

1 **A zebrafish model of developmental joint dysplasia: Manipulating the larval**
2 **mechanical environment to drive the malformation and recovery of joint shape.**

3

4 **Short title – Zebrafish joint shape recovery**

5

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14

15 **Abstract**

16 Developmental dysplasia of the hip (DDH), a malformation of the acetabulum, is a frequent
17 cause of early onset osteoarthritis. The disease encompasses a spectrum of severities,
18 some of which are more amenable to treatment. Embryonic immobilisation significantly
19 impairs the development of joint shape however the impact of this malformation to the
20 function and growth of the joint in the short to medium term is unclear. We developed a
21 novel model of developmental joint dysplasia using the zebrafish jaw joint to identify the
22 mechanisms regulating cellular plasticity and ability to recover joint shape and function.
23 Larval zebrafish were immobilised either pharmacologically or using targeted ablation of jaw
24 muscles to induce an altered joint shape. Following restoration of muscle activity we
25 dynamically monitored the joint shape and function in individuals at cellular resolution
26 impossible in other vertebrate species. Reflecting the variability of the human condition we
27 found a proportion of joints will recover both their shape and function, while others will not;
28 despite coming from a genetically homogenous population. This allowed us to study what
29 controls likelihood of recovery; we identified a number of cellular changes that predict
30 likelihood of functional recovery, including position of precursor cells, and specific patterns of
31 proliferation, migration and differentiation in joints and associated connective tissues. These
32 factors together predict recovery better than severity of malformation alone. Using Finite
33 Element Analysis we studied the mechanics of joints representative of ones that recover and
34 those that fail to identify differences in patterns of strain that could explain the cellular
35 behaviours that underpin likelihood of recovery. Thus, this model would enable the study of
36 the short to long term impact of altered joint shape on function and could help to identify the
37 changes that render an individual more receptive to treatment and therefore may potentially
38 be indicative of long term joint health.

39

40 **Introduction**

41 The complex shape of a synovial joint is a vital component of its function; governing its range
42 of motion and capacity to efficiently transmit load. Congenital joint shape defects, such as
43 developmental dysplasia of the hip (DDH) increase an individual's risk of osteoarthritis (OA)
44 even if treated successfully; although some subtle forms of DDH can resolve spontaneously
45 [1]. DDH, caused by a shallow acetabulum leading to a joint prone to subluxation is
46 associated with factors that constrain foetal and postnatal movement including breech
47 presentation and swaddling [2]. DDH has a dramatic impact on the hip joint but more subtle
48 variations in joint shape have been associated with increased risk of developing
49 osteoarthritis [3,4]. Thus, joint shape is an important regulator of joint fitness. Current
50 treatment for DDH using a Pavlic harness relies on postnatal remodelling and growth to
51 modify the shape of growing joint, however, success is linked to early treatment and failure
52 to surgical intervention [1]. The mechanisms through which these abnormal joint shapes are
53 corrected, and the factors governing the optimal window for treatment remain unclear.

54 Mechanoregulatory signals generated by muscle contraction are vital to the development of
55 its characteristic 3D structure [5-9] and have been suggested as primary driver of DDH
56 development [10]. Studies in the chick [6,11,12], mouse [13-15] and zebrafish [5,15,16] have
57 shown that loss of muscle activity leads to malformed joints lacking features such as
58 ligaments, articular cartilage, meniscus and articular processes. Gradients of shear, tension
59 and compression are created during muscle contractions and cells are differentially
60 responsive to these varying mechanical signals altering their cell behaviour or differentiation
61 in response to the changing mechanical force [17-19]. Mechanical stimulus is also a major
62 modifier of disease progression in OA. Immobilising murine knee joints after surgical
63 destabilisation, reduces expression of OA associated genes and protects these joints from
64 degeneration [20] and also slows tissue damage in patients with established OA [21,22].
65 However, immobilisation can also drive the degeneration of healthy cartilage [23]. Thus,
66 mechanical stimulus wrongly applied can be detrimental to joint health. How aberrant

67 mechanical stimuli, generated by the movement of a malformed joint might be integrated into
68 the regulatory framework governing postnatal growth in order to achieve joint recovery is
69 poorly understood.

70 Various models of DDH exist; including spontaneous canine models [24,25], cast/swaddling
71 models in rats and rabbits [26,27] and foetal immobilisation [10]. Canine and cast/swaddling
72 models do not recapitulate the initial development of DDH as the hip is normal at birth. The
73 chick immobilisation model can induce a shallow acetabulum however joint degeneration
74 has not been confirmed. Here we present a zebrafish model that facilitates the study of the
75 early developmental steps leading to a subluxed joint and the testing of factors underpinning
76 likelihood of recovery. Zebrafish develop externally making them tractable for drug treatment
77 or laser surgery. Zebrafish have synovial joints and have been demonstrated to develop
78 phenotypes resembling osteoarthritis as they age [28,29]. Most importantly the availability of
79 transgenic lines labelling the developing skeleton enables studies of cell behaviour in live
80 larvae [39]. We hypothesise that restoration of movement, following immobilisation, drives
81 morphogenetic changes in the zebrafish jaw and joint remodelling. To test this, we induced
82 jaw joint malformation using a period of pharmacological immobilisation [5] or by muscle
83 ablation, then allowed movement to resume and tracked the changing jaw shape, cell
84 behaviour and differentiation. Laser ablation has been used to study repair mechanisms
85 after injury [30] and was used here to sever the adductor mandibularis. We show that
86 immobilised larvae that recover have a significantly different joint shape to those that fail to
87 recover; and they successfully remodel the joint and associated tissues including the
88 ligament and enthesis. We also demonstrate that recovery potential is underpinned, in part,
89 by changes to cell orientation, migration and proliferation. Using finite element analysis to
90 explore the mechanoregulatory environment experienced by recovering and subluxed larvae
91 we identify differences in patterns of strain that could explain the cellular behaviours that
92 underpin likelihood of recovery.

93

94 **Results**

95 *Resumption of movement following immobilisation leads to changes in jaw placement.*

96 The anaesthetic MS222 (tricaine) induces flaccid paralysis in zebrafish and has been shown
97 to lead to malformation of the jaw joint and changes to joint cell behaviour [5,15,16]. To
98 determine the capacity of these malformed joints to recover functionality larvae were treated
99 with MS222 for 48hrs from 3-5dpf then allowed to resume movement. To rule out secondary
100 drug effects and to investigate the impact of targeted change to jaw mechanics we also
101 developed a model in which we bilaterally ablated the adductor mandibularis, which is
102 responsible for jaw closure, using a laser. Following ablation, muscle regrowth was visible
103 by 1 day post ablation (dpa) and muscle fibres reconnected the elements and were
104 contractile by 2dpa (at 5dpf) (S1 Fig).

105 Following either anaesthesia or muscle ablation the jaw joint was subluxed in all larvae such
106 that it was rendered unable to close (S1-3 Video) at 5dpf (Fig 1B-C, 1-2). Underlying this
107 loss of function was a significant change to the shape of the MC and jaw joint which lacked
108 the complementary surfaces of control joints (Fig 1A-C, 3-4). Rather, the MC overlapped the
109 palatoquadrate on the medial side of the joint (Fig 1A-C, 3-4, arrowhead). Anaesthesia and
110 muscle ablation also caused flattening and thickening of the anterior tip of the MC, close to
111 the mandibular symphysis (Fig 1B3, C3, double headed arrow). Following removal from
112 MS222, treated larvae moved their jaws significantly more frequently than control larvae (Fig
113 1D), potentially due to mild hypoxia. Although all AM-ablated larvae resumed movement,
114 indicating regeneration, they had significantly fewer jaw movements than controls (Fig 1D)
115 (S1 Fig), likely due to the reduced contractile ability of the regenerating fibres at the stages
116 observed.

117

118 By 7dpf the majority of immobilised and of ablated larvae recovered from the subluxed
119 position, such that they could now fully close the mouth (Fig 1E-I, 1,2 arrows). Larvae with a

120 “recovered” jaw position (Fig 1F, H) displayed complementary joint surfaces, and an
121 appropriately positioned retro-articular process (RAP) (Fig 1F,H), resembling the
122 appearance of unmanipulated controls. The remaining larvae retained their subluxed jaw
123 position (Fig 1G1-2, I1-2, arrows) with overlapping joint surfaces (Fig 1G3-4, I3-4
124 arrowhead).

125 Severity of joint malformation correlates with timing and duration of anaesthesia treatment
126 [5]. We varied the timing and duration of treatment to determine if these factors also
127 determine ability to recover joint shape and function following resumption of movement. We
128 immobilised zebrafish for 24, 48 or 56 hours from 72 or 96hpf (Fig 1J) and monitored the
129 percentage of larvae that recovered following drug wash out and resumption of muscle
130 activity (Fig 1K). Recovery was significantly affected by the timing of treatment. When
131 movement resumed prior to 4dpf 95% of larvae recovered by 7dpf, a significantly higher
132 percentage than the worst performing treatment group (those larvae immobilised for 56
133 hours) from which only 69 % recovered (Fig 1K). Following ablation 84% of larvae
134 recovered, however, due to low sample number (n=30) these were not included in statistical
135 tests. These results indicate that there is sufficient joint plasticity at 5dpf to enable most
136 larvae to recover functionality, however earlier resumption of movement significantly
137 improves chance of recovery.

138 *Anaesthesia significantly alters the overall shape of the Meckel’s cartilage*

139 To investigate the impact of immobilisation and restored movement on MC shape we used
140 geometric morphometric analysis. In addition to immobilised and ablated larvae we included
141 Myod^{-/-} which lack jaw musculature [31] to model continued jaw joint paralysis. Control,
142 Myod^{-/-}, larvae anaesthetised from 3-5dpf and larvae with ablated AM were imaged live at
143 5dpf then each larva re-imaged at 7dpf. At 7dpf immobilised and AM-ablated larvae were
144 classified as “Subluxed” or “Recovered”. Outlines of the MC at 5dpf (Fig 2A) were analysed
145 using npManova and between-groups Principal Component Analysis (bgPCA) [16] to
146 quantify how MC shape differs between the groups. Each principal component (PC)

147 describes a trend in how the shapes within the analysis vary. Thus, similar shapes occupy
148 similar positions along a PC axis while different shapes occupy opposing ends. A shape is
149 described fully by all components as they may be similar in one component but differ in
150 another.

151 Loss of muscle activity had a significant effect on the shape of the MC at 5dpf. The
152 anaesthetised, ablated and Myod^{-/-} larvae were all significantly different from control larvae
153 (Fig 2B, S1 Table) and occupied different quadrants in PC1 and PC2 of the morphospace
154 relative to control. PC1 accounts for 78.4% of variation in shape, separates the groups
155 based on increasing MC width near the mandibular symphysis and degree of joint overlap
156 (Fig 2D, F). PC2 accounts for 15.5% of the variation and captures the shape of the MC arch;
157 control and Myod mutants are more gently arched than anaesthetised or ablated larvae.
158 Ablated and anaesthetised larvae collocate while Myod mutants are dramatically different to
159 controls. At 5dpf there was no significant difference in shape between larvae that will recover
160 and those that remain subluxed at 7dpf (Fig 2B, D, F S1 Table). As such, MC shape at 5dpf
161 does not reliably predict likelihood of recovery.

162 Resuming movement had a significant impact on the shape of the larval jaw at 7dpf. PC1
163 and PC2 accounted for 73.4% and 14.3% of variation between the groups; describing similar
164 trends as at 5dpf (Fig 2E). The shapes of recovered jaws at 7dpf were significantly different
165 from those that remained subluxed (Fig 2C, S2 Table) occupying an intermediate position in
166 the morphospace plot, overlapping both the control and subluxed domains (Fig 2E, G).
167 These data indicate that within a pool of similarly treated larvae a subgroup can recover a
168 functional joint and the resultant recovered shape differs significantly from the shape of
169 those that remain subluxed.

170 *Differences in cell orientation, shape and size precede recovery.*

171 Morphospace analysis highlighted the impact of altered load on mandibular symphysis and
172 jaw joint shape. The differentiation of cells at both sites is affected by loss of muscle activity

173 [5,15,16]. As chondrocytes mature their appearance changes from small spherical cells to
174 larger ellipsoids [32]. Therefore, chondrocyte differentiation state can be inferred by
175 measuring their size and circularity. A perfect circle has a circularity of 1, with decreasing
176 values for elongated cells. We compared the shape and size of chondrocytes in the anterior
177 MC in control, Myod^{-/-}, anaesthetised and ablated larvae, with the latter two groups
178 subdivided by their status at 7dpf. Cells flanking the mandibular symphysis were outlined
179 and circularity and area calculated for each cell (Fig 3A-J). Due to the small number of AM
180 ablated larvae that remained subluxed all further analysis on AM-ablated larvae were carried
181 out on jaws that were classed as recovered at 7dpf. At 5dpf midline chondrocytes were
182 significantly smaller and rounder in Myod^{-/-} mutants and larvae that remain subluxed than
183 controls and larvae that recover. At 7dpf only chondrocytes in larvae that remain immobile,
184 i.e. Myod^{-/-}, were significantly rounder than control, suggesting that movement drives
185 maturation of this cell population.

186 Immobility leads to alterations to joint cell orientation and size [5]. To determine whether
187 restoration of normal cell size and orientation is achieved during recovery we measured the
188 chondrocytes located at the joint (Fig 3O-Q, S2 Fig). At 5dpf joint chondrocytes in
189 anaesthetised larvae that will recover were statistically indistinguishable to controls; while
190 chondrocytes in those that failed to recover, Myod^{-/-} and AM-ablated larvae, were
191 significantly smaller than the chondrocytes of controls (Fig 3P). No significant difference in
192 cell size was seen between any of the groups at 7dpf; due to the variability of control cell
193 size (Fig 3Q). Analysis of the orientation of the major axis of joint cells relative to the
194 palatoquadrate surface, revealed significant differences in orientation of cells at 5dpf on both
195 the medial and lateral joint sides in larvae that fail to recover relative to controls, whereas
196 joint cells from larvae that will recover do not differ from controls. By 7dpf the orientation of
197 cells in subluxed treated larvae are no longer significantly different from controls and
198 recovered larvae, whereas Myod^{-/-} larvae, which receive no mechanical input remain
199 significantly different to controls (S2 Fig B, C). This data demonstrates that variation in size

200 and orientation of cells located at the joint and symphysis at 5dpf are associated with ability
201 to recover; and that reapplication of mechanical stimulus allows cells to reorientate.

202 *Movement drives restored expression of joint associated genes*

203 Loss of muscle activity in amniotes leads to cartilage fusion in the joint territory driven by
204 loss of joint specific markers in favour of the expression of *Collagen 2 (Col2)* and other
205 cartilage markers [6,12,33]. We used the relationship between *Col2a1*, a marker of mature
206 chondrocytes, and *Sox10*, a marker of neural crest [34] expressed in immature craniofacial
207 chondrocytes and joint precursor cells (Fig 4A), to determine if joint boundaries re-emerge
208 during recovery. At 3dpf cells expressing *Sox10* were seen across the whole joint, with
209 particularly strong expression on the medial side where the RAP forms (Fig 4A 2, 3, 6). At 5
210 and 7dpf cells in the joint line, tip of the RAP and 2-4 cells flanking the mandibular
211 symphysis express high levels of *Sox10* and much lower levels of *Col2a1* (Fig 4 A4, 5, 7).

212 To alleviate variation in the intensity of *Sox10* or *Col2a1* due to copy number variation or
213 imaging parameters we extracted the anti-correlation component of the “product of the
214 difference of the mean” (PDM) [35] of the confocal dataset. In 5dpf controls *Sox10* was
215 expressed across the joint line in cells where *Col2a1* was not expressed; these cells were
216 clearly identifiable in the PDM (Fig 4B, arrows). In comparison immobilisation lead to a
217 significant reduction in the size and definition of the joint domain, particularly apparent in
218 *Myod* mutants (Fig 4B, D). The magnitude of the anti-correlation component, indicative of
219 how different the two channels were, was also significantly reduced in *Myod*^{-/-} larvae at 5dpf
220 (S3 Fig).

221 At 7dpf the relative expression of *Sox10* and *Col2a1* remained significantly disrupted in
222 subluxed and *Myod*^{-/-} larvae (Fig 4C, E), whereas the joint region of recovered larvae and
223 AM-ablated larvae were no longer significantly different to controls (Fig 4E). Thus, larvae
224 capable of recovery reacquire a group of cells with high *Sox10* low *Col2a1*, suggestive of

225 immaturity/plasticity in the joint, whereas larvae that fail to recover or remain paralysed fail to
226 maintain these joint specific cells.

227 *Recovery is driven by changes to cell proliferation and migration.*

228 We hypothesised that proliferation and/or migration of these *Sox10* high *Col2* low cells might
229 drive joint recovery. Cell proliferation following the resumption of movement was measured
230 by treating larvae with BrdU from 5-7dpf, at which time the larvae were sorted into recovered
231 and subluxed groups, stained immune-fluorescently and quantified (Fig 5C, D). BrdU
232 incorporation was significantly increased, relative to control, on both the medial and lateral
233 side of the joint in subluxed larvae. In contrast, proliferation was only increased on the lateral
234 side of the joint in recovered larvae (Fig 5 D). BrdU+ cells were present in larger numbers in
235 the lateral joint margin (Fig 5A arrowhead) in both groups relative to control.

236 Cell migration during recovery was assayed by photoconversion of kaede-expressing cells in
237 zebrafish carrying *Tg(Sox10:GAL4-VP16)* and *Tg(UAS:Kaede)* transgenes. Kaede protein is
238 green in its native state but is irreversibly photoconverted to red using UV light. 8-10 cells
239 were photoconverted at 5dpf on either the medial or the lateral side of joints in larvae that
240 were anaesthetised and in controls. These larvae were then reimaged at 7dpf to track the
241 behaviour of the photoconverted cells. The spread of cells across the joint was calculated as
242 the change in the area of the photoconverted domain (Fig 5E, F), measured from max
243 projections between 5 and 7dpf (Fig 5B, red outline). Cellular migration was significantly
244 increased on the lateral side of recovered but not subluxed larvae (Fig 5E, F). There was
245 also evidence of cellular reorientation on the lateral side of recovered joints that was not
246 apparent in subluxed larvae (Fig 5B arrowhead). In comparison, no changes to cellular
247 orientation or spread of photoconverted cells were detected on the medial side. This data
248 indicates that cells at the joint retain the capacity to adapt to the mechanical environment
249 and sculpt the joint by modifying regional patterns of cell migration and proliferation.

250 *Joint associated structures such as ligaments and their insertions are restored in recovered*
251 *larvae.*

252 Immobilisation during development has been shown to have a significant impact on the
253 structure of joint associated tissues such as ligaments and their attachments [6,11,36]. The
254 RAP, the insertion site of the interoperculomandibular ligament (IOM) is significantly
255 impacted by loss of mechanical load (Fig 1,2) [5,16]. *Col10a1:citrine (ColX)* was expressed
256 by osteoblasts in the ligament entheses (Fig 6A ,E) [37] and at a lower level throughout the
257 IOM ligament (Fig 6A, E). It was used to assess the impact of immobilisation (Fig 6 A-D) and
258 recovery (Fig 6E-I) on osteoblast number in the RAP and ligament width. Immobilisation
259 caused a significant reduction in osteoblast number in the RAP at 5dpf (Fig 6C). By 7dpf the
260 number of osteoblasts at the RAP of subluxed, but not recovered larvae, were significantly
261 fewer than controls (Fig 6 E-H).

262 At 5dpf, the width of the IOM ligament was significantly increased in immobilised larvae
263 reflecting a loss of the tight cohesive structure characteristic of ligaments (Fig 5D). At 7dpf
264 the subluxed cohort remained marginally larger but this was not significant, possibly due to
265 the small sample number (n=4). We verified the recovery of the ligament by comparing the
266 number of ligament cells that incorporated BrdU between 5 and 7dpf in control, subluxed
267 and recovered larvae. Larvae that recover proliferated significantly more between 5 and 7dpf
268 than either control or subluxed larvae. Therefore, larvae that successfully recover a
269 functional shape also repair the degenerative impact of immobilisation on the interzone and
270 joint associated tissues such as ligaments.

271 *Recovery potential is associated with regional differences in the pattern, magnitude and*
272 *orientation of mechanical stimuli within the jaw*

273 Mechanical forces act as a form of positional information with cells behaving in a regionally
274 specific manner dependent on its mechanical environment. We hypothesised that joint
275 shape, cellular orientation and differentiation could feed into the mechanical environment

276 ,which in turn regulates the cells, producing a feedback loop resulting either in success or in
277 failure. Renewed movement is required for the initiation of shape recovery and may drive the
278 rapid correction seen in recovered larvae. Finite element (FE) models of representative 5dpf
279 control, anaesthetised recovered and subluxed larva were analysed to identify potential
280 differences in the pattern of biophysical stimuli experienced by larvae that recover and those
281 that remain subluxed. The range of motion of the tip of the jaw in control and anaesthetised
282 larvae was 53.3 (+/- 18.4) μm and 57.71(+/- 22.5) μm respectively, which was replicated by
283 the displacement of the FE models during the closure step (Fig 7A-C). The insertion of the
284 IOM (interosseous membrane) ligament at the jaw joint generates a distinct peak in strain,
285 which is located at the tip of the RAP in controls (Fig 7D, J, Fig S6A). This peak correlates
286 with the position of *Col10a1* expressing osteoblasts; which are mechanically responsive and
287 reduced in anaesthetised larvae (Fig 6). The mandibular symphysis is also a site of
288 increased mechanical load in models of 5dpf control and recovered larvae (Fig7, D-E,J-K
289 arrow head) but not the subluxed model. The orientation/direction of the principal strains
290 within a tissue are important regulators of cell orientation. In the MC the orientation of
291 maximum principal strain, as indicated by the direction of the arrows (Fig 7G-I), closely
292 follows the curvature of the cartilage with a distinct peak at symphysis. Thus cells within the
293 MC are orientated perpendicular to these strains. While the magnitude of the loads are
294 reduced, relative to control, it is still possible to identify a distinct peak at the symphysis in
295 the model of recovery which is absent in the subluxed (Fig 7I).

296 We identified distinct differences in the regional behaviour of cells between larvae that
297 remain subluxed and those that successfully recover. FE modelling has been shown to
298 predict changes to joint shape and cellular behaviour in the developing jaw joint [5]. We used
299 the FE models to identify if larvae that recover also exhibit distinctly different patterns of
300 biophysical stimuli. In controls, the medial side of the joint is highly loaded (Fig 7D, J, black
301 arrowhead). Recovery is generated by a specific pattern of cell proliferation and migration
302 that varies medial-laterally. In joints that recover there is a significant increase in both

303 proliferation and migration on the lateral side of recovering joints that is absent from those
304 that remain subluxed. To determine if the magnitudes and pattern of loading differed
305 between regions of the model we extracted the absolute maximum principal strain for 10
306 nodes on the medial and lateral side of the joint (Fig 7M) and graphed the results (Fig 7N).
307 In all three models the medial side experiences variable but negative absolute maximum
308 strain indicating a dynamic compressive environment. In comparison, the lateral side of both
309 control and recovered models oscillates between positive (tensional) and negative
310 (compressive) strain while the subluxed surface remains under a lower magnitude negative
311 (compressive) strain.

312

313 **Discussion**

314 Here, we present a novel zebrafish model of developmental joint dysplasia; characterising
315 the cellular mechanisms underpinning the recovery of joint function in malformed joints.
316 Immobilised joints lack the complex interlocking joint shape characteristic of synovial joints,
317 which maintain joint position and range of motion, therefore recapitulating many of the
318 features of DDH [8]. Here we demonstrate that the resumption of movement in immobilised
319 zebrafish joints can be used to examine the impact of suboptimal joint shape on joint health,
320 raising the prospect of following manipulated joints throughout the life of the individual.

321 The zebrafish jaw joint, between the MC and Palatoquadrate, was the focus of this study.
322 This joint is synovial and can develop osteoarthritis [38] and has all the key features of
323 mammalian skeleton including articular cartilage, joint cavity, synovium, tendon and
324 ligaments [39,40]. The zebrafish also has a growing array of genetic tools [41] and is
325 amenable to live imaging making it ideal for longitudinal studies. Tricaine immobilisation
326 alters the shape of the jaw joint and MC by altering cell differentiation and proliferation [5,15]
327 and is similar to the method used in the chick model of DDH [10]. Laser muscle ablation
328 induced a similarly malformed jaw joint without the systemic issues associated with drug

329 treatments. We found a significant proportion of both the anaesthetised and ablated larvae
330 could recover a normal jaw position and approximately normal joint shape while those that
331 fail to recover remain subluxed and have a significantly different jaw shape, reflecting the
332 variability seen in human cases of joint dysplasia.

333 DDH symptoms can range from a mild joint laxity that can resolve itself, up to full subluxation
334 requiring reduction. Prognosis is correlated with severity of the malformation of the
335 acetabulum and age of treatment [1]. We hypothesised, that within a similarly treated group,
336 jaw joint shape would be an indicator of recovery. However, morphometric analysis indicated
337 that the shape of the MC at 5dpf was a relatively poor indicator of likelihood of recovery. As
338 a simplified 2D analysis was performed, it is possible that 3D aspects of the shape that might
339 influence recovery were not captured by the analysis. The relationship between the shape
340 and position of the palatoquadrate surface relative to the MC could play a substantial role in
341 recovery and wasn't included in the morphometric analysis.

342 While shape alone could not predict which larvae would recover, chondrocyte behaviours at
343 the mandibular symphysis and joint could. The orientation of cells in the joint is significantly
344 disrupted by immobilisation [5]. At 5dpf, cells in the mandibular symphysis and jaw joint of
345 larvae that remain subluxed were rounder and less differentiated than those that recover.
346 Interestingly, by 7dpf cell orientation is corrected in all samples bar the *Myod* mutants. The
347 larval response to immobilisation and recovery is a spectrum determined by differences in
348 cell behaviour.

349 Embryonic immobilisation is associated with loss of the articular surfaces, joint fusion and
350 the degeneration of structures such as meniscus and patella [33]. 5dpf immobilised larvae
351 lost the definition of the interzone, which gives rise to the articular cartilage [42]. By 7dpf the
352 anti-correlative relationship between *Sox10* and *Col2a1* expression in the interzone of
353 recovered larva was redefined but remained abnormal in joints that fail to recover (subluxed
354 and *Myod* mutants). The development of ligaments [6,33] and their insertion sites (Entheses)
355 are particularly vulnerable to loss of mechanical stimulus [43]. In immobilised and ablated

356 larvae at 5dpf the interoperculo-mandibular ligament was wider and looser and ossification at
357 the entheses was significantly reduced. By 7dpf recovery ameliorated this impact
358 significantly. Thus, movement corrects not only the shape of the joint but also drives the
359 recovery of vital joint associated structures.

360 Skeletal morphogenesis requires preferential regional growth, a feature lost during
361 immobilisation. It is achieved by the local moderation of cell migration, proliferation, cell
362 shape and matrix synthesis [33]. Cellular reorientation and shape change are a feature of
363 recovery however immobilisation has been shown to alter patterns of cell proliferation [7,44].
364 BrdU incorporation and Kaede photoconversion identified a specific pattern of cell
365 proliferation, migration and growth on the lateral side of the joint that was only present in
366 larvae that recover. These local differences in cell behaviour may be driven by specific
367 biomechanical differences between recovered and subluxed larvae.

368 We developed Finite Element (FE) models based on 3D confocal datasets of control and
369 anaesthetised larvae at 5dpf. The two chosen anaesthetised larvae were representative
370 examples of those that recover and those that remain subluxed. We identified variations
371 between the mechanical stimuli experienced by these anaesthetised larvae, which may in
372 turn drive the restoration of normal joint shape and function. FE results suggest that cells in
373 the Meckel's cartilage orientate themselves perpendicular to the main direction of strain and
374 that this signal is stronger in larvae that successfully recover. This is in keeping with reports
375 that cells align nearly perpendicular to dynamically varying stresses [45]. The
376 mechanoregulation of chondro-progenitor and chondrocyte proliferation and biosynthesis
377 has been extensively studied in a variety of culture system including explants and monolayer
378 in response to a wide range of mechanical stimuli including compression, tension,
379 hydrostatic pressure and fluid flow [46]. The response of cells to a particular load is
380 dependent on the type of load, its mode of application i.e static or cyclic and the magnitude
381 of this load. Cyclical loading of chondrocytes cultured in alginate beads had a positive effect
382 on chondrocyte metabolism increasing cartilage matrix synthesis while static load was

383 degenerative[47]. Cyclical loading impacts the differentiation of cells, increasing Indian
384 hedgehog expression and stimulating chondrocyte proliferation[19]. Stretch also stimulated
385 the proliferation of immature chondrocytes, but not that of hypertrophic chondrocytes [48].
386 Ultimately load appears to differentially regulate the behaviour of cells[18]. Thus
387 morphogenesis is regulated through a combination of intrinsic regulatory signals produced
388 by the tissue itself e.g. Wnt or Bmp signalling, and extrinsic signals produced by mechanical
389 stimuli. These extrinsic stimuli will in turn alter the expression of intrinsic regulators creating
390 a vicious circle of degeneration amplifying the original malformations. Thus the difference
391 between success and failure may be initially quite minor but could be exacerbated by this
392 “intrinsic-extrinsic” feedback loop. It also demonstrates that successful recovery is very
393 dependent on the individual, their specific cellular orientation, joint shape and mechanical
394 environment. This is corroborated by the long term prognosis of DDH which even after
395 treatment is variable[49].

396 It is possible that higher vertebrates, which lack the regenerative capacity of zebrafish [50],
397 would not respond as dramatically to the resumption of movement, though even in humans
398 cases of DDH can resolve spontaneously with no intervention [1]. As skeletal plasticity is
399 preserved in higher vertebrates we believe the zebrafish model could provide useful insight
400 into the mechanism regulating this plasticity. Using either anaesthesia or muscle ablation it
401 will be possible to target specific windows of plasticity during joint development and identify
402 what processes are disrupted and ultimately determine how some larvae are better
403 positioned for recovery, in the hope of identifying why the prognosis from some DDH
404 patients is so severe.

405 **Materials and Methods**

406 **Zebrafish husbandry and lines.**

407 Zebrafish were housed as previously described [51]. Animal experiments were approved by
408 Bristol University animal ethics committee and the UK Home Office. All transgenic and

409 mutant lines have been previously described: TgBAC(*col2a1a:mCherry*)^{hu5910} [52]; Myod^{fh261}
410 [31]; Tg(-4725*sox10:GFP*)^{ba4} [53]; *symhc:EGFP* [54]; Tg(*Sox10:Gal4-VP16*) [55];
411 Tg(UAS:Kaede) [56]; TgBAC(*col10a1a:Citrine*)^{hu7050} [57]. Sample numbers used are listed in
412 supplementary S 3 Table.

413 **Pharmacological treatment and imaging**

414 Fish were treated for 24 hours beginning 72 hours post fertilisation (hpf) and 96hpf, 48 hrs
415 from 72hpf, or 56 hours from 72hpf (Fig 1E) with 0.1mg/ml MS222 (Tricaine
416 methanesulfonate)(Sigma), diluted in Danieau's buffer refreshed twice daily. Jaw position
417 was assessed daily between 5 and 7dpf (Fig. 1C) and the percentage recovery calculated
418 from at least 4 plates of 30 larvae. Tracked cohorts were imaged on 5 and 7dpf.

419 **Live imaging**

420 Larvae were sedated, embedded ventrally in 0.5% LMP agarose (Sigma) and fluorescence
421 visualised on a Leica SP8 system using a 10x objective. After imaging larvae recovered in
422 fresh Danieau's buffer.

423 **Muscle Ablation**

424 The Tg(*symhc:EGFP*) transgenic line was used to visualise muscle position. Anaesthetised
425 3dpf larvae were mounted ventrally in 0.2% LMP agarose in Danieau's and adductor
426 mandibulae (AM) muscles bilaterally ablated with a 440nm nitrogen Micropoint laser
427 connected to a Zeiss Axioplan microscope. Ablation success was confirmed by imaging after
428 ablation. As *symhc:eGFP* is expressed in slow fibres, the efficacy of laser ablation was
429 verified in 3dpf fixed larvae using fluorescent immunohistochemistry [58] against GFP
430 (ab13970 abcam, 1:200) and skeletal myosin (A4.1025 DSHB, 1:200) (S4 Fig).

431 **Jaw movement movies**

432 Movies were taken as previously described [5]. Larvae were imaged at 30msec per frame for
433 500 frames and the number of mouth movements and jaw displacement was recorded.
434 Results were analysed by Anova (SPSS).

435 **Analysis of shape variation**

436 Changes to the Meckel's cartilage (MC) shape were quantified using two-dimensional (2D)
437 geometric morphometrics [16]. Outlines of the MC of 5dpf and 7dpf controls, anaesthetised
438 and ablated larva were digitised and analysed by non-parametric MANOVA (npManova) and
439 between-groups Principal Components Analysis (bgPCA). The anaesthetised and ablated
440 larvae were subdivided by the functional position of their jaw (subluxed or recovered) at
441 7dpf.

442 **Cell orientation, area and shape**

443 Separate Z-projections of 2-3 consecutive slices were created from *Col2a1* labelled images
444 of the MC through the mandibular symphysis and jaw joint. Cell area and the circularity of a
445 minimum of 10 cells between the insertion points of the intermandibularis anterior muscle
446 were analysed [16]. Cell orientation and area were measured for a minimum of 6 joints per
447 condition at 5 and 7dpf [5]. Chondrocytes were subdivided into those located within 3 cell
448 widths of the medial or lateral side of the joint. Graphs were produced and analysed using
449 PAST [59] and PRISM (Version 7).

450 **Sox10 Col2a1 colocalisation**

451 Larvae carrying *Col2a1:mcherry* and *sox10:GFP* transgenes were anaesthetised for 48hrs
452 from 3dpf, allowed to recover, imaged at 5 and 7dpf and processed using a custom MATLAB
453 tool. Stacks were binarised using a threshold to remove background noise. The product of
454 difference from mean (PDM) of each image was calculated [35]. In this data positive values
455 correspond to correlation and negative to anti-correlation between the expression of *Sox10*
456 and *Col2a1*, after each has been normalised by average brightness. The anti-correlation
457 dataset was transformed to a positive image, the joint region selected in 2D and the

458 boundary projected across all slices of the image stack. Within each boundary, pixels above
459 the intensity threshold were fit with an alpha shape [60] yielding a measure of the enclosed
460 volume.

461 **Kaede protein photoconversion**

462 Control and anaesthetised double transgenic Tg(*Sox10:GAL4-VP16*) Tg(UAS:Kaede) larvae
463 were mounted ventrally at 5dpf and 8-10 Kaede expressing cells on the medial or lateral side
464 of the joint were photoconverted [58]. Following photoconversion, larvae were kept
465 individually, reimaged at 7dpf and jaw position assessed. Maximum projections of the red
466 channel were prepared [61] and the area of the photoconverted region was calculated
467 before and after recovery.

468 **BrdU immunostaining**

469 Control and 48hrs anaesthetised larvae were treated with 3mM BrdU (Sigma) diluted in
470 Danieau's solution from 5-7dpf. BrdU incorporation was visualised by immunohistochemistry
471 [58] using mouse anti-BrdU (Sigma, 1:100) and rabbit anti-collagen II (Abcam, 1:200), prior
472 to imaging on confocal SP8. BrdU positive cells within the jaw joint cartilage, were counted
473 and divided into the medial and lateral side of the joint.

474 **Finite Element Modelling**

475 Representative confocal images of 5dpf recovered and subluxed larvae expressing
476 *Col2a1:mcherry* were converted into 3D finite element models for comparison with
477 previously published 5dpf control models using Hypermesh (Version 10, Altair Engineering)
478 [5]. All models had a Young's modulus of 1.1 MPa for the cartilage and 0.75MPa for the
479 interzone and mandibular symphysis, and Poisson's ratio of 0.25 [as described in 5]. Muscle
480 attachments were added to the FE-models for mouth closure (adductor mandibulae) and
481 opening (protractor hyoideus and intermandibularis) using confocal datasets for reference for
482 attachment sites (Fig S5 A,C,E, O-Q, Table S5). Spring elements were used to simulate the
483 Interoperculomandibular ligament (Fig S5, O-Q, S4 Table) [7]. Anaesthesia impacts the

484 function of both the muscle and ligaments. In the chick Immobilisation causes to a reduction
485 in muscle size and its ability to transmit forces [62,63]. The magnitudes of the forces applied
486 to the model were therefore adjusted to 75% of the normal estimation [derived from 63].
487 Immobilisation of the mouse cruciate ligament also reduced its stiffness and peak force[64].
488 The stiffness of the spring was reduced to 75% (1.3E-0.4 kN/mm). Immobilisation disrupts
489 the definition of the interzone. While a clear interzone could be identified in control and
490 recovered images it was not possible to identify a clear interzone spanning the joint space in
491 the subluxed dataset, therefore the joint was modelled as a fusion with a collar of interzone
492 (Fig S5 O). The geometrically linear models of approximately 2 million tetrahedral elements
493 were imported from into Abaqus FE-software (v6.14 Simulia, Dassault Systèmes), the spring
494 elements were assigned and the model was analysed (v6.10.2 Simulia, Dassault Systèmes).

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675

676 **Figure legends**

677 Fig 1. Restoration of muscle contraction is sufficient to drive the recovery of jaw function and
678 shape.

679 Zebrafish expressing Tg(*Col2a1aBAC:mcherry*) were imaged laterally (1,2) and ventrally (3-
680 4) at 5dpf (A-C) and 7 dpf (E-I). Immobilisation and ablation caused joint subluxation
681 (arrows, B1-2, C1-2) and flattening of mandibular symphysis (ms, B3-4, C3-4). The number

682 of mouth openings per 500 frames at 5dpf in anaesthetised larvae, after wash out, and
683 ablated larvae (D) analysed by 2-way anova. 7dpf anaesthetised and ablated larva
684 subdivided by jaw position (F-I). Time course of percentage recovery following varying
685 anaesthesia treatment analysed using Kruskal-Wallis test (K). (a) $p=0.009$ “24hrs 3-4” vs “48
686 hrs”, (b) $p=0.008$ “24 hrs 3-4” vs “24 hrs 4-5”, (c) $p=0.049$ “24hrs 3-4” vs “56 hrs” and (d)
687 $p=0.002$ “24hrs 3-4” vs “56 hrs”. m; Meckel’s cartilage, ch; ceratohyal, pq; palatoquadrate,
688 RAP; retro-articular process.

689 Fig 2. Shape cannot be used to predict recovery in anaesthetised or ablated larvae however
690 restored movement significantly altered the shape of the Meckel’s cartilage by 7dpf.

691 Graphic representation of results of npManova (B, C) of 2D outline of
692 Tg(*Col2a1aBac:mcherry*) larvae (A). Principal component analysis of 5 (D) and 7dpf (E)
693 treatment grouped by colour and subdivided by jaw position at 7dpf. Selected joint examples
694 arranged according to PC1 and 2 at 5 dpf (F) and 7dpf (G) showing the increasing thickness
695 of the MC (double arrow), overlapping joint shape (arrow) and altered curvature of the
696 cartilage (PC2, double arrow).

697 Fig 3. Chondrocyte maturation is less affected in the mandibular symphysis and jaw joint of
698 larvae that recover.

699 Confocal images of the mandibular symphysis of 5dpf and 7dpf control, *myod*^{-/-},
700 anaesthetised and ablated zebrafish cartilage visualised using the Tg(*Col2a1aBAC:mcherry*)
701 transgenic line (A-J). Confocal image of a representative jaw joint (O). Cell circularity (K, L)
702 and cell area (M, N, P, Q) of 10 cells, outlined in red, between the insertion of the
703 intermandibularis muscle ($n=6$ per condition) (A-N) and below intercalation in the joint (O).
704 1.00 represents a perfect circle.

705 Fig 4. Restored movement drives the restoration of the joint line.

706 Ventrally imaged zebrafish expressing Tg(*Col2a1aBAC:mcherry*) and Tg(-
707 *4725sox10:GFP*)^{ba4} at 3, 5 and 7dpf and laterally at 3 and 5dpf. High magnification confocal

708 images of the joint line of 5dpf (B) and 7dpf (C). Ablated and anaesthetised larvae which
709 were subdivided by their jaw position at 7dpf. Joint volume (D, E) derived from the “product
710 of the difference of the mean” (PDM) of the joint (white box). JL; joint line, SymI; mandibular
711 symphysis PQ; palatoquadrate, mc; Meckel's cartilage, Ch; ceratohyal.

712 Fig 5. Cell proliferation and migration is differentially regulated in zebrafish that recover
713 relative to those that remain subluxed.

714 BrdU incorporation (green) between 5-7dpf (A). Arrows show BrdU+ cells within the
715 cartilage. Arrowhead shows BrdU+ incorporation outside the cartilage. Cells expressing
716 Tg(Sox10:GAL4-VP16) and Tg(UAS:Kaede) are green until photoconverted. Red
717 photoconverted cells located on the medial or lateral sides of the joint at 5dpf (left panels),
718 reimaged at 7dpf (middle) with detail of photoconverted cells (right). Arrowhead indicates
719 migration, p indicates proliferation. Number BrdU+ cells on the medial and lateral side of the
720 joint in control, anaesthetised recovered and subluxed zebrafish. Fold change in total area of
721 cells (red outline in B) expressing photoconverted red kaede in control, anaesthetised
722 recovered and subluxed. The impact of side and treatment on BrdU+ counts and Kaede
723 growth analysed by MANOVA.

724 Fig 6. Recovery restores joint associated structures.

725 Confocal images of control and anaesthetised TgBAC(Col10a1a:Citrine)^{hu7050} and
726 Tg(Col2a1aBAC:mcherry) were imaged ventrally at 5dpf (A-B'') and again at 7dpf (A-G''). At
727 7dpf they were split into recovered and subluxed by jaw position. Detail images of RAP (')
728 and RAP rotated approximately 90° ("). Counts of ColX expressing osteoblasts located at the
729 RAP (arrows in detail images) at 5dpf (C) and 7dpf (H). Width of the Interoperculomandibular
730 ligament (IOM), measured at 3 sites along the ligament at 5dpf (D) and 7dpf (I). Counts of
731 BrdU incorporation into the IOM between 5dpf and 7dpf, prepared within 40µm of its
732 insertion into the RAP in control and anaesthetised larvae split by jaw position at 7dpf (J-K).
733 Analysed by Anova.

734 Fig 7. Finite element (FE) analysis reveals distinct differences between the patterns of
735 biophysical stimuli

736 FE model simulating jaw displacement during closure in models of a 5 dpf control and anaesthetised
737 larvae that recover (B) or remain subluxed (C). Displacement is shown as the block colour model
738 relative to the semi-transparent model of the jaw original position, with the direction of motion
739 indicated as an arrow. Finite element models of maximum principal strain created from confocal
740 stacks of 5 dpf control (D,G, J) and anaesthetised larvae that recovered (E,H, K) or remained
741 subluxed (F,I,L) during closure (D-I) and opening (J-L). Each panel shows a ventral view, enlarged
742 image of the joint (Jj) and mandibular symphysis (MS). Strain was elevated at the symphysis (green
743 arrowhead), RAP (red arrow) and on the lateral side of the joint (black arrow heads) (D-F, J-L). The
744 orientation and magnitude of the strains are indicated by the direction, size and colour of arrows in the
745 plane through the mandibular symphysis (G-I). Graph of the average absolute maximum principal
746 strain during opening and closure (N), extracted from 10 elements on the medial and lateral side of
747 the joint in a plane located at the midpoint of the joint (redline M).

748 *Supplementary Figures:*

749 S1 Fig. Muscle repair post ablation. *Smyhc:egfp* and *Col2a1:mcherry* transgenic zebrafish
750 were bilaterally ablated at 3dpf and imaged at 4dpf and 5dpf. Yellow arrowhead indicate
751 ablation site and repairing muscle.

752 S2 Fig. Cell orientations on the medial (A, green cells) and lateral (A, red cells) in wild type,
753 anaesthetised recovered and subluxed and *myod*^{-/-} and bilaterally ablated zebrafish jaw
754 joints. Orientation angle, calculated from the longest axis of the cell (A, insert) of
755 chondrocytes 5dpf (B) and 7dpf (C) zebrafish in the Meckel's cartilage element of the joint,
756 plotted on circular histograms (rose plots), where 0° lies on the medial side of the joint and
757 180° at the lateral side of the joint. n=6 joints per experimental condition (1 or 2 refers to
758 number of joints per blue wedge). Histogram bins equal 20°. The red line marks mean
759 orientation and the green line marks the 95% confidence interval and analysed by MANOVA.

760 S3 Fig. Differential expression of *Sox10: GFP* and *Col2a1:mCherry* is lost in the joints of *Myod-*
761 *-/-* zebrafish. The intensity of product of the difference of the mean (PDM) between *Sox10: GFP*
762 and *Col2a1:mCherry* double transgenic in control, anaesthetised recovered and sublaxed,
763 *Myod* mutants and double ablated zebra fish.

764 S4 Fig. Muscle fibres numbers reduced after ablation. Confocal images of control (A,C,E)
765 and adductor mandibularis (AM) ablated (B,D,F) transgenic *smyhc;EGFP* (A-B) zebrafish
766 following immunohistochemistry for total Myosin (C-D) show significant reduction in the
767 reduction in the number of fibres (G). Percentage of Fish divided by the success of ablation
768 (H). Arrowhead indicates adductor mandibularis.

769 S5 Fig. Building Finite element (FE) models.

770 3D surfaces were generated from confocal images of 5dpf *Smyhc:egfp* and *Col2a1:mCherry*
771 control (A) and anaesthetised larvae (C,E) were used to generate FE models (O-Q).
772 Anaesthetised larvae were subdivided by their jaw position at 7dpf (C, E). By 7dpf the
773 recovered larva (F) was significantly different in shape to the sublaxed (D). Sections through
774 the jaw joint (J,K,N indicated by white line) show a continuous interzone in the joint (J', N',
775 arrowhead) and mandibular symphysis (MS) (G, I, arrowhead) of controls and the recovered
776 larva at 5dpf but not the sublaxed. A spring modelled the Interoperculomandibular ligament
777 (IOM) between the retro articular process (RAP) and Hyoid bone (P-R, yellow line). 50% of
778 maximum muscle loads (measured in Newtons (N)) were applied at the origin and insertion of the
779 muscle. The adductor mandibularis (AM) (O',P',Q', blue line) was applied to model mouth closure. To
780 capture the wider insertion of the intermandibularis (IM) and protractor hyoideus (PH) (O) the forces
781 were applied across 10 nodes to model of mouth opening (O''-Q''). White dots indicate the area of
782 applied constraints in either the x,y, or z orientation (O).

783

784 S6 Fig. Finite element (FE) models of minimum principal strain (E_{min}).

785 Finite element models of minimum principal strain (compressive) created from confocal stacks of 5 dpf
786 control (A,D) and anaesthetised larvae that recovered (B, E) or remained subluxed (C,F) during
787 closure (A-C) and opening (D-F). Each panel shows a ventral view, enlarged image of the joint (Jj)
788 and mandibular symphysis (MS). Strain was elevated RAP (red arrow).

789

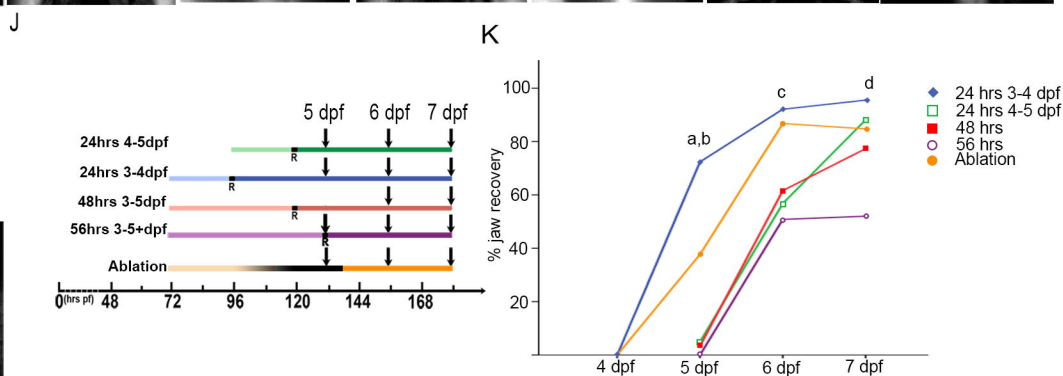
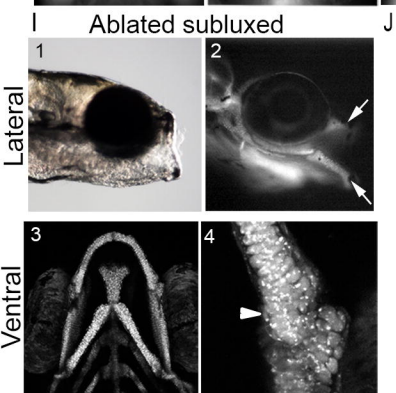
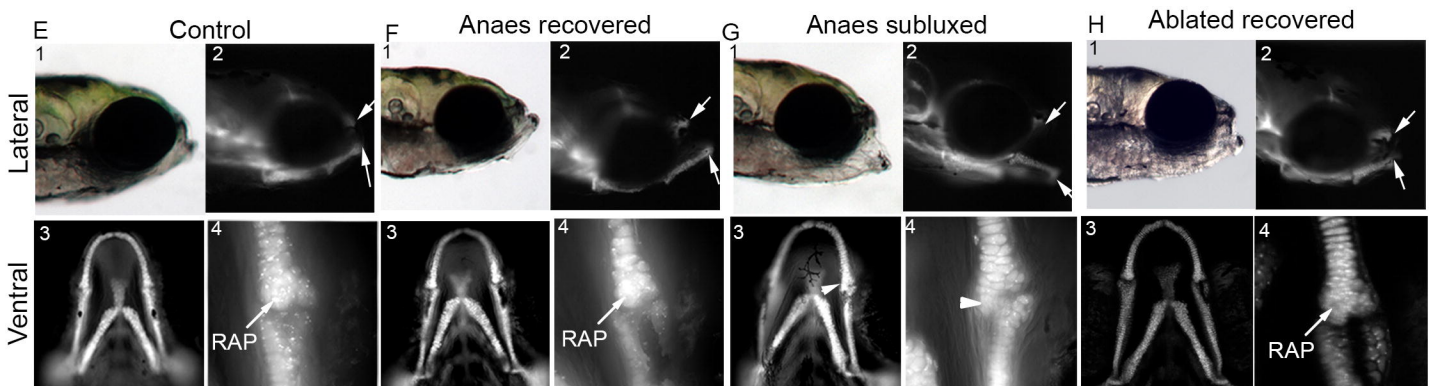
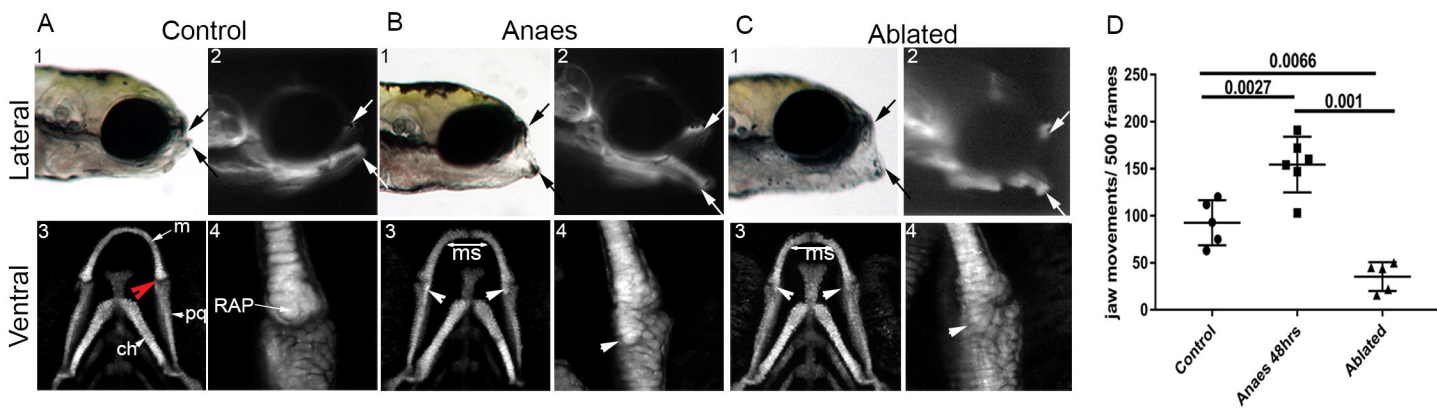
790 S1 Video. Movie of jaw movement at 5dpf control larvae expressing *Col2a1:mcherry*.

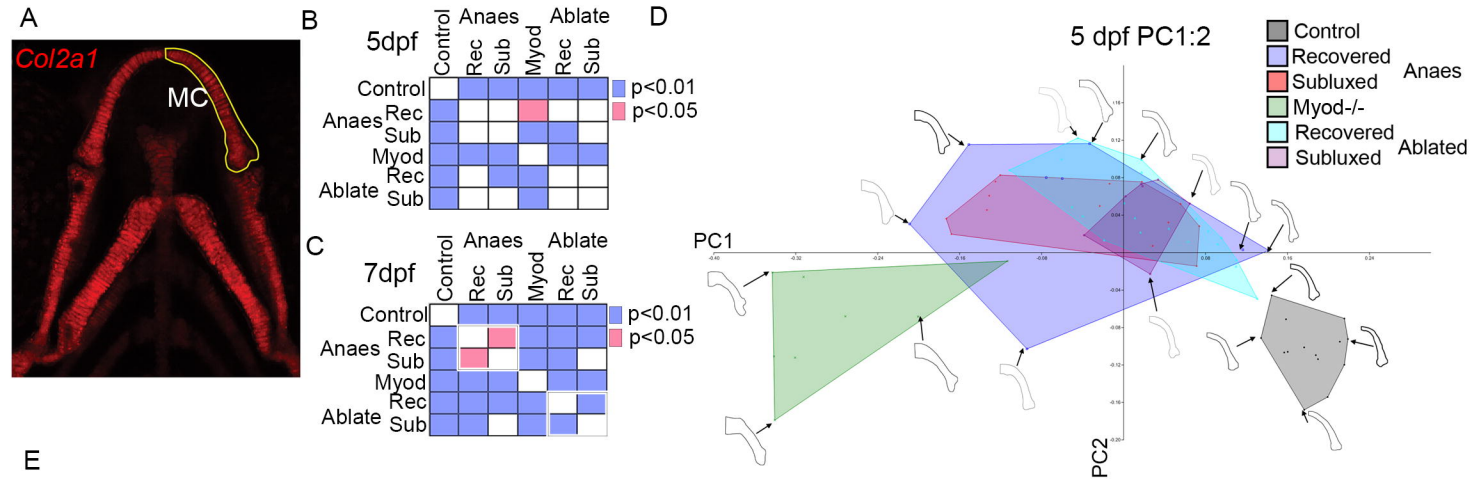
791 S2 Video. Movie of jaw movement at 5dpf anaesthetised larvae expressing *Col2a1:mcherry*.

792 S3 Video. Movie of jaw movement at 5dpf in double ablated larvae expressing

793 *Col2a1:mcherry*

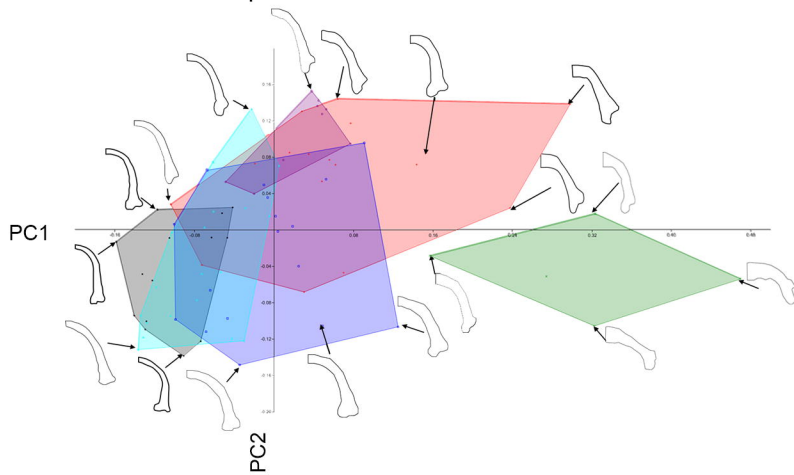
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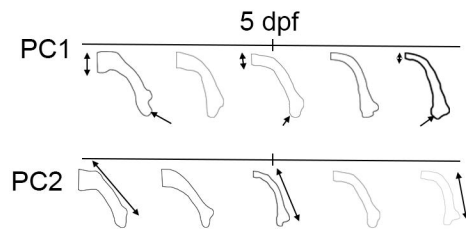


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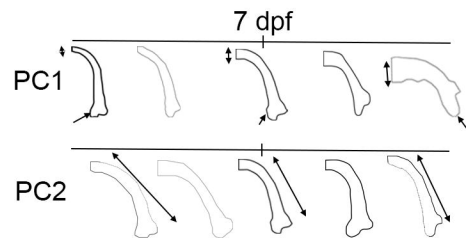
7 dpf PC1:2



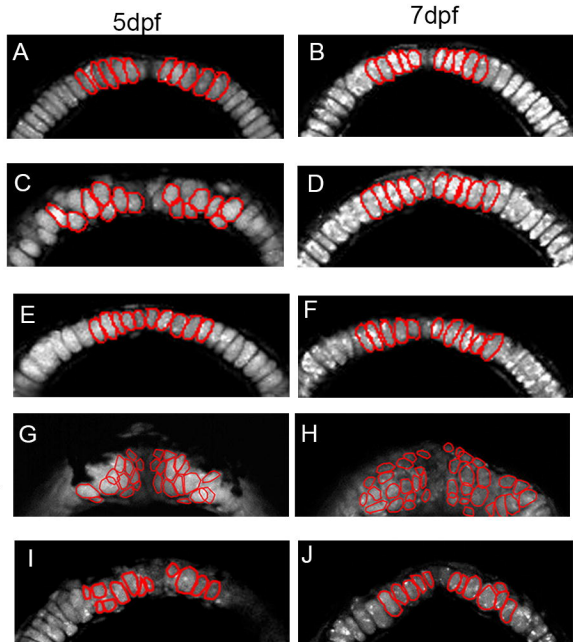
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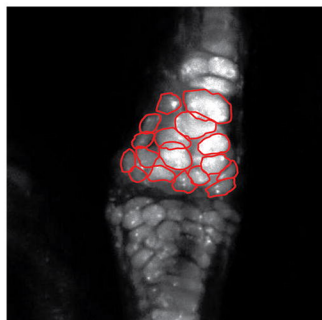
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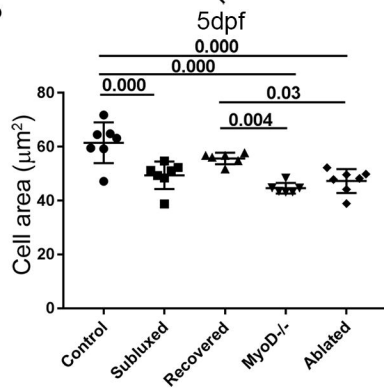
Control
Subluxed
Recovered
MyoD^{-/-}
Ablated



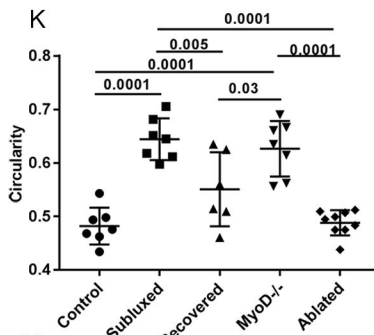
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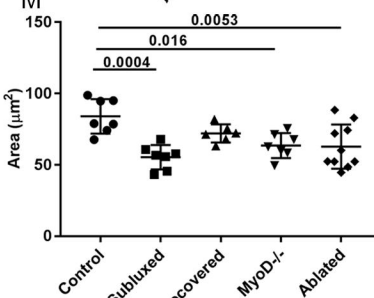
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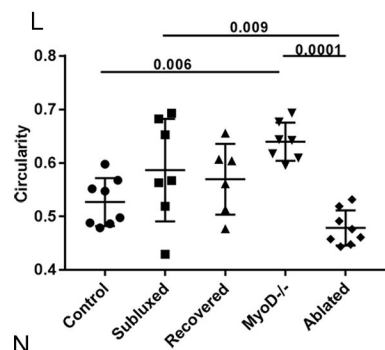
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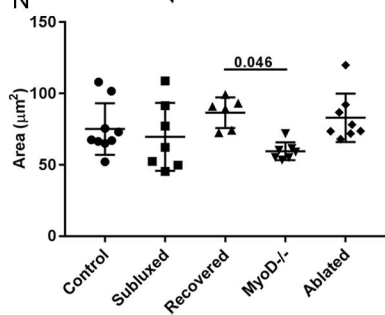
M



7dpf



N



Q

