1 VISUALIZING MINERALIZATION IN DEFORMED OPERCULAR BONES OF
2 LARVAL GILTHEAD SEA BREAM (*SPARUS AURATA*)

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19 Mineralisation in deformed opercles

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30 **Summary**

31 During the rearing process of gilthead sea bream (*Sparus aurata*), abnormal development of
32 the opercular bone is particularly common (Koumoundouros et al. 1997). In order to
33 alleviate its occurrence in rearing facilities, it's crucial to identify the very first physical
34 signs of deviation in normal skeletal development. Nano-CT-scanning was tested for its
35 applicability to quantify deviations in bone mineralization levels. Seven opercles were
36 dissected from larvi of 65 days post hatching, randomly sampled at the commercial sea
37 bream hatchery Maricoltura di Rosignano Solvay (Livorno, Italy). The samples were nano-
38 CT-scanned and computationally reconstructed. Mineralization intensity was colorcoded
39 using Amira software, resulting in a detailed visualization of opercular morphology and
40 mineralization patterns. In conclusion, nano-CT-scanning promises to be a good tool to both
41 describe morphology and detect mineralization levels in the early onset of deformities.

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57 **Introduction**

58 After 20 years of successful aquaculture research, skeletal deformities remain a major
59 cutback in fish production worldwide (Fraser & Nys, 2005). During the rearing process of
60 gilthead sea bream (*Sparus aurata*), being the most intensively cultivated fish species in the
61 Mediterranean region, abnormal development of the opercular bone is particularly common
62 (Koumoundouros et al. 1997). Affected individuals show an inward folding of the operculum
63 (fig. 1a) and significantly diminished growth rates, consequently downgrading the marketing
64 image of the product (Beraldo et al. 2003). As the abnormality develops at an early larval
65 stage (17 days post hatching (DPH) and most probably earlier, Galeotti et al. 2000),
66 identifying physical criteria that allow detection of the very first deviations in normal
67 skeletal development are crucial to alleviate its occurrence in rearing facilities. One of
68 potentially informative criteria is the level of hydroxyl-apatite mineralization of the opercula.
69 In order to explore this potential, two different tools are tested for their applicability to
70 characterize those criteria: (1) nano-CT scanning and (2) histological stainings (Masson's
71 trichrome and toluidine blue). Here, the results of the nano-CT scanning will be discussed.

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73 **Materials and methods**

74 A total of 1141 specimens of gilthead sea bream (*Sparus aurata*) were collected from a
75 commercial hatchery (Maricoltura di Rosignano Solvay), and fixed in a 4% NaH_2PO_4 -
76 Na_2HPO_4 buffered formalin. We selected a range of phenotypically normal to severely
77 deformed *S. aurata* larvi (65 DPH, average standard length (LS) $16,8 \pm 1,5$ mm, from
78 Maricoltura di Rosignano Solvay, Italy). Although the deformity is already visible at 17
79 DPH (Galeotti et al. 2000), we started the research with the analysis of 65 DPH old
80 specimens. At 65 DPH, the deformity is clearly visible with the naked eye, so that an
81 unambiguous CT-image can be acquired and serve as a frame of reference. In a next phase,
82 younger specimens will be analyzed and compared with the frame of reference. Seven
83 opercles were dissected, dehydrated, mounted on gum and subjected to a nano-CT-scan
84 (resolution of the CT-sections were approximately 1/1000 of the longest axis of the bone).

85 CT-scanning was performed at the UGent CT-facility (www.ugct.ugent.be) (Masschaele et
86 al., 2007). The specimens were scanned using a directional tube head, using an a:Si flat panel
87 (Varian Paxscan 2520) detector with CsI scintillator. The raw data were processed and
88 reconstructed using the in-house developed CT software Octopus (Vlassenbroeck et al.,
89 2007) and rendered with Amira 4.1 (Mercury Systems).

90 To visualize levels of mineralization densities, the contrast level of the CT-images was
91 optimized to remove voxel information from soft tissue. Colour gradients were applied to
92 visualize the different levels of mineralization densities in the bone tissue (fig. 1d-f). Four
93 parts of the opercle were used for comparison, along a horizontal plane (fig. 1i-m): at the
94 level of the distal process of the opercle (fig. 1j), at the centre point of the opercular
95 articulation (fig. 1k), at midway of the ventral plate of the opercle (fig. 1l), and at the lowest
96 quartile of the ventral plate of the opercle (fig. 1m). Apart from mineralization densities, also
97 differences in level of trabeculation of the bone tissue were compared.

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99 **Results and discussion**

100 The ossification pattern of a phenotypically normal opercle at 65 DPH shows a rather well
101 developed opercular bone, comprising a plate-like ventral part with solid bone and rather
102 homogenous mineralization levels (fig. 1j-m). At the mid-region of this ventral plate, the
103 caudal rim can show some level of outward folding, whereas the anterior rim is more densely
104 mineralized. Additionally, the anterior rim shows medially directed trabecular projections
105 that are completely lacking caudally. At the level of the articulatory facet, trabeculation is
106 the most extensive, with a horizontal crest (at the medial face of the bone) running caudally
107 up to the distal tip. Dorsal to this crest, the opercular bone is plate-like with a rather
108 homogenous distribution of minerals.

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110 After colour gradient application, the volume-rendered 3D models indicate a denser
111 mineralization in deformed opercles (overall more brightly coloured, fig. 1f, h) than in
112 normal opercles (fig. 1e, g). This increased mineralization could be induced by an osteogenic
113 response to increased mechanical loading of the opercle, as a result of this folding. In order
114 to find out what occurs first: the aberrant mineralization or the folding of the opercle, larger
115 samples of younger ontogenetic stadia will be scanned.

116

117 **Conclusion**

118 Focusing on traits that characterize bone and ossification may yield a powerful tool to detect
119 early onset of deoperculation, and thus allow to test the effect of different rearing conditions
120 on its prevalence. Several of these traits can be suggested for monitoring, such as bone
121 shape, collagen content or mineralization content. In this study, we tested the applicability of
122 nano-CT-scanning with graphical 3D-volume rendering as a tool to detect aberrant patterns
123 of mineralization in bone as a proxy for early onset of opercular deformation. It provides a
124 detailed 3D image of the gill cover morphology and enables visualization of differences in
125 mineral density. The obtained results indicate that performing quantitative analysis on
126 different levels of mineralization, using nano-CT-scanning, is feasible and may allow
127 statistical testing for effects of specific rearing conditions that are targeted for in order to
128 reduce the occurrence of opercular deformations. Once the earliest signs of deviation from
129 normal skeletal development are pinpointed, the obtained histological characters can allow
130 us to correlate body shape characters with opercular deformations at the earliest stages.
131 Verhaegen et al. (2007) could significantly separate deoperculated from normal specimens at
132 69 DPH, based on small differences of the external morphology. Body shape is a potential
133 screening criterion to be applied in hatcheries.

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158 **Figure legends**

159 **Fig. 1:** (a) Habitus of 65 DPH old larva with deformed right opercle; (b) normal phenotype of
 160 dissected opercle; (c) phenotype of highly deformed opercle; (d) reconstruction of nano CT-scanned
 161 65 DPH larva with deformed right opercle with colourmap threshold, ranging from low (red) to high
 162 (white) electron density (mineralization); (e-f) volume renderings of two gill covers, visualizing
 163 mineral density by same colour mapping of normal gill cover (e) and severely deformed gill cover
 164 (more densely mineralised) (f); (g-h) axial sections through the opercles as indicated by the blue
 165 lines, show highly bended regions being more densely mineralized; (i-m) phenotypically normal
 166 opercle of 65 DPH old larva (reconstruction from CT-data) (i), with mineralization density showed in
 167 horizontal sections at four levels (j, k, l, m).

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