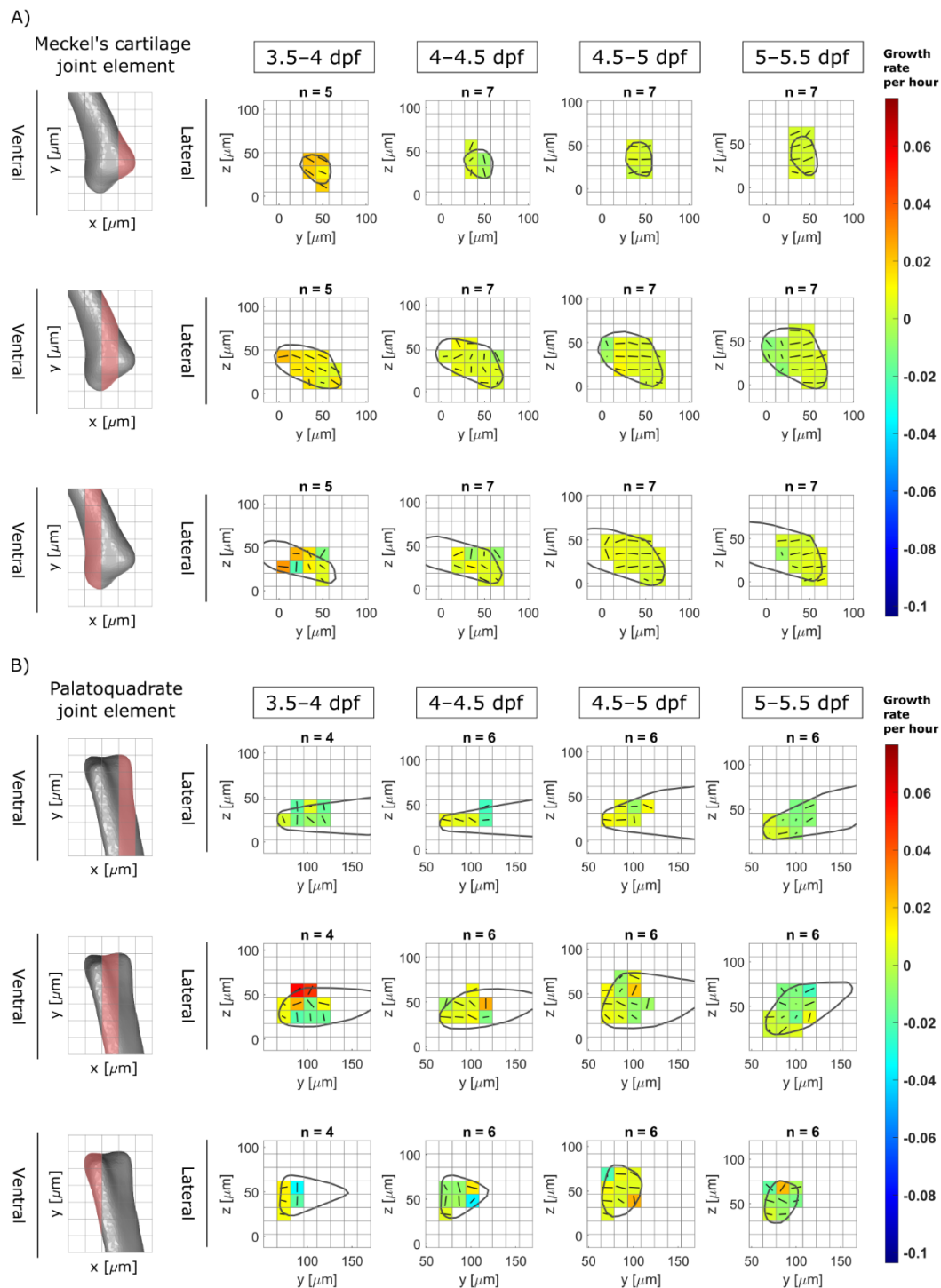


Figure S1.2: Maps showing growth rates along the second direction for growth (median axis of the ellipsoid) and their associated directions for each time window (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf) in the anterior Meckel's cartilage (A) and posterior Palatoquadrate (B) joint elements in the lateral plane. Growth rates are represented by colours while the direction is shown by solid black lines. Results are displayed across the rudiment's depth; views in the ventral plane of each section are displayed on the left panels.

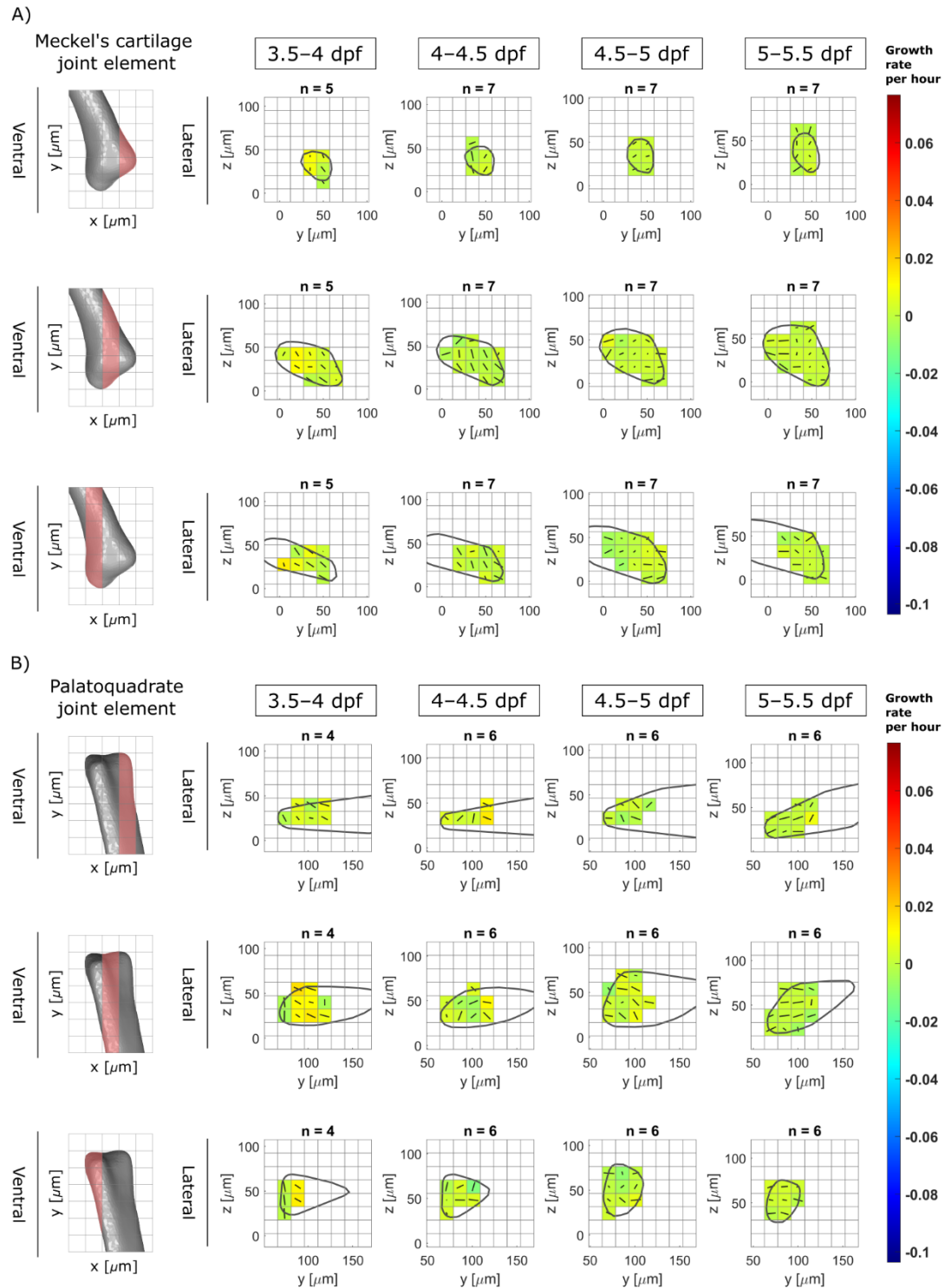


Figure S1.3: Maps showing growth rates along the third direction for growth (minor axis of the ellipsoid) and their associated directions for each time window (3.5–4, 4–4.5, 4.5–5 and 5–5.5 dpf) in the anterior Meckel's cartilage (A) and posterior Palatoquadrate (B) joint elements in the lateral plane. Growth rates are represented by colours while the direction is shown by solid black lines. Results are displayed across the rudiment's depth; views in the ventral plane of each section are displayed on the left panels.

Appendix I.4. Cell rearrangements at the Meckel's cartilage anterior tip region

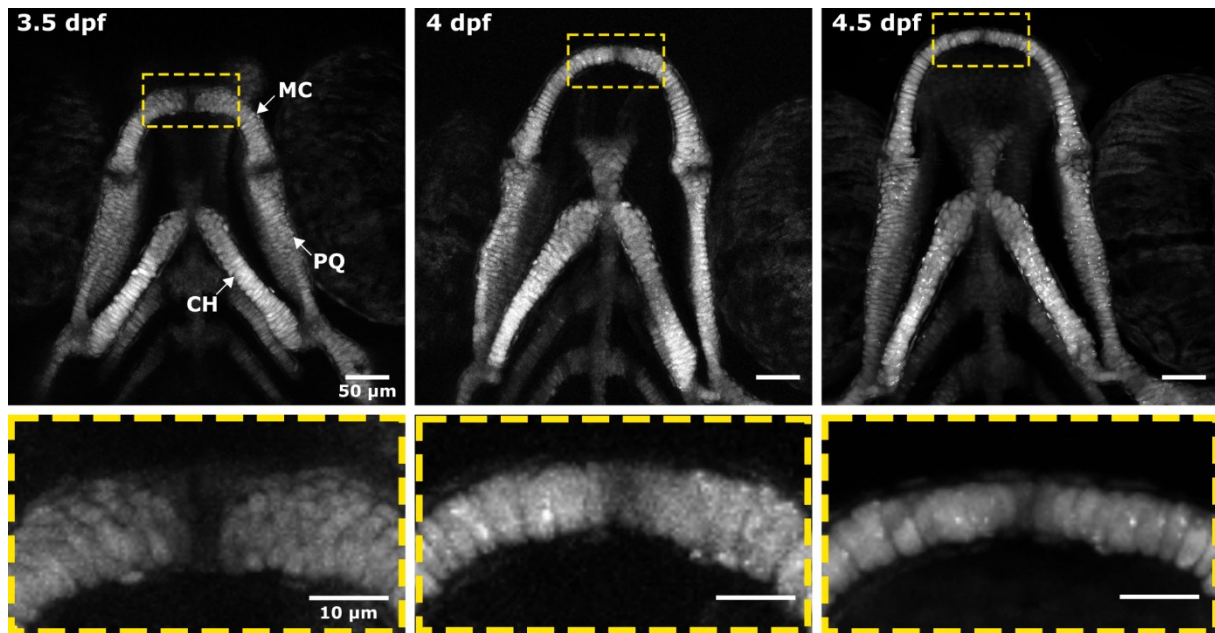


Figure S1.4: Maximum projection of ventral confocal image stacks expressing *Col2a1aBAC:mcherry* of representative specimens aged 3.5, 4 and 4.5 dpf with magnification of the MC anterior tip region showing the morphological changes and cell rearrangements occurring in this region. The most dramatic rearrangements in cell organisation at the anterior tip of the MC occurred between 3.5 and 4 dpf. MC: Meckel's cartilage, PQ: palatoquadrate, CH: ceratohyal.

Appendix I.5. Growth predictions when growth orientations or growth rates heterogeneity are suppressed

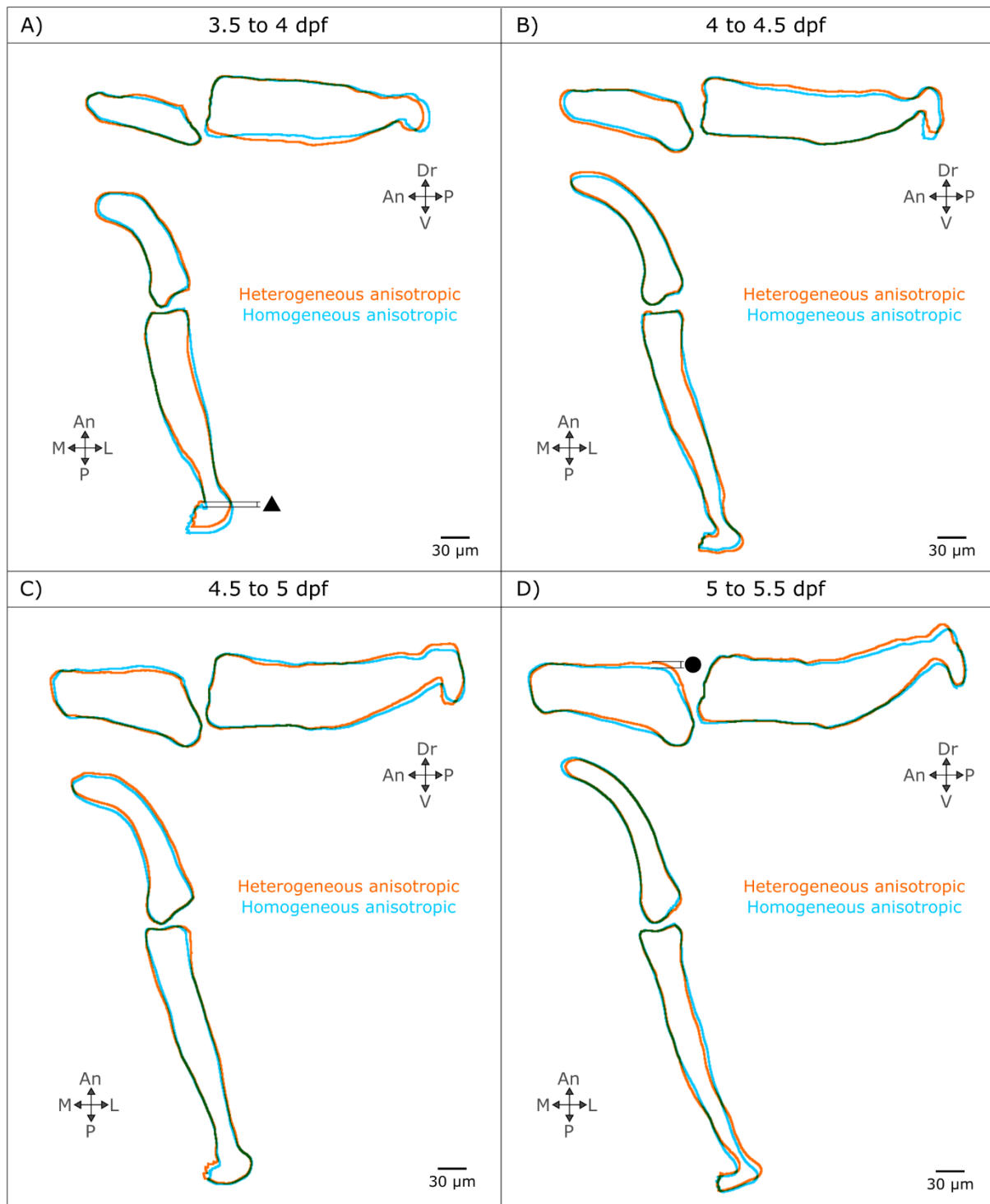


Figure S1.5: **Growth predictions obtained from homogeneous anisotropic growth fields are compared with the “full” simulation for each time window.** The black symbols denote the shape features which have been altered when growth heterogeneity has been removed (triangle: Palatoquadrate (PQ) length, diamond: Meckel’s cartilage (MC) length, square: MC width, semi-circle: PQ depth, circle: MC depth). An: anterior, Dr: dorsal, L: lateral, M: medial, P: posterior, V: ventral.

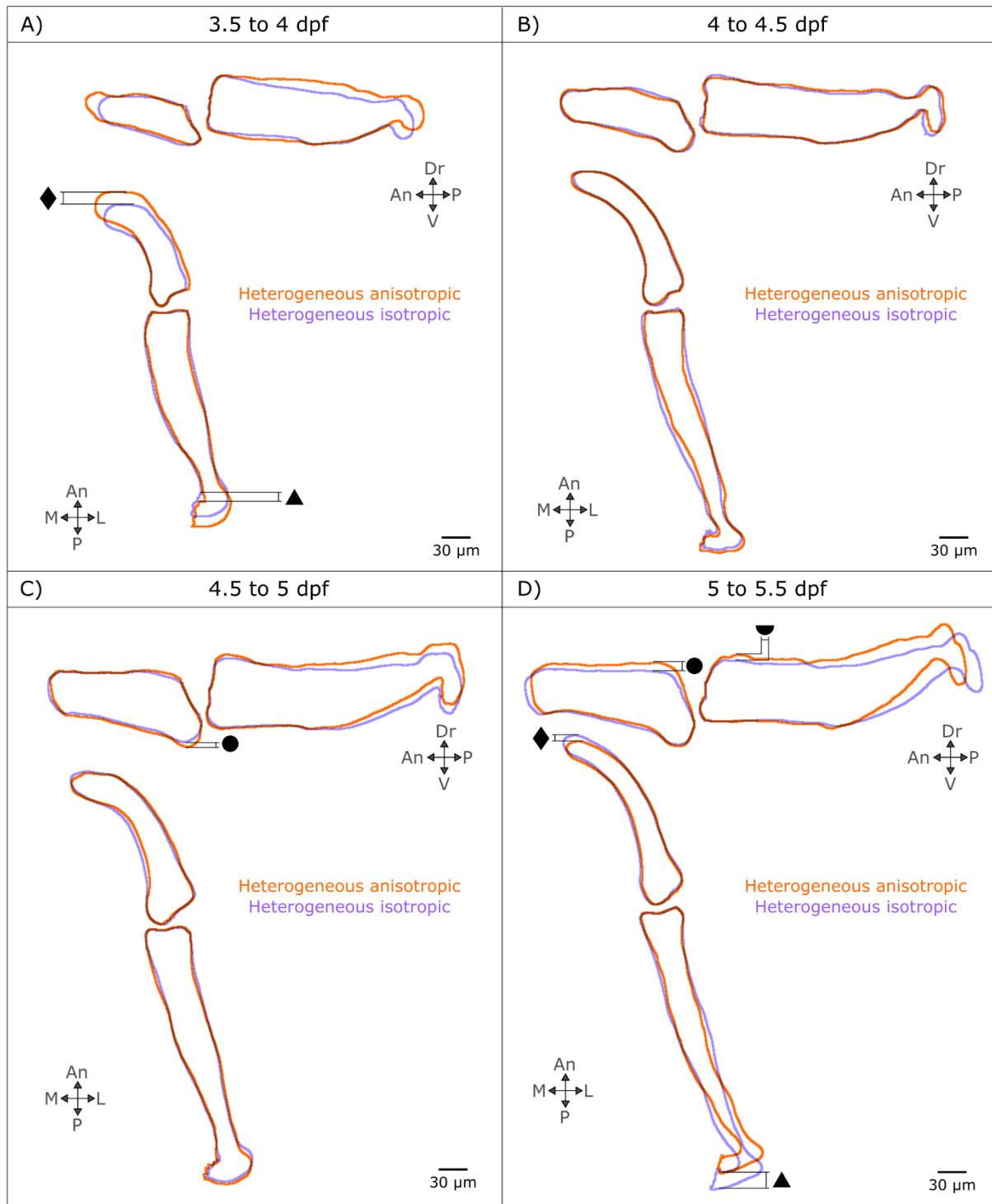


Figure S1.6: **Growth predictions obtained from heterogeneous isotropic growth fields are compared with the “full” simulation for each time window.** The black symbols denote the shape features which have been altered when growth orientation has been removed (triangle: Palatoquadrate (PQ) length, diamond: Meckel’s cartilage (MC) length, square: MC width, semi-circle: PQ depth, circle: MC depth). An: anterior, Dr: dorsal, L: lateral, M: medial, P: posterior, V: ventral.

Appendix II: Chapter 4 supplementary material

Appendix II.1. Comparisons between the material properties of immature and hypertrophic regions

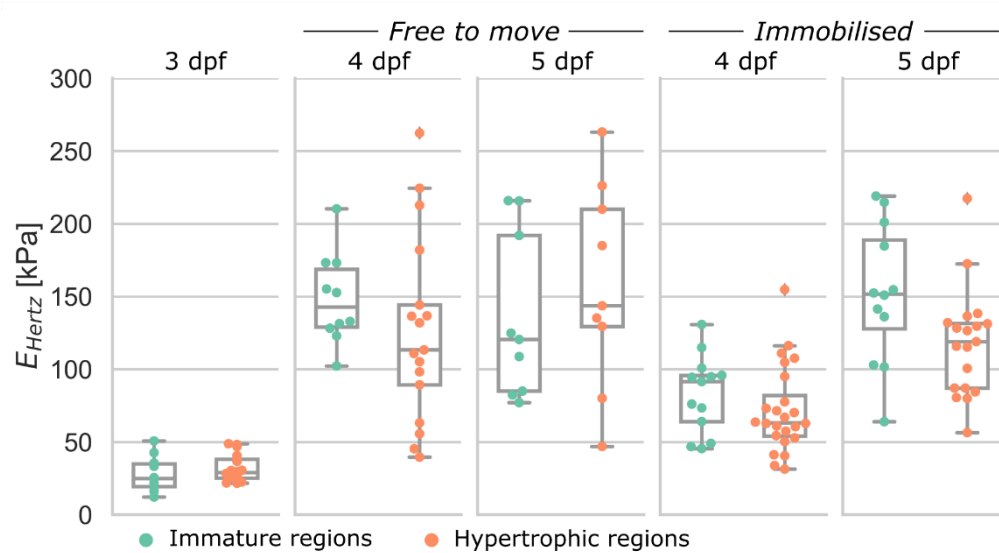


Figure S2.1: **Hertzian modulus E_{Hertz} estimations from nano-indentation measurements taken in immature and hypertrophic regions in 3 dpf, free-to-move 4 and 5 dpf and immobilised 4 and 5 dpf larvae.** No significant difference was detected between immature and hypertrophic regions.

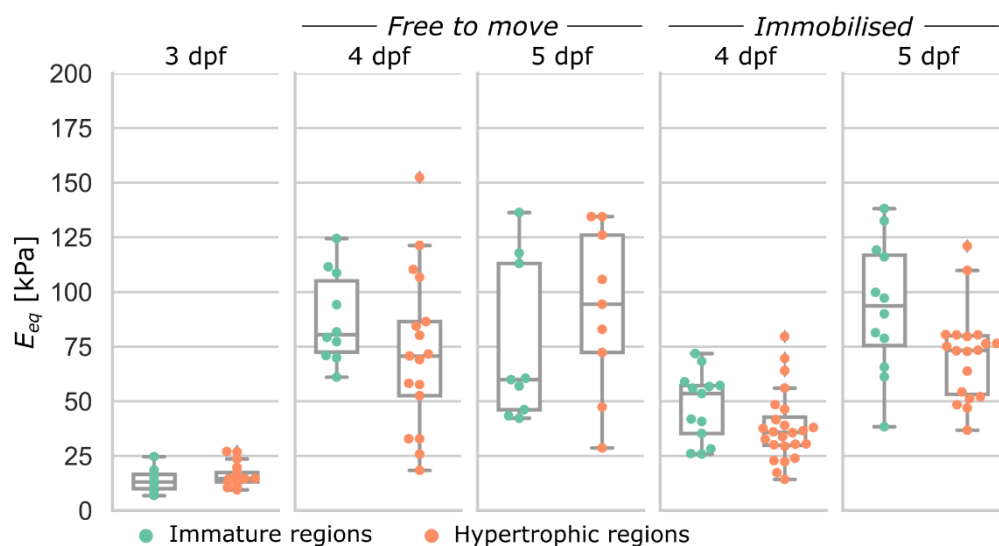


Figure S2.2: **Equilibrium modulus E_{eq} estimations from nano-indentation measurements taken in immature and hypertrophic regions in 3 dpf, free-to-move 4 and 5 dpf and immobilised 4 and 5 dpf larvae.** No significant difference was detected between immature and hypertrophic regions.

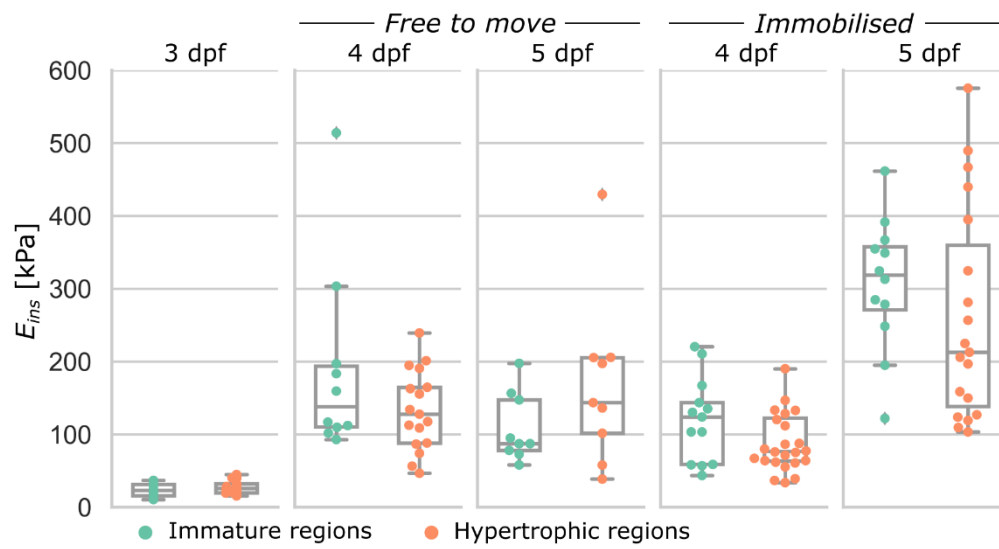


Figure S2.3: Instantaneous modulus E_{ins} estimations from nano-indentation measurements taken in immature and hypertrophic regions in 3 dpf, free-to-move 4 and 5 dpf and immobilised 4 and 5 dpf larvae. No significant difference was detected between immature and hypertrophic regions.

Appendix II.2. Statistical analyses of jaw joint cartilage biomechanical moduli

		<i>Free-to-move</i>			<i>Immobilised</i>	
		3 dpf	4 dpf	5 dpf	4 dpf	5dpf
<i>E_{Hertz}</i>	Immature regions Mean ± SD (kPa)	28.06 ± 12.27	148.23 ± 31.33	135.79 ± 56.95	82.91 ± 26.61	151.96 ± 47.67
	Hypertrophic regions Mean ± SD (kPa)	31.96 ± 9.11	126.55 ± 63.90	157.74 ± 69.90	71.72 ± 29.60	117.82 ± 36.74
	p-values	0.508	0.597	0.468	0.064	0.087
<i>E_{ins}</i>	Immature regions Mean ± SD (kPa)	23.58 ± 9.38	189.03 ± 130.79	108.79 ± 46.87	119.55 ± 57.26	307.45 ± 90.74
	Hypertrophic regions Mean ± SD (kPa)	27.22 ± 9.21	132.95 ± 54.62	168.36 ± 115.65	109.47 ± 117.15	261.00 ± 146.48
	p-values	0.797	0.508	0.157	0.249	0.695
<i>E_{eq}</i>	Immature regions Mean ± SD (kPa)	13.81 ± 5.29	87.86 ± 20.88	75.10 ± 36.60	47.68 ± 15.72	93.15 ± 30.12
	Hypertrophic regions Mean ± SD (kPa)	16.06 ± 5.23	72.39 ± 36.11	91.77 ± 37.82	38.11 ± 16.01	71.14 ± 20.80
	p-values	0.867	0.799	0.374	0.061	0.157

Table S2.1: Lists of p-values obtained with paired t-test or Wilcoxon signed-rank tests for significant differences in elastic moduli E_{Hertz} , E_{ins} and E_{eq} between measurements taken in immature regions and hypertrophic regions for all groups.

<i>E_{Hertz}</i>		<i>Free-to-move</i>			<i>Immobilised</i>	
		3 dpf	4 dpf	5 dpf	4 dpf	5dpf
Mean ± SD (kPa)		28.06 ± 12.27	148.23 ± 31.33	135.79 ± 56.95	82.91 ± 26.61	151.96 ± 47.67
<i>Free-to-move</i>	3 dpf	-	< 0.001	0.003	< 0.001	< 0.001
	4 dpf		-	0.975	< 0.001	0.999
	5 dpf			-	0.142	0.956
<i>Immobilised</i>	4 dpf				-	0.003
	5 dpf					-

Table S2.2: Lists of p-values obtained with ANOVA and Games Howell or Kruskal-Wallis and Dunn's tests with Bonferroni adjustments for significant differences in Hertzian moduli E_{Hertz} in free-to-move and immobilised larvae over time (measurements taken in the joint region, immature cartilage). Bold indicates significant difference between comparison groups ($p < 0.05$).

E_{ins}		<i>Free-to-move</i>			<i>Immobilised</i>	
		3 dpf	4 dpf	5 dpf	4 dpf	5dpf
Mean $\pm$ SD (kPa)		23.58 $\pm$ 9.38	189.03 $\pm$ 130.79	108.79 $\pm$ 46.87	119.55 $\pm$ 57.26	307.45 $\pm$ 90.74
<i>Free-to-move</i>	3 dpf	-	0.001	0.002	< 0.001	< 0.001
	4 dpf		-	1	1	0.621
	5 dpf			-	0.962	< 0.001
<i>Immobilised</i>	4 dpf				-	0.003
	5 dpf					-

Table S2.3: Lists of p-values obtained with ANOVA and Games Howell or Kruskal-Wallis and Dunn's tests with Bonferroni adjustments for significant differences in instantaneous moduli E_{ins} in free-to-move and immobilised larvae over time (measurements taken in the joint region, immature cartilage). Bold indicates significant difference between comparison groups ($p < 0.05$).

E_{eq}		<i>Free-to-move</i>			<i>Immobilised</i>	
		3 dpf	4 dpf	5 dpf	4 dpf	5dpf
Mean $\pm$ SD (kPa)		13.81 $\pm$ 5.29	87.86 $\pm$ 20.88	75.10 $\pm$ 36.60	47.68 $\pm$ 15.72	93.15 $\pm$ 30.12
<i>Free-to-move</i>	3 dpf	-	<0.001	0.003	< 0.001	< 0.001
	4 dpf		-	1	< 0.001	0.962
	5 dpf			-	1	1
<i>Immobilised</i>	4 dpf				-	< 0.001
	5 dpf					-

Table S2.4: Lists of p-values obtained with ANOVA and Games Howell or Kruskal-Wallis and Dunn's tests with Bonferroni adjustments for significant differences in equilibrium moduli E_{inf} in free-to-move and immobilised larvae over time (measurements taken in the joint region, immature cartilage). Bold indicates significant difference between comparison groups ($p < 0.05$).

Elastic fraction f		Free-to-move			Immobilised	
		3 dpf	4 dpf	5 dpf	4 dpf	5dpf
Mean $\pm$ SD (kPa)		13.81 $\pm$ 5.29	87.86 $\pm$ 20.88	75.10 $\pm$ 36.60	47.68 $\pm$ 15.72	93.15 $\pm$ 30.12
Free-to-move	3 dpf	-	> 1	> 1	0.673	0.010
	4 dpf		-	> 1	0.875	0.015
	5 dpf			-	0.011	< 0.001
Immobilised	4 dpf				-	> 1
	5 dpf					-

Table S2.5: Lists of p-values obtained with ANOVA and Games Howell or Kruskal-Wallis and Dunn's tests with Bonferroni adjustments for significant differences in elastic fraction f in free-to-move and immobilised larvae over time (measurements taken in the joint region, immature cartilage). Bold indicates significant difference between comparison groups ($p < 0.05$).

Appendix III: Chapter 5 supplementary material

Appendix III.1. Jaw joint growth orientations

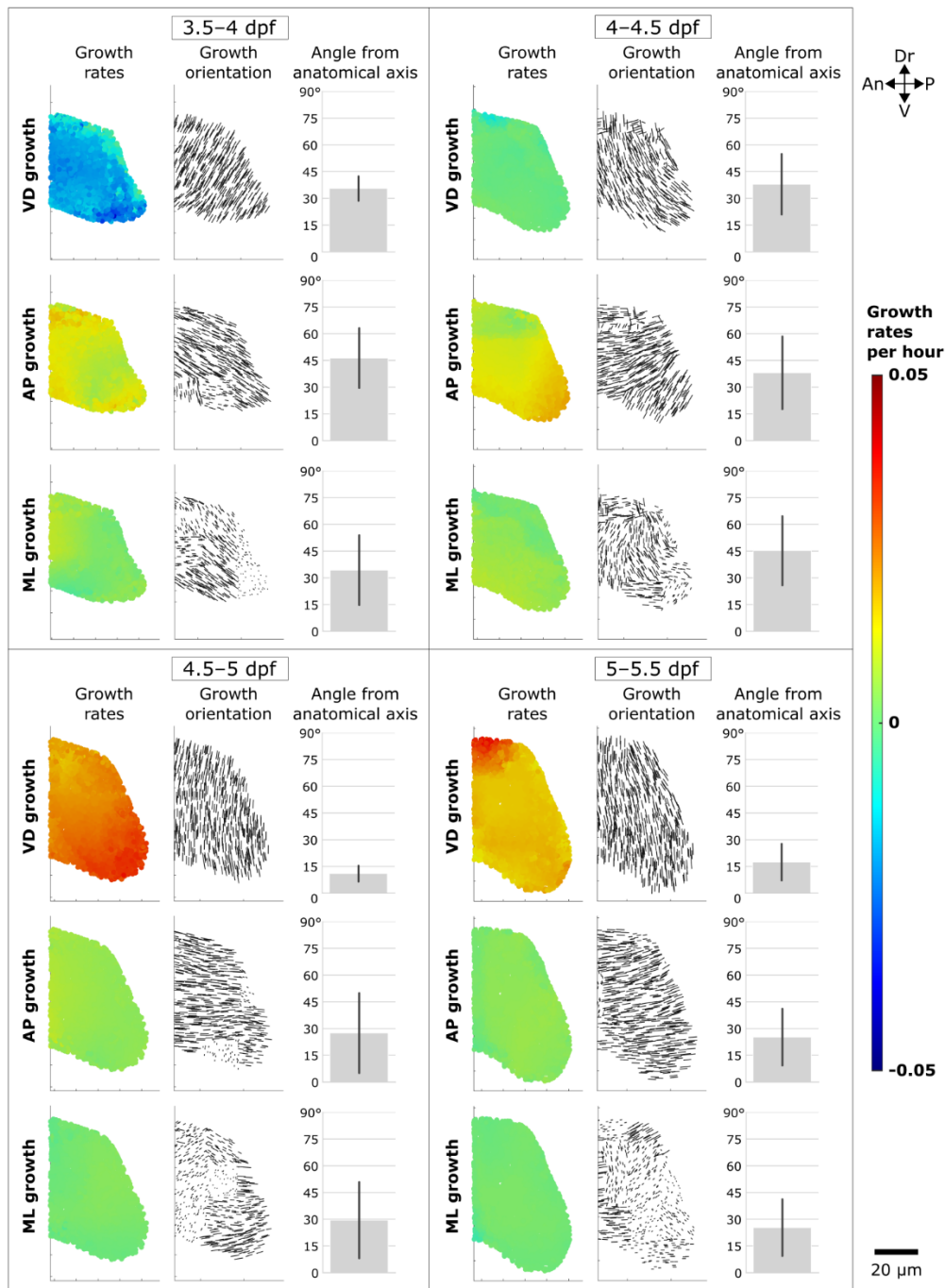


Figure S3.1: **Growth orientations in free-to-move larvae.** Maps showing ventrodorsal (VD)/anteroposterior (AP)/mediolateral (ML) growth rates (colours) and orientations (solid lines) in the MC joint element. Growth orientations correspond to the axis of the local growth ellipsoid (major, median or minor axis). Results are displayed in one section in the mid lateral plane. The angles between the growth orientations and the associated anatomical axis (e.g between VD growth orientations and VD anatomical axis) are shown in bars. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

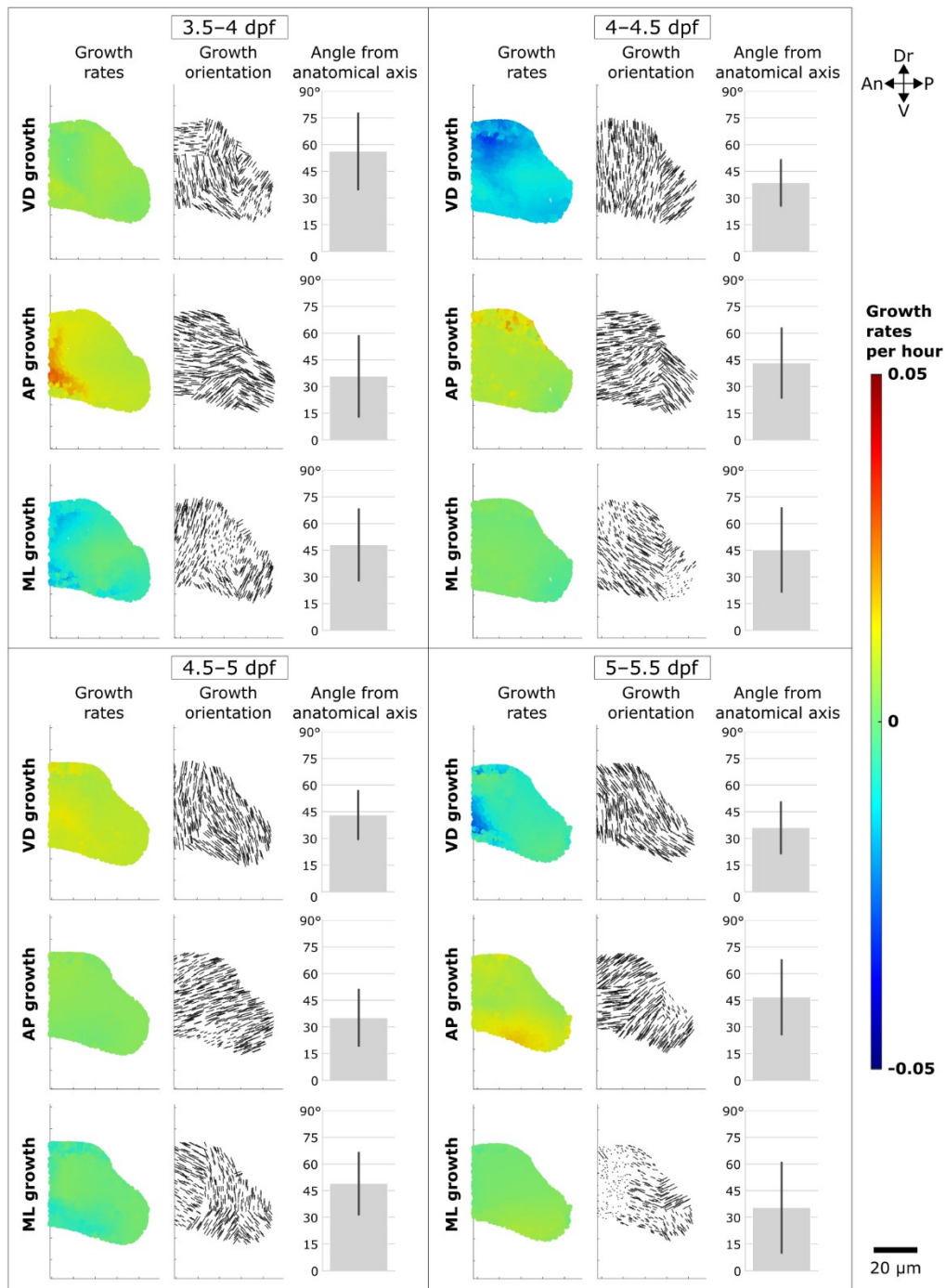


Figure S3.2: **Growth orientations in immobilised larvae.** Maps showing ventrodorsal (VD)/anteroposterior (AP)/mediolateral (ML) growth rates (colours) and orientations (solid lines) in the MC joint element. Growth orientations correspond to the axis of the local growth ellipsoid (major, median or minor axis). Results are displayed in one section in the mid lateral plane. The angles between the growth orientations and the associated anatomical axis (e.g between VD growth orientations and VD anatomical axis) are shown in bars. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

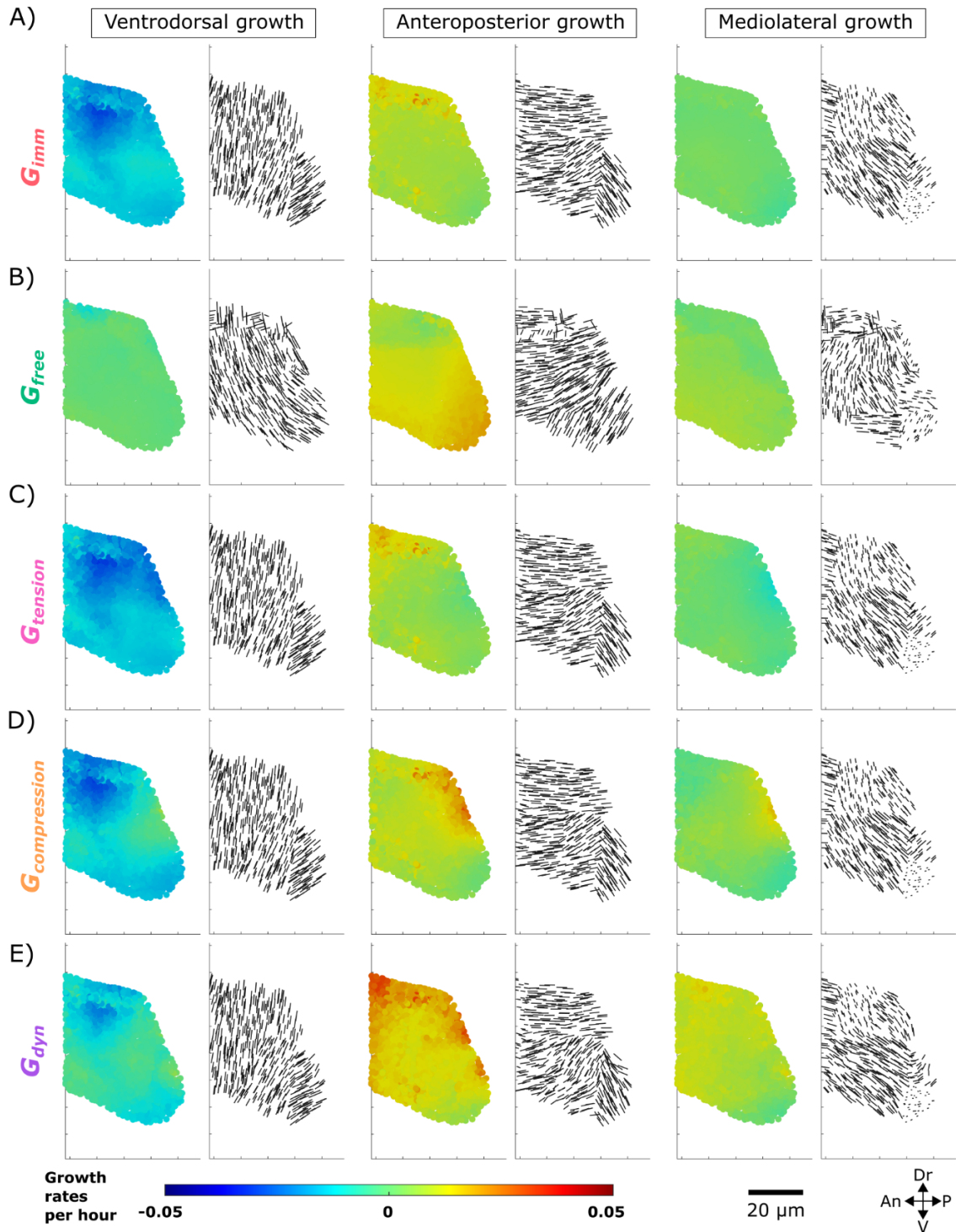


Figure S3.3: 4 to 4.5 dpf mechanobiological growth maps orientations. (A) G_{imm} , (B) G_{free} , (C) $G_{tension}$, (D) $G_{compression}$ and (E) G_{dyn} growth maps from 4 to 4.5dpf in the free-to-move MC joint element. Maps showing ventrodorsal/anteroposterior/mediolateral growth rates (colours) and growth orientations (solid lines). Growth orientations correspond to the axis of the local growth ellipsoid (major, median and minor axes). Results are displayed in one section in the mid lateral plane. Mechanoregulatory growth modulating variables $a = 2e9 \text{ N}^{-1}\text{s}^{-1}$, $b = 3e9 \text{ N}^{-1}\text{s}^{-1}$ and $c = 10e10 \text{ m.N}^{-1}$. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

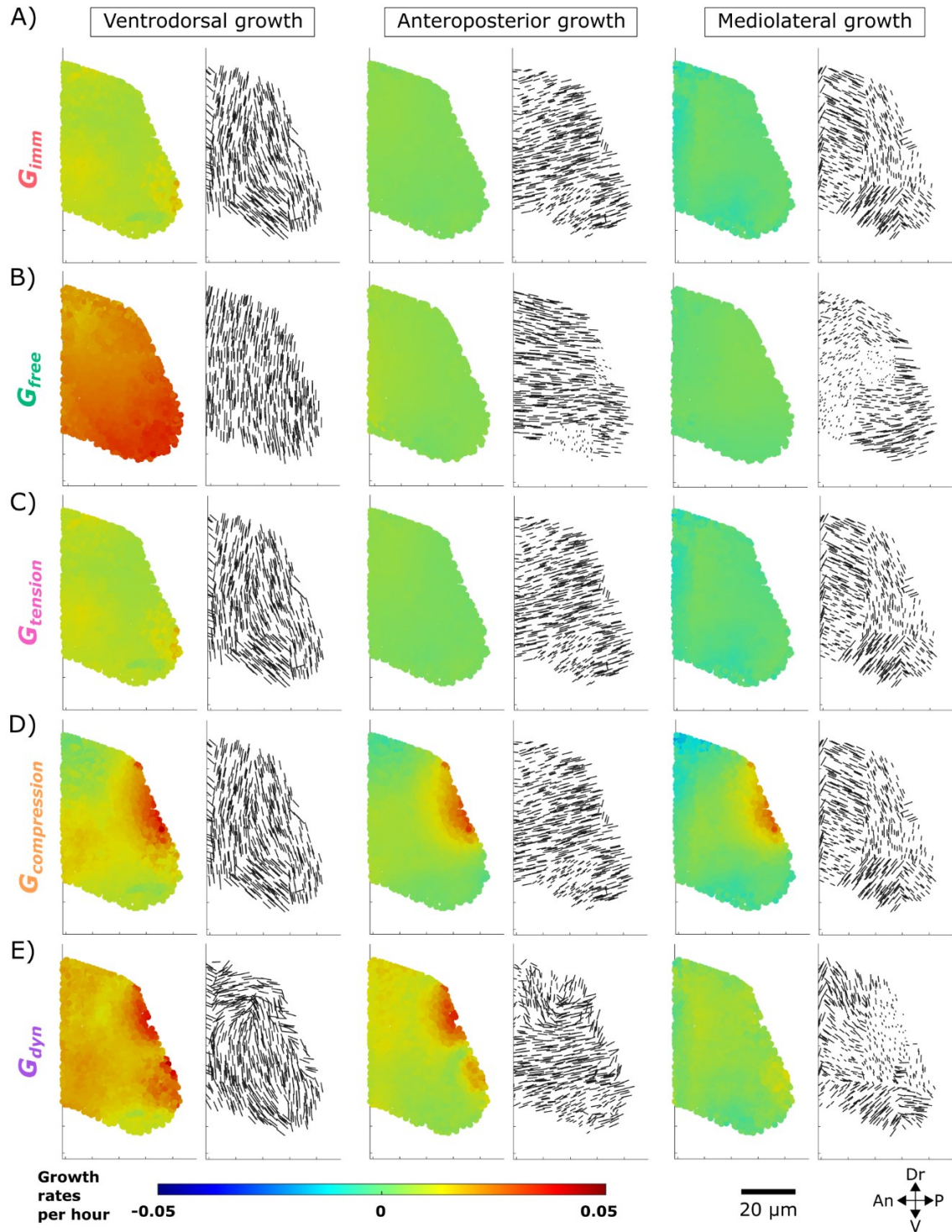


Figure S3.4: **4.5 to 5 dpf mechanobiological growth maps orientations.** (A) G_{imm} , (B) G_{free} , (C) $G_{tension}$, (D) $G_{compression}$ and (E) G_{dyn} growth maps from 4.5 to 5dpf in the free-to-move MC joint element. Maps showing ventrodorsal/anteroposterior/mediolateral growth rates (colours) and growth orientations (solid lines). Growth orientations correspond to the axis of the local growth ellipsoid (major, median and minor axes). Results are displayed in one section in the mid lateral plane. Mechano-regulatory growth modulating variables $a = 2e9 \text{ N}^{-1}\text{s}^{-1}$, $b = 3e9 \text{ N}^{-1}\text{s}^{-1}$ and $c = 10e10 \text{ m}\cdot\text{N}^{-1}$. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

Appendix III.2. Comparison between linear elastic and viscoelastic material properties in jaw movement simulations

Jaw movements were simulated at 4 dpf using successively linear elastic or viscoelastic material properties. In this appendix, details on how the viscoelastic parameters were determined from nano-indentation measurements are provided. A brief comparison of pressure fields when linear elastic or viscoelastic parameters were used is also shown.

Methods

Nano-indentation measurements were analysed as detailed in Chapter 4. A typical force-indentation curve is shown in Figure S3.1A. The material was represented as a spring (elastic modulus E_0) in parallel to a Maxwell solid (elastic modulus E_1 and time constant τ_1) to allow tissue relaxation (Figure S3.1B). The relaxation function is:

$$E(t) = E_0 + E_1 e^{-t/\tau_1}$$

E_0 , E_1 and τ_1 were obtained from fitting the relaxation function to nano-indentation measurements in Python ([Van Rossum and Drake Jr 1995](#)) as shown in Figure S3.1C, and the average across sample was used. A Poisson ratio $\nu = 0.3$ was assumed (value which was previously used for AFM testing of the larval zebrafish jaw cartilage ([Lawrence et al. 2021](#))). In Abaqus, Prony series parameters in the time domain were used:

$$g_1 = 1 - E_0 / (E_0 + E_1)$$

$$k_1 = g_1$$

Resulting parameters are shown in Table S3.1. Jaw opening and closure were simulated with each step sets to 1s with time increments of 0.1s.

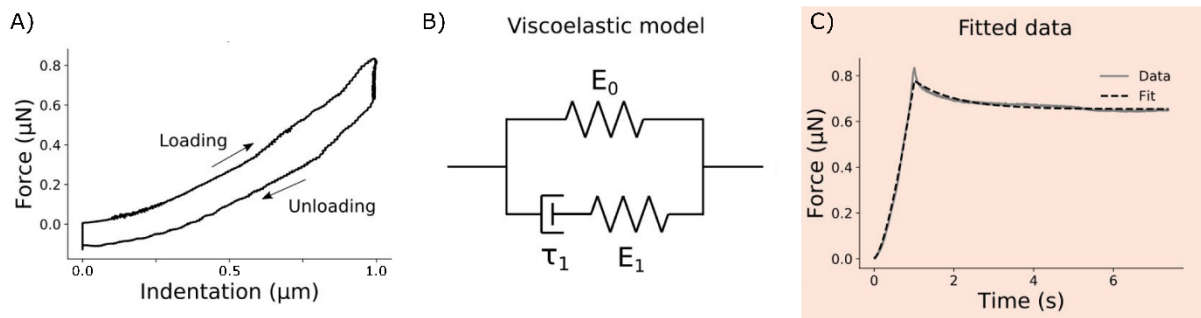


Figure S3.5: **Analyses of nano-indentation measurements.** A) Output force-indentation curve displaying hysteresis. B) The viscoelastic model consists of a spring (elastic modulus E_0) in parallel to a spring (elastic modulus E_1) and a dashpot (time constant τ_1) in series. C) The relaxation function associated with the viscoelastic model is fitted to experimental data to characterise the material's behaviour during load- and hold-phases. Adapted from (Levillain et al. 2021)

Linear elastic	E_{Hertz} [kPa]	142.0
	ν	0.3
Visco elastic	E_{ins} [kPa]	148.9
	ν	0.3
	E_0 [kPa]	62.7
	E_1 [kPa]	51.9
	τ_1 [s^{-1}]	1.87
	g_1	0.45

Table S3.1: Linear elastic and visco elastic parameters used in Abaqus simulations of jaw movement at 4 dpf.

Results

Using viscoelastic rather than linear elastic material properties the jaw displacement from peak opening to peak closure increase of 1.3 %. Pressure fields at the level of the jaw however remained similar with subtle changes of the order of magnitude of 10^{-14} . Pressure fields at peak opening and peak closure with both material properties are shown in Figure S3.2.

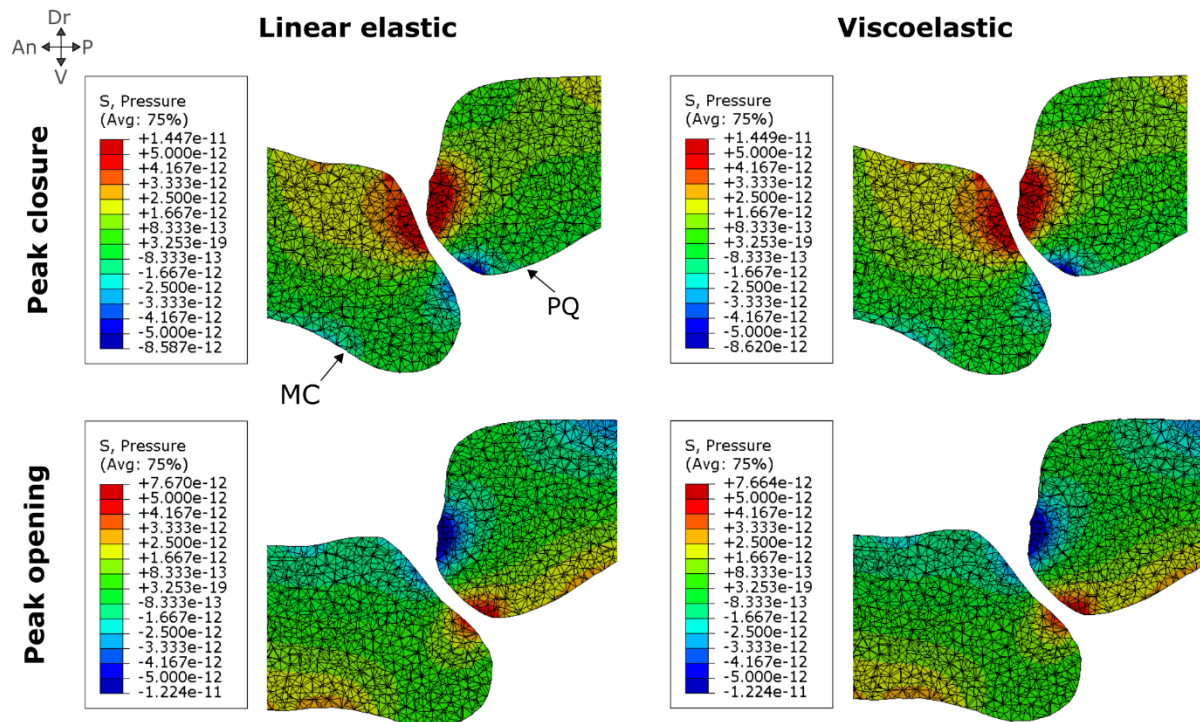


Figure S3.6: Pressure fields at the level of the joint at peak closure and peak opening when linear elastic or viscoelastic material properties are used. One section in the lateral plane is shown. An: anterior, Dr: dorsal, MC: Meckel's cartilage, P: posterior, PQ: palatoquadrate, V: ventral.

Appendix III.3. Errors to the direction during muscle loading

	Maximum relative displacement between muscle insertion points [μm]		Distance between muscle insertion points [μm]		Error to the direction [°]	
	4 dpf	4.5 dpf	4 dpf	4.5 dpf	4 dpf	4.5 dpf
adductor mandibularis (am)	4,39E+00	4,67E+00	1,96E+02	2,06E+02	1,28E+00	1,30E+00
interhyal (ih)	1,25E-02	1,31E-02	1,08E+02	1,17E+02	3,94E-03	3,78E-03
intermandibularis posterior (imp)	1,42E+01	1,39E+01	1,82E+02	1,99E+02	7,47E+00	6,80E+00
intermandibularis anterior (ima)	N/A	N/A	8,29E+01	9,55E+01	N/A	N/A

Table S3.2: Error to the direction during jaw motion simulations for each muscle, expressed in degrees.

Appendix III.4. Comparison between hydrostatic strain and stress fields

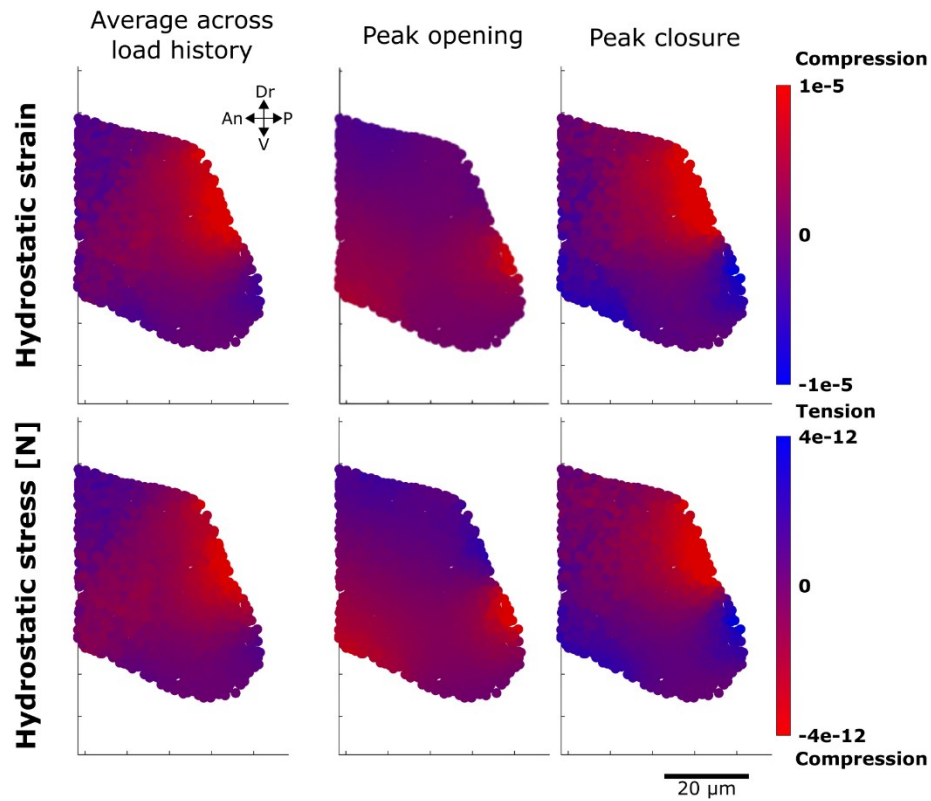


Figure S3.7: **Hydrostatic strain and stress patterns are alike.** Hydrostatic stress and strain fields in the 4dpf free-to-move MC joint element. Average fields across full joint motion, at peak closure and at peak opening are shown. Results are displayed in one section in mid the lateral plane. An: anterior, Dr: dorsal, P: posterior, V: ventral.

Appendix III.5. Mesh convergence study

A mesh convergence study was conducted on 4 dpf jaw movement simulations to ensure that the extracted stress fields did not visibly vary when a mesh with more elements was used. Mesh convergence study for growth simulation using the methodology described in Chapter 5 was conducted in Appendix I.2. Initial shape assembly at 4dpf was remeshed in 3-matic with twice as many elements as shown in Figure S3.8A. Jaw opening and closure simulations at 4 dpf were performed in Abaqus CAE following the methodology described in Chapter 5 using either the baseline mesh assembly used throughout this thesis or the x2 remeshed assembly. The resulting hydrostatic stress fields when either mesh was used showed no visible differences as shown in Figure S3.8B. The baseline mesh was therefore considered suitable when modelling jaw movements using the methodology described in Chapter 5.

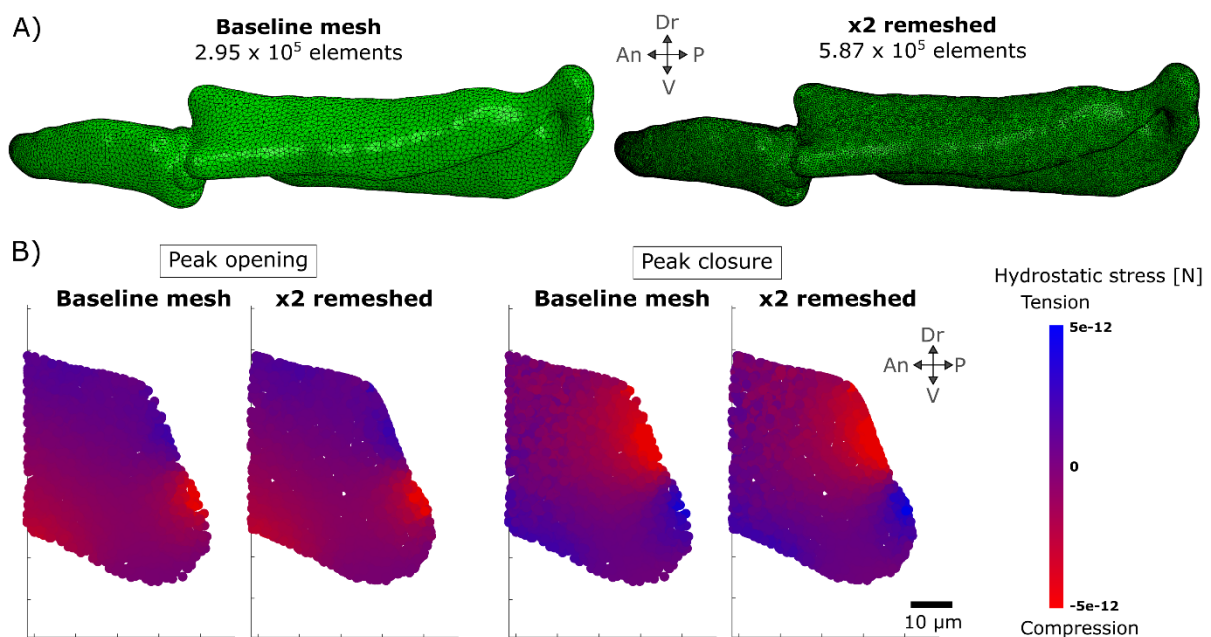


Figure S3.8: **Mesh convergence study revealed no difference in extracted hydrostatic stress field in the anterior joint element when simulating jaw movements at 4 dpf using twice as many mesh elements.**

A) Initial assembly (whole jaw) at 4dpf with baseline mesh or meshed with twice as many elements (x2 mesh). B). Hydrostatic stress fields in the anterior joint element at peak opening and peak closure using either mesh. An: anterior, Dr: dorsal, P: posterior, V: ventral.

Appendix III.6. Sensitivity analyses of mechanoregulatory growth modulating variables

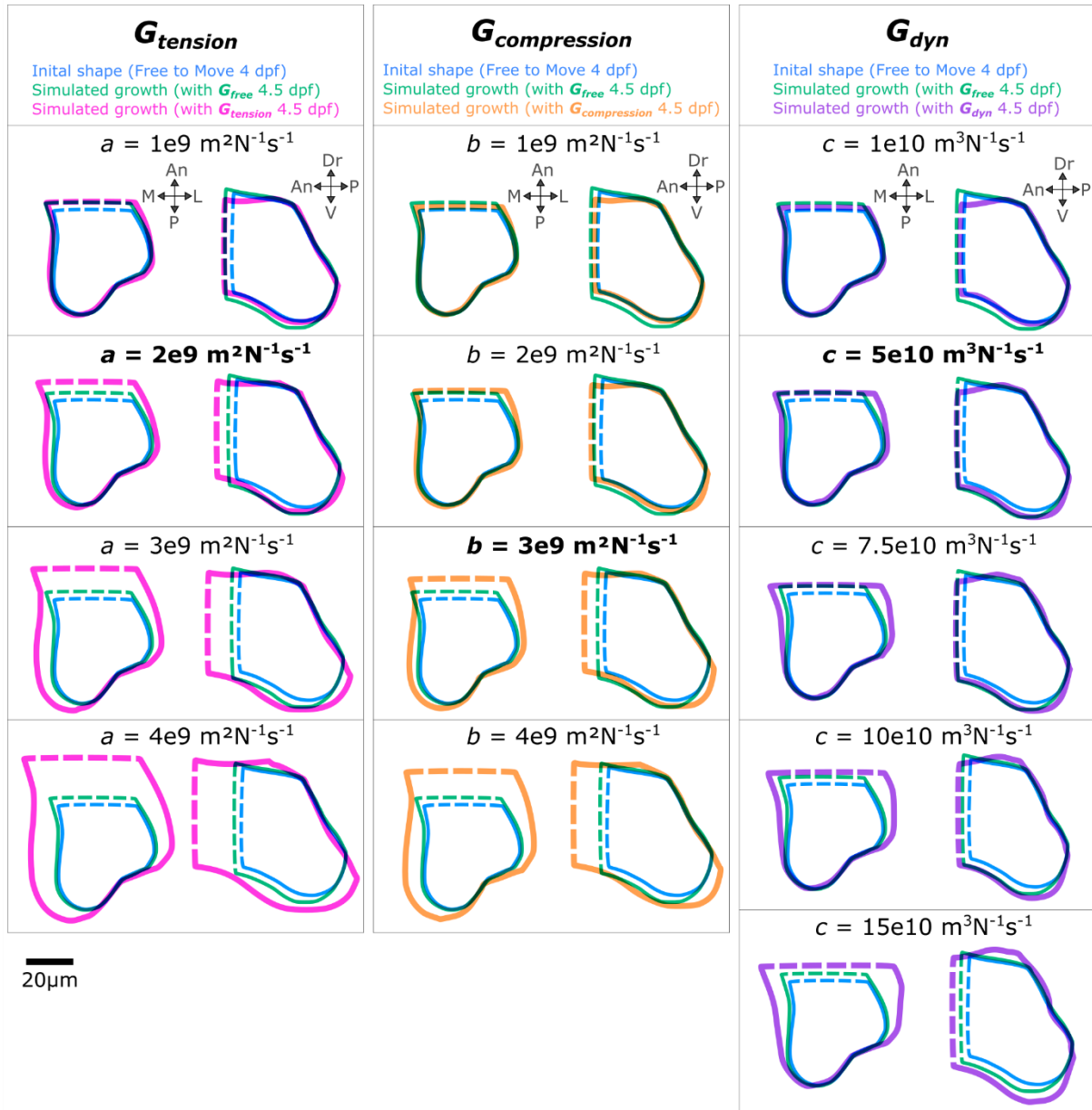


Figure S3.9: Sensitivity analyses of mechanoregulatory growth modulating variables affecting growth rates from 4 to 4.5 dpf. Shape outlines of growth simulations using $G_{tension}$, $G_{compression}$ and G_{dyn} with the modulating variables a , b and c being incrementally increased. The modulating variable enabling rescue of the MC depth at 4.5 dpf was chosen and is highlight in bold. An: Anterior, Dr: Dorsal, L: Lateral, M: Medial, P: Posterior, V: Ventral.

Appendix III.7. Statistical analyses of jaw joint growth rates

Mean $\pm$ SD	<i>Time window</i>			
	3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Ventrodorsal growth	-2.1 $\pm$ 1.3 E-02	0.4 $\pm$ 2.4 E-03	2.6 $\pm$ 0.6 E-02	1.9 $\pm$ 0.3 E-02
Anteroposterior growth	1.1 $\pm$ 0.7 E-02	1.3 $\pm$ 0.7 E-02	0.5 $\pm$ 0.2 E-02	0.4 $\pm$ 0.1 E-02
Mediolateral growth	0.3 $\pm$ 0.4 E-02	0.3 $\pm$ 0.5 E-02	0.2 $\pm$ 0.2 E-02	0.1 $\pm$ 0.1 E-02

Table S3.3: List of means and standard deviations for free-to-move growth rates for each group.

Mean $\pm$ SD	<i>Time window</i>			
	3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Ventrodorsal growth	0.4 $\pm$ 0.3 E-02	-1.5 $\pm$ 0.6 E-02	1.1 $\pm$ 0.4 E-02	-0.7 $\pm$ 0.5 E-02
Anteroposterior growth	1.1 $\pm$ 0.2 E-02	1.1 $\pm$ 0.8 E-02	0.4 $\pm$ 0.2 E-02	1.0 $\pm$ 0.5 E-02
Mediolateral growth	-0.6 $\pm$ 0.6 E-02	0.2 $\pm$ 0.3 E-03	-0.3 $\pm$ 0.5 E-02	0.3 $\pm$ 0.2 E-02

Table S3.4: List of means and standard deviations for immobilised growth rates for each group.

p-values	<i>Time window</i>			
	3.5 to 4 dpf	4 to 4.5 dpf	4.5 to 5 dpf	5 to 5.5 dpf
Ventrodorsal growth	< 0.001	< 0.001	< 0.001	< 0.001
Anteroposterior growth	3.560	0.061	0.160	< 0.001
Mediolateral growth	< 0.001	0.361	< 0.01	0.372

Table S3.5: Lists of p-values obtained with Mann-Whitney U-test and Bonferroni adjustments for significant differences between free-to-move and immobilised growth rates within each time window and each axis. Bold indicates significant difference between comparison groups ($p < 0.05$).

Appendix IV: Chapter 6 supplementary material

Appendix IV.1. Mouse sample parameters

Specimen		Measurements		Parameters	
Type	Number	Width [μm]	Ratio	Grid size [μm]	Neighbours search distance [μm]
TS23 wt	1	709.17	1.19	118.69	10.33
	2	603.61	1.01	101.02	10.84
	3	748.51	1.25	125.27	10.04
	4	597.52	1.00	100.00	8.26
TS23 muscleless-limb	1	592.13	0.99	99.10	13.25
	2	717.64	1.20	120.10	14.49
	4	539.10	0.90	90.22	15.72
TS24 wt	1	816.16	1.37	136.59	13.95
	2	806.19	1.35	134.92	14.16
	3	853.40	1.43	142.82	10.51
	4	865.26	1.45	144.81	10.6
TS24 muscleless-limb	1	624.87	1.05	104.58	15.36
	2	723.57	1.21	121.10	10.71
	3	842.87	1.41	141.06	10.97
	4	936.93	1.57	156.80	14.53
TS25 wt	1	1027.16	1.72	171.90	13.82
	2	1231.95	2.06	206.18	12.37
	3	1252.15	2.10	209.56	13.44
	4	1825.70	3.06	305.55	17.93
TS27 wt	1	1348.34	2.26	225.66	12.83
	2	1393.45	2.33	233.21	13.58
	3	1350.78	2.26	226.06	13.95
	4	1582.76	2.65	264.89	14.13
TS27 muscleless-limb	1	1321.28	2.21	221.13	15.68
	2	1296.81	2.17	217.03	13.74
	3	1349.18	2.26	225.80	20.05
	4	761.83	1.27	127.50	13.55

Table S4.1: Parameters used to create ROIs and identify neighbouring cells in each mouse sample

Appendix IV.2. Decomposition of cell processes' contributions to growth

In this appendix, the fraction of $V_{c,e}$ which is due to an increase of l_{cell} solely (when l_{ecm} is assumed constant over time) is assessed.

The average length $\langle l_{ecm} \rangle$ was calculated in each ROI at each timepoint. In each ROI, when calculating the texture matrix $M_{c,e}$, the quantity $\Delta l_{ecm} = \langle l_{ecm} \rangle_{t+1} - \langle l_{ecm} \rangle_t$ was subtracted from every link vector l at $t+1$ to approximate a constant ECM volume. The resulting $V_{c,e} (constant ECM)$ (with an approximate constant l_{ecm}) was of same magnitude than $V_{c,e} \frac{V_{cell}}{V_{cell} + V_{ecm}}$ when l_{ecm} was not altered. Given that subtracting Δl_{ecm} from every link vector l was a considerable approximation, the quantity $V_{c,e} \frac{V_{cell}}{V_{cell} + V_{ecm}}$ seemed a reasonable candidate when assessing which fraction of $V_{c,e}$ was due to an increase of l_{cell} solely.

Additional tests were carried on and $V_{c,e} (constant cell size)$ was estimated by subtracting $\Delta l_{cell} = \langle l_{cell} \rangle^{t+1} - \langle l_{cell} \rangle^t$ from every link vector l at $t+1$ to approximate a constant cell volume. As previously, the resulting $V_{c,e} (constant cell size)$ was of the same magnitude as $V_{c,e} \frac{V_{ecm}}{V_{cell} + V_{ecm}}$ when l_{cell} was not altered. The sum of $V_{c,e} (constant ECM)$ and $V_{c,e} (constant cell size)$ was of the same magnitude as $V_{c,e}$, the slight difference between the two terms being a consequence of the considerable approximations with no biological meaning.

Consequently, the quantity $V_{c,e} \frac{V_{cell}}{V_{cell} + V_{ecm}}$ was preferably used when estimating which fraction of $V_{c,e}$ was due to an increase of l_{cell} solely. Reciprocally, the fraction of $V_{c,e}$ due to an increase of l_{ecm} solely is approximated as $V_{c,e} \frac{V_{ecm}}{V_{cell} + V_{ecm}}$.

Similarly, the quantities $P_{c,e} \frac{P_{cell}}{P_{cell} + P_{ecm}}$ and $P_{c,e} \frac{P_{ecm}}{P_{cell} + P_{ecm}}$ are approximations of the fractions of $P_{c,e}$ which are due to an increase of respectively l_{cell} and l_{ecm} solely. The same tests than for $V_{c,e}$, as detailed above, were performed and the same conclusions were drawn.

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