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1 **Individual monitoring of gonad development in the European flat oyster *Ostrea edulis* by in**
2 **vivo magnetic resonance imaging**

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12

13 **Abstract**

14 Previous studies have shown that magnetic resonance imaging is a very appropriate non-invasive
15 technique for quantifying the growth of somatic and gonadic tissues and sex determination in the
16 Pacific oyster, *Crassostrea gigas*. Despite a thinner gonad, harder to distinguish than that of the
17 Pacific oyster, we showed in this study that it was possible to distinguish and quantify the
18 development of the gonad of the European flat oyster, *Ostrea edulis*, using a 3D MRI sequence
19 with a suitable finer resolution. Compared to T2-weighted images, theoretically the most
20 appropriate for good anatomical description, T1-weighted images were more suited to gonad
21 observation. The gonad development was quantified by the number of voxels with higher grey
22 level. Larvae were then depicted in the intervalvar cavity of spawning females. MRI imaging is a
23 non-invasive method that is well suited to the description of gametogenesis in the European flat
24 oyster.

25

26 *Keywords:* Oyster, *Ostrea edulis*, Gonad development, Magnetic Resonance Imaging

27 1. Introduction

28 The European flat oyster, *Ostrea edulis*, is a native European species and traditionally is an
29 attractive product for consumption in a range of European countries. This species is highly
30 valued and sought after on the European seafood market. However, supplies are limited because
31 of declines in fisheries due to recruitment failure and the occurrence of diseases caused mainly
32 by parasites and environmental stress.

33 Aquaculture may counteract decline in availability and, in terms of biodiversity, it is also
34 important to sustain the production of healthy and genetically differentiated populations along
35 European coasts. Spat production of flat oysters has been a challenge over the years compared to
36 the more easily cultured introduced species such as Pacific oyster, *Crassostrea gigas*. Research
37 focused on the flat oyster is therefore essential to maintain large enough production to meet
38 demands and retain the market share. However, the ecology and physiology of this bivalve are
39 not fully understood, and consequently monitoring of growth and reproduction, both in the field
40 and in hatcheries, is still based on empirical factors.

41 Investigation of soft tissues in bivalves classically relied on the removal of the hermetic shell that
42 protects the animal (Chavez-Villalba et al., 2002; Fabioux et al., 2005; Dridi et al., 2007). This
43 has provided much valuable information, but has two major disadvantages: these methods are
44 time consuming and necessarily destructive. Practical anaesthesia and sampling protocols have
45 been developed in some bivalves such as Pacific oyster, enabling sex determination and
46 maturation stage observation without sacrificing animals (Namba et al., 1995; Suquet et al.,
47 2009). However, the assessment of biometric parameters (gonad wet weight and total body
48 weight) facilitating the determination of condition index, is prevented.

49 Non-invasive and quantitative procedures have therefore been developed and have proved
50 promising after preliminary trials (Pouvreau et al., 2006). Non-invasive characterization of
51 gonad maturation and sex determination of the Pacific oyster by Magnetic Resonance Imaging
52 (MRI) has already been successfully applied using longitudinal relaxation T1-weighted MRI
53 sequences (Davenel et al., 2006; Hatt et al., 2009). MRI is the most appropriate technique for
54 quantification of the growth of somatic and gonadic tissues because this method is non-invasive
55 and successive sampling may be performed on the same individuals, thus facilitating descriptive
56 studies such as gametogenesis.

57 We report here the ability of MRI to monitor gonad development in individual European flat
58 oysters, *Ostrea edulis*, until spawning. The European flat oyster is a larviparous species, the
59 larvae of which are released after 7 to 10 days of development. Compared to the Pacific oyster,

60 the fecundity of the European flat oyster is lower (1 to 2 million eggs; Walne, 1964), and smaller
61 gonad size is reported. Any MRI protocol must therefore take into account these reproduction
62 features.

63

64 **2. Materials and methods**

65 *2.1 Origin of animals and preparation*

66 Three hundred flat oysters (4 years of age, *Bonamia* and *Marteilia* free), originating from
67 Norway, were transferred to the Argenton experimental hatchery (West Brittany). Oysters were
68 maintained from April to August 2009, in 700L flow-through tanks at 19°C with permanent
69 light, and continuously fed according to Chávez-Villalba et al. (2002). .

70 Twelve non-anaesthetised oysters were brought 250 km to Cemagref in Rennes (Brittany,
71 France) on five dates (April 7, April 29, May 13, June 2, July 10, 2009) for MRI investigations
72 without any specific anaesthetization procedure. Before MRI measurements, animals were
73 soaked in sea water to expel any air bubbles which can cause artefacts on images. Sea water was
74 maintained at room temperature and shell opening was facilitated by the addition of
75 phytoplankton. After expelling air bubbles, oyster shells were maintained closed with a rubber
76 band during MRI scanning.

77 *2.2 MRI measurements*

78 In this new study, NMR measurements were performed on the European flat oyster at Cemagref
79 (Rennes, Brittany, France) with a Siemens Avento imager operating at 1.5T (60MHz) equipped
80 with a “knee” probe which allowed the simultaneous investigation of two oysters. The stronger
81 magnetic field of this machine allowed us to obtain more precise images with higher spatial
82 resolution and to monitor the development of the very thin gonad observed in individual flat
83 oysters. Each oyster was scanned using two high resolution 3D MRI protocols (Table 1). A T2
84 weighted Turbo 3D Spin Echo sequence provided 48 contiguous images, highlighting water in
85 the shell and chambers and pericardial cavities (Fig. 1a). A T1-weighted Turbo Gradient Echo
86 sequence provided 52 contiguous images, highlighting animal tissues and particularly gonadic
87 tissues (Fig. 1b).

88

89 2.3 *Image analysis*

90 Examination of raw images obtained from an MRI scanner can lead to erroneous interpretations
91 of variations in the grey level in the images: several sources of spatial inhomogeneities in the
92 apparatus related to the inhomogeneity of the magnetic field and the geometry of the transmitting
93 and receiving probes lead to undesirable variations in intensity in the images. Using
94 homogeneous doped water phantoms as reference, we calculated that the grey levels of the oyster
95 placed in the lower part of the MRI probe were 7% lower than the grey levels of the oyster
96 placed in the upper part. The grey levels of the image, originating from the lower part of the raw
97 images, were therefore multiplied by 1.07 after automated segmentation of the raw images in
98 two under-images to separate each of the two oysters, to obtain comparable grey levels for all the
99 oysters scanned.

100 Although it was possible to obtain a reliable assessment of the development of somatic and
101 gonadic tissues by visualizing the images, we tried to reduce the influence of the human input by
102 exploiting the overall histograms of voxels in all the images and setting thresholds to separate
103 tissues, while counting the somatic index reflecting the volume occupied by flesh and the gonad
104 index reflecting the volume occupied by the gonad.

105 **3. Results**

106 *3.1 Choice of MRI sequence to monitor gonad development and tissue evolution*

107 Although the T2-weighted images (Fig. 1a) offered higher resolution and better signal to noise
108 ratio and were theoretically the most appropriate for accurate anatomical description, the best
109 contrasts for the characterization and segmentation of the gonad were obtained with T1-weighted
110 images (Fig. 1b). In particular, the highlighting of the gonad before spawning was very
111 significant, while the gonad gave a low signal that was difficult to differentiate from the signal of
112 the background and the signal of the shell in T2-weighted images. Therefore, in addition to

113 visual observation and the method successfully used previously to segment MRI T1-weighted
114 images of the Pacific oyster, we sought to characterize the gonad development of each oyster
115 with a gonad index (the number of voxels of grey level > 150) and the flesh occupation by a
116 somatic index (the number of voxels of grey level > 90) to eliminate background, shell and
117 seawater in images (Fig. 2).

118

119 *3.2 Monitoring of gonad development and detection of spawning of gametes*

120 Four of the twelve oysters died during the experiments: oyster 9 died after April 29, oyster 7
121 after May 13 and oysters 5 and 6 after June 2. The most representative T1-weighted images of
122 the gonads of 10 of the 12 oysters scanned at the five dates is reported in Figure 3, including
123 oysters 5 and 6. Female gametes liberated in the intervalvar cavity, and probably fertilized by
124 externally released sperm, were clearly visible for oysters 1, 2, 4 and 11 at different dates: larvae
125 appeared as a grey volume in the gills. At the date preceding this stage when larvae were
126 detected, the grey level of pixels corresponding to the gonad was very high and clearly visible in
127 the images. We were therefore also able to determine the stage preceding spawning for oysters 5,
128 8 and 12, despite the fact that one corresponding animal died thereafter (oyster 5), or the larvae
129 were not visible after their expulsion into the surroundings following an incubation period in the
130 branchial chamber of 8-10 days, and probably because the time interval between the two
131 successive samples was too long for these individual oysters. No gonad development was
132 discernible for oysters 3, 6 and 10.

133 It was possible to estimate the gonad development by counting the number of voxels at grey
134 level > 150 (Fig. 4a and 4b). The number reached 5000 to 6200 voxels for oyster 1, 2, 8, 11 and
135 12 just before spawning, i.e. a gonad volume between 1.10 and 1.37 cm³. Volume was only
136 significantly lower (0.44 cm³) for oyster 5 just before death.

137 By counting voxels at grey levels > 90 (Fig. 4c and 4d), it was possible to propose a raw somatic
138 index. The index clearly increased from April to July for oysters 3 and 10 (Fig. 4d) but these

139 oysters showed no apparent development of the gonad during this period. It was the same for
140 oysters 2 and 4 after spawning, the stage at which this index was the lowest. The index did not
141 vary significantly during the period of gonad maturation before spawning.

142 **4. Discussion**

143 This new study confirms the potential of nuclear magnetic resonance in the non-invasive
144 phenotype characterization of bivalves. Because of the development of a thick gonad, we have
145 previously shown the possibility of monitoring and measuring the development of the gonad in
146 the Pacific oyster with a low-field 0.2T MRI imager and with medium spatial resolution
147 ($0.94*0.94*4 \text{ mm}^3$) (Davenel et al., 2006). In this study tracking individual European flat
148 oysters, we individually monitored a dozen oysters between April and July 2009 during gonad
149 maturation. Despite the development of a thinner gonad, using a higher field (1.5T) with better
150 spatial resolution ($0.47*0.47*1 \text{ mm}^3$) we were able to monitor and quantify gonad development
151 until spawning.

152 We confirmed that T1-weighted MRI sequences are particularly well suited to highlight the
153 gonad of the European flat oyster. It was possible during monitoring to visualize the incubation
154 of larvae in the intervalvar cavity, providing evidence that the gonad observed days earlier
155 belonged to a female animal. Our previous studies on the Pacific oyster showed that the female
156 gonad provided a clearer signal than the male gonad. The gonad index proposed to monitor the
157 development of the gonads just before spawning and visual observation showed that seven of the
158 ten oysters presented in Fig. 3 would clearly develop a female gonad during the observation
159 period. The three others did not seem to have developed a gonad, unless it was a male gonad
160 with a weaker signal than that of a female gonad and little different from that of the viscera. This
161 hypothesis could not be confirmed during this study because we did not want to sacrifice any
162 animal.

163 The somatic index adopted in this study, currently validated to reflect only the weight of flesh in
164 the Pacific oyster (Davenel et al., 2006), was lowest when the animal had just spawned. In the
165 following period, it was more often growing progressively and then seemed to slow down during
166 the gonad development phase. While the female gonad provided a clearer signal during this
167 phase, the signal from the viscera tended to decrease. It is possible that there is accumulation of
168 reserves before the gonad development phase and transfer of a proportion of the reserves
169 accumulated in the organs to the gonad during gonad development. We have previously
170 demonstrated that the somatic index proposed here in the case of the Pacific oyster (based on the
171 number of voxels with grey levels higher than 90) is well correlated with flesh weight and thus
172 mainly reflects the volume occupied by the flesh (Davenel et al, 2006). The grey level intensity
173 in T1-weighted MRI images is highly correlated with dry matter content of tissues and
174 particularly increased by the presence of lipids.

175 In conclusion, non-invasive imaging using MRI provides very valuable information for the
176 individual monitoring of gonad development in the European flat oyster. Further studies,
177 supplemented by biopsy and dissection, should be undertaken to characterize the development of
178 the male gonad and to develop a new somatic index more suited to reflecting the accumulation of
179 reserves including lipids. This would improve the existing relationship between the grey level in
180 MRI image and dry matter content of the different tissues of the oyster.

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185
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217 **Captions for figures**
218 Table 1. Parameters of the two 3D MRI sequences used to describe and quantify flesh and gonad
219 development.
220 Fig. 1. The same four most representative transverse images through the body of flat oysters
221 *Ostrea edulis* from the 48 images of the T2-weighted Turbo 3D Spin Echo sequence (a),
222 and the 52 images of the T1-weighted Gradient Echo sequence (b). Main organs identified:
223 adductor muscle (am), viscera (vi), gills (gi), gonad (go), pericardial cavity (pc), mantle
224 (ma), shell chamber (ch).
225 Fig. 2. Grey-level histograms from the 52 images from the T1-weighted Spoiled Gradient Echo
226 for oyster 12 at the five dates of MRI scanning.
227 Fig. 3. The most representative MRI scanning images of gonad development of 10 laboratory
228 conditioned flat oysters collected at five successive dates from April to July 2009 (S:
229 spawning period)
230 Fig. 4. Evolution of the gonad index (a, b) and the somatic index (c, d) of 6 oysters with apparent
231 development of the gonad (a,c) and 4 oysters without apparent development of the gonad
232 (b,d) at five dates of MRI scanning during April-July 2009.
233

234 **Table 1:** Davenel, Individual monitoring of gonad development in the European flat oyster

235

236 Parameters of the two 3D MRI sequences used to describe and quantify flesh and gonad
237 development.

238

MRI sequence	T2-weighted 3D Turbo Spin Echo	T1-weighted Spoiled Gradient Echo
Slice number	48	52
Slice thickness (mm)	1	1
FOV (mm x mm)	75x100	75x120
Matrix	192x256	160x256
Pixel size (mm x mm)	0.39x0.39	0.47x0.47
TR (ms)	2000	11
TE (ms)	150	4.76
Flip angle (°)	170	20
Bandwidth (Hz/Pixel)	168	200
Excitation number	1	6
Acquisition time	13 min	8 min

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240 **Fig 1- Davenel, Individual monitoring of gonad development in the European flat oyster**

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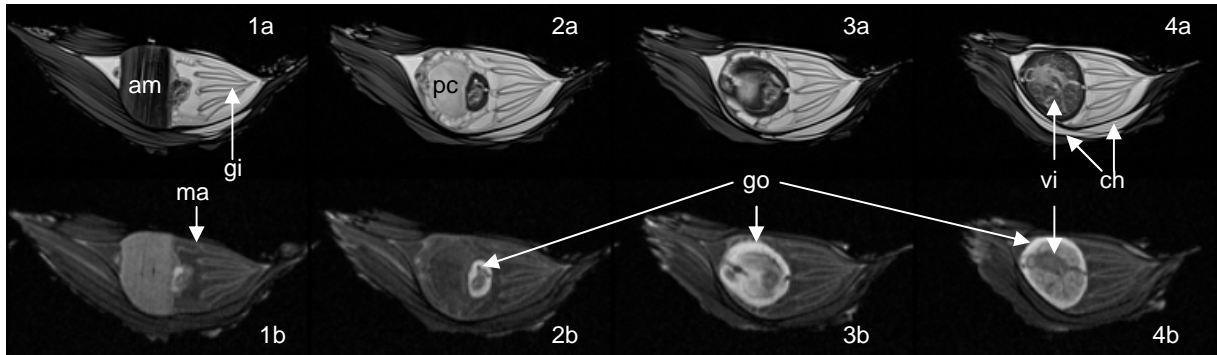
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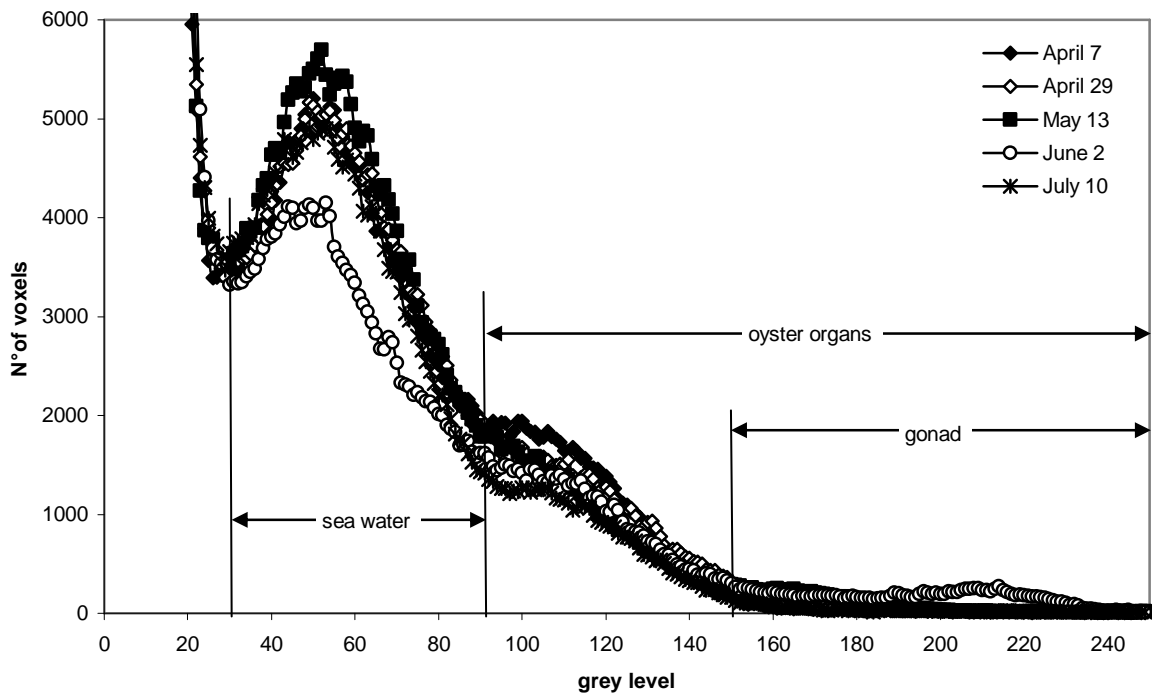


251 **Fig 2: - Davenel, Individual monitoring of gonad development in the European flat oyster**

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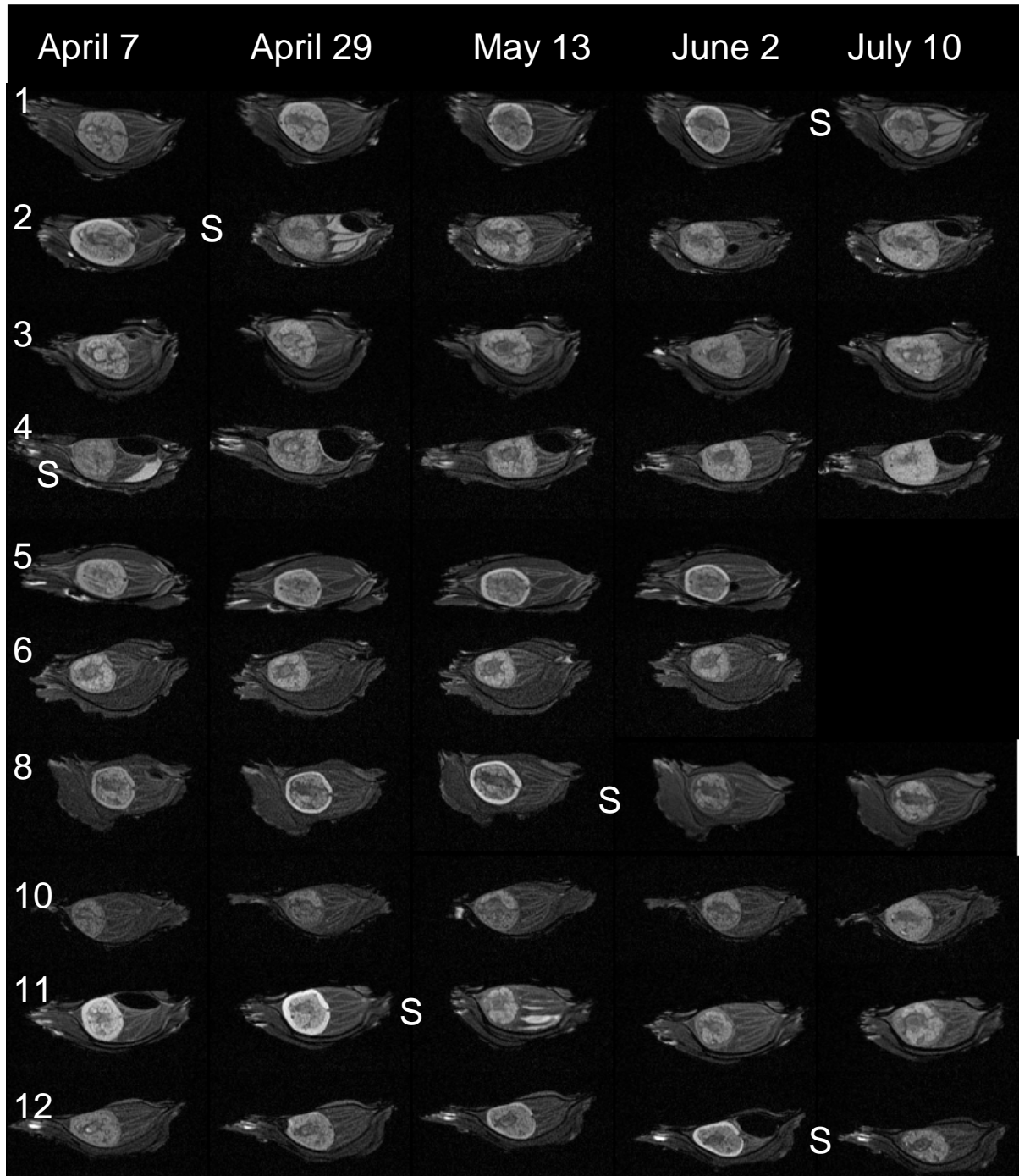


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Fig 3: - Davenel, Individual monitoring of gonad development in the European flat oyster



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Fig 4- Davenel, Individual monitoring of gonad development in the European flat oyster

