Please note that this is an author-produced PDF of an article accepted for publication following peer review. The definitive publisher-authenticated version is available on the publisher Web site

Aquaculture
June 2009, Volume 291, Issues 1-2, Pages 74-77
http://dx.doi.org/10.1016/j.aquaculture.2009.03.008
© 2009 Elsevier B.V. All rights reserved.

Archimer
Archive Institutionnelle de l'Ifremer
http://www.ifremer.fr/docelec/

NMR relaxometry as a potential non-invasive routine sensor for characterization of phenotype in *Crassostrea gigas*

Armel Davenel[a, c, *], Stéphane Pouvreau[b], Mireille Cambert[a, c], Marc Suquet[b] and François Mariette[a, c]

[a] Cemagref, UR TERE, 17 avenue de Cucillé - CS 64427, F-35044 Rennes, France
[b] Ifremer, UMR100, LPI, Station Expérimentale d'Argenton, 29840 Argenton en Landunvez, France
[c] Université Européenne de Bretagne, France

*: Corresponding author : Armel Davenel, Tel.: +33 2 2348 2160; fax: +33 2 2348 2115, email address : armel.davenel@cemagref.fr

Abstract:

MR imaging is the most appropriate non-invasive technique for quantifying the growth of somatic and gonad tissues and to determine sex in the Pacific oyster, *Crassostrea gigas*. However, this technique is too costly for field studies where oysters are used as bioindicators of environmental quality or to be applied routinely in hatcheries. We have tested the ability of low Nuclear Magnetic Resonance relaxometry, a much less expensive technique, to obtain phenotype parameters that can be used to monitor the physiological state of oysters. NMR measurements were carried out at three different periods using a low field spectrometer equipped with a 50 mm diameter probe to investigate 60 oysters in their first year of maturity, which were then dissected to measure internal shell cavity volume and dry flesh weight and to determine sex and gonad development. The NMR results showed that it was possible to determine both internal shell cavity volume and dry flesh weight in less than one minute with very high determination R^2 coefficients (0.95 and 0.94, respectively). The results showed also that it was possible to identify sex and gonad development, with success rates of 93% and 83%, respectively. For oysters with dry weight above 0.7 g, the success rate in identifying sex was 100%. Further studies are required to design an NMR probe that is appropriate for larger oysters and to improve sex discrimination and prediction of gonad development with larger study groups.

Keywords: Phenotype characterization; *Crassostrea gigas*; NMR; Growth; Sex identification

33 ## 1. Introduction

34 With an annual production of above 117,500 metric tons in 2006, production of the Pacific
35 oyster *Crassostrea gigas* is economically important in French aquaculture. However, the ecology
36 and physiology of this bivalve are not fully understood, and consequently monitoring of its
37 growth and reproduction, both in the field and in hatcheries, is still based on empirical factors.
38 Investigation of soft tissues in marine mollusks, especially in marine bivalves, classically relies
39 on destructive methods, since a hermetic shell protects the animal. For example, anatomical
40 structures are generally studied after opening and dissection by means of histological sections,
41 with a resolution of 4-5 µm (Chavez-Villalba et al., 2002; Didri et al., 2007). The evolution of
42 gametogenesis can be quantitatively assessed with the help of image analysis (Chavez-Villalba et
43 al., 2003; Fabioux et al., 2005), and sex may be determined after anesthesia using magnesium
44 chloride and tissue sampling using needles (Namba et al., 1995). However, analysis of
45 physiological and biochemical changes requiring large volume tissue samples necessarily
46 involves the sacrifice of numerous specimens and the preparation and analysis of several
47 samples. These standard techniques provide valuable information on marine mollusk biology but
48 have two main disadvantages which are limitations for many studies: these methods are very
49 time consuming and they are necessarily destructive. Non-invasive and quantitative procedures
50 have therefore been developed, and after preliminary trials (Pouvreau et al., 2006) Nuclear
51 Magnetic Resonance (NMR) imaging (MRI) has proved promising.

52 Non-invasive characterization of gonad maturation and determination of the sex of Pacific
53 oysters by Magnetic Resonance Imaging (MRI) has already been successfully tested using
54 longitudinal relaxation T1-weighted MRI sequences (Davenel et al., 2006). MRI is the most
55 appropriate technique for quantification of the growth of somatic and gonad tissues and to
56 determine sex. However, this technique is too costly for field studies and particularly for routine
57 use in hatcheries. Preliminary results (Davenel et al., 2006) of the characterization of various

58 oyster tissue samples by NMR relaxometry, a much less expensive technique, showed that the
59 T1 relaxation times of gonad and muscle were shorter than those of the heart, other viscera and
60 gills which are bathed in seawater, itself characterized by very high T1 and T2 relaxation time
61 values (more than 1600ms). These experiments ascertained that the NMR technique was able to
62 differentiate the ovaries (T1 of about 207± 21 ms) from the testes (T1 of about 456± 50 ms) and
63 muscles (T1 of about 461± 13 ms). However, the NMR technique did not make it possible to
64 differentiate the male gonad and muscle on the basis of the T1 value alone. We report here the
65 ability of NMR to obtain phenotype parameters that can be used to monitor the physiological
66 state of entire oysters in their shells by multivariate data analysis techniques on NMR transverse
67 T2 relaxation data weighted by longitudinal T1 relaxation using different relaxation delays.
68

69 2. Materials and methods

70 *2.1 Origin of animals and preparation*

71 NMR measurements were carried out at three different periods (May 7, June 4 and June 29 2007)
72 on 31 diploid (2N) and 25 triploid (3N) *Crassostrea gigas* oysters in their first year of maturity
73 to limit their size to the 52mm diameter of the NMR probe. They were bred by the IFREMER
74 Shellfish Laboratory located in Argenton near Brest (Brittany, France) and brought to Cemagref
75 in Rennes (Brittany, France) for NMR investigations without any specific anesthetization
76 procedure (distance 250 km). Before NMR measurements, animals were soaked in sea water to
77 expel any air bubbles. Sea water was kept at room temperature with the addition of
78 phytoplankton to facilitate the opening of oysters.

79 After NMR measurements, each oyster was measured and weighed using a standard method.
80 First, individual total mass (*i.e.* shell plus tissue), was weighed to the nearest 0.1g on an
81 electronic Sartorius balance (TW). Oysters were then opened and the flesh was drained for 15
82 min on absorbent paper and weighed using an electronic Sartorius balance to the nearest 0.001g
83 to determine fresh flesh weight (FW). The difference between total weight (TW) and flesh
84 weight (FW) provides an estimate of the internal shell cavity (ISC) expressed as mass unit (g).

85 Gonad development (GD) was visually estimated at four qualitative stages from stage 0 (no
86 visible gonad tissues) to stage 3 (well developed gonad tissues). A biopsy was then taken from
87 each gonad and analyzed under a light microscope to determine sex. The presence of
88 spermatozoa (2-3 µm) or oocytes (30-50 µm) in the sample indicated if oysters were male (M) or
89 female (F), respectively. Triploid oysters that were asexual were annotated 3N. Finally dry flesh
90 weight (DW) was measured after a 72-hour freeze-drying cycle.

91 *2.2 NMR measurements*

92 NMR measurements were performed at Cemagref (Rennes, Brittany, France) with an OXFORD
93 MQA 6005 spectrometer operating at 0.12T (5MHz) equipped with a 52mm vertical diameter
94 probe which allowed investigation of oysters in their first year of maturity (< 45 g total weight).
95 NMR data comprised the intensities of echoes originating from an NMR pulse sequence that
96 acquired two Carr-Purcell-Meiboom-Gill (CPMG) spin echo trains with different relaxation
97 delay (RD) times between each signal accumulation reflecting combined T1 and T2 relaxation
98 times (Table 1). The 15000ms RD time was chosen to obtain the full T2 relaxation signal of sea
99 water in the internal shell cavity without any T1 weighting. The 400ms RD time corresponded to
100 the T1-weighting parameter used in previous MRI experimentations (Davenel et al., 2006) to
101 obtain the best contrast between testes and ovaries. Low-field NMR measures the spin-spin (T2)
102 and spin-lattice (T1) relaxation of hydrogen nuclei (protons) in mobile or less mobile molecules.

103 *2.3 Statistical analysis*

104 Ideally, after data treatment based on Levenberg-Marquardt or maximum entropy
105 decomposition, low field NMR measurements lead to a distribution of spin-spin relaxation times
106 with peaks corresponding to resonance states of hydrogen nuclei that may be related to specific
107 classes of molecules in the sample, each class characterized by its state (liquid/solid), its
108 diffusion rate or its type of bonding or interaction with other molecules. However, if the rate of
109 chemical exchange of protons between different classes is faster than the time scale of the NMR
110 experiment, two or more classes may be indistinguishable from each other and the exact number

111 and types of classes cannot be measured. We therefore chose to use other approaches based on
112 chemometric techniques to extract information from the NMR CPMG relaxation curves.

113 *Determination of quantitative phenotype characteristics (ISC, FW and DW).*

114 Because the first echo intensity of the non-T1-Weighed CPMG sequence with long RD
115 (15000ms) is proportional to the total proton density of the sample, we tested the simple linear
116 relationship between ISC and the intensity of this variable, and also the intensity of the first echo
117 of the T1-Weighed CPMG sequence with shorter RD value (400ms). Protons in the flesh were
118 water protons interacting with macromolecules or were constituents of lipids and their T2
119 relaxation parameters were shorter than sea water protons. Consequently we tested the linear
120 relationship between flesh weight (FW) or dry weight (DW) and a combination of the intensity
121 of the first echo and the intensity of a longer echo time, strongly weighted by the transverse
122 relaxation of the sea water protons.

123 The traditional univariate linear and multiple regression methods are of limited value to handle
124 the large amount of co-linear data points acquired by CPMG sequences. Multivariate data
125 analysis (chemometric) techniques include algorithms that can handle large co-linear data
126 structures. In this context, the main advantage of chemometric tools is that they are able to deal
127 with spectral or relaxation information, such as NIR and NMR (multivariate co-linear data) by
128 reducing data to a few latent factors (LF). Correlations between quantitative phenotype and
129 NMR data were evaluated using the partial least squares (PLS) predictive regression method on
130 relaxation data originating from the CPMG sequence for each RD value (CPMG-400, CPMG-
131 15000) separately and in combination with NMR data (CPMG-400-15000).

132 *Determination of qualitative phenotype characteristics (SEX, GD).*

133 First non-supervised principal component analysis (PCA) was performed to reduce the
134 dimensions of the original CPMG data matrices retaining the maximum amount of variability.
135 This provided a new set of variables (principal components, PCs) which facilitated the discovery
136 of patterns hidden in the dataset. A stepwise linear discriminant analysis (LDA), a supervised

137 method used for classification purposes, was then performed on PC variables. LDA renders a
138 number of orthogonal linear discriminant functions equal to the number of classes minus one.
139 This method maximizes variance between categories and minimizes variance within classes.
140 Three sex classes were defined: male (M), female (F) and triploid or asexual oysters (3N), and
141 the four qualitative stages described above were used for classification according to gonad
142 development of diploid oysters.

143 Stepwise PLS and LDA procedures were combined with the leave-one-out cross-validation
144 procedure to seek subsets of synthetic LF or PC variables that were the most useful to evaluate
145 predictive errors (RMSEP) of quantitative phenotype characteristics or to discriminate between
146 classes and estimate the clearly classified percentages of qualitative characters. All of the
147 chemometrics discussed were performed using the statistical package R (R Foundation for
148 Statistical Computing, Vienna, 2006: www.R-project.org).

149

150 3. Results

151 *3.1 Determination of quantitative phenotype characteristics (ISC, FW and DW).*

152 In terms of the relationships between the quantitative phenotype characteristics themselves,
153 measured by the standard destructive methods, it should be noted that fresh weight (FW) and dry
154 weight (DW) were highly correlated (R^2: 0.94), demonstrating the good repeatability of the
155 method used to measure fresh weight. Internal shell cavity (ISC) volume was moderately
156 correlated with FW and DW (R^2: 0.56).

157 Simple univariate correlation between internal cavity volume (ISC) and the intensity of the first
158 echo (echo1) of the CPMG-15000 relaxation curve showed that it was possible to determine ISC
159 with a very high correlation coefficient (R^2 of 0.95). In the range 4.7g to 16g, ISC was predicted
160 with a standard error of prediction of 0.62g (Table 2).

161 To eliminate the influence of the sea water protons on the NMR signal, slightly more
162 sophisticated relationships were necessary to predict FW and DW. The results showed that the
163 best combination was to subtract the intensity of a spin echo strongly affected by sea water
164 protons from the first spin echo of the relaxation curve. All the possible combinations were
165 tested. After optimization, the value resulting from the subtraction of the intensity of the echo at
166 64 ms from the beginning of the relaxation curve from the first echo of the CPMG-400 data
167 appeared to be the best correlated with DW and FW, with correlation coefficients of 0.94 and
168 0.91, respectively. In the range 0.22g to 2.10g, DW was predicted with a standard error of
169 prediction of less than 0.1g (Table 2, figure 1). FW was predicted with a standard error of
170 prediction of less than 0.54g in the range 1.28g to 8.90g (Table 2).
171 Several combinations of latent variables resulting from the PLS procedure showed good
172 correlations but did not provide a very significant improvement in the relationships with the
173 different quantitative phenotype characteristics such as DW and FW despite the combinations of
174 5 or 6 latent variables (Table 3). However, the combination of only two latent variables resulting
175 from the CPMG-400 curves alone provided a potentially acceptable prediction of ISC (R^2: 0.93;
176 RMSEP: 0.78g).

177 *3.2 Determination of qualitative phenotype characteristics (SEX, GD).*

178 Stepwise linear discriminant analysis (LDA) of each CPMG curve result separately showed that
179 SEX could be classified with a success rate of 59% and 71% with CMPG-400 and CPMG-
180 15000, respectively. Based on a combination of the results from both CPMG curves, LDA
181 showed a very significant increase in the success rate (to 93%) to predict SEX (Table 4).
182 Discriminant function 1, explaining 78% of the variance, could be interpreted as well correlated
183 with ploidy, and discriminant function 2 clearly separated 2N males and females (Figure 2).
184 Stepwise linear discriminant analysis based on the combination of data from both CPMG curves
185 showed that the potential of NMR relaxometry to discriminate gonad development was good,
186 with 84% of 2N oysters correctly classified (Table 5).

187 ## 4. Discussion

188 These preliminary results show that the low field NMR relaxometry technique is a potentially
189 non-invasive routine sensor for phenotype characterization in *Crassostrea gigas.* The technique
190 provides access to two important numerical representations of the quality of soft bivalve tissue:
191 the weight of the soft tissue and the meat condition index based on the percentage of the internal
192 shell volume occupied by soft body tissue. The most promising result was the estimation of the
193 fresh or dried soft tissue weight by simple bilinear regression based on the CPMG-400 curve,
194 obtained in less than a minute. Using 400 ms RD time, the weight of the very mobile hydrogen
195 atoms (protons) in the CPMG-400 relaxation signal was considerably reduced compared to less
196 mobile protons of macromolecules constituting the dry soft tissue matter, characterized by a
197 longitudinal relaxation T1 parameter that was shorter than for seawater protons. Seawater
198 protons were also characterized by a longer transverse T2 relaxation parameter: subtraction of
199 the intensity of a longer echo time, such as the 64 ms echo time, strongly weighted by the sea
200 water protons from the intensity of the first echo time, provided an indicator that was correlated
201 with the quantity of protons in the macromolecules of the dry soft tissue matter. However, Figure
202 2 shows that the dry weight of oysters with high gonad development could be slightly
203 underestimated. This could be related to the effects of differences in the biochemical
204 composition of soft tissue: gonad tissues are high in lipids (Matus de la Parra et al. 2003) which
205 have higher proton density than glycogen and proteins. Clearly this method would be less precise
206 than the gravimetric method based on lyophilization, but the new method has the advantage of
207 being fast and easy to use for routine investigations and its non-destructive nature should allow
208 precise individual follow-up studies of large oyster collections.
209 The NMR technique also appears to be able to provide an acceptable determination of ISC that
210 can be used to calculate a condition index. ISC is clearly correlated with the intensity of the first
211 echo of the non-weighted CMPG-15000 relaxation curve which indicates the quantity of all the
212 protons from soft tissues and seawater in the NMR probe. The main difficulty is being sure that

213 the internal shell cavity volume is full of seawater and that shell valves remain closed during
214 NMR scanning. An NMR sensor with a horizontal probe would be more appropriate to reduce
215 the risk of water loss. Early methods sought to measure the internal shell cavity by volumetric
216 methods but these are slow and difficult to perform accurately. A faster and easier gravimetric
217 method was also developed: shell cavity capacity was well correlated with the difference
218 between whole oyster weight and empty shell weight after drying for 24 h (Laurence and Scott,
219 1982). However, with this method, any water contained within the shells themselves (not
220 between them) was included in the internal shell cavity weight (Abbe and Albright, 2003). The
221 same limitation exists with the NMR method which quantifies all the protons contained in the
222 NMR probe. Differences in shell morphology and fouling community structure may influence
223 shell porosity and reduce the precision of the method.
224 Exploitation of all the data originating from both CMPG relaxation curves by more sophisticated
225 chemometric methods provided encouraging results that also showed the good potential of NMR
226 to determine sex and identify gonad development. However, given the small number of
227 individuals and the difficulty in visually appreciating gonad development, further studies with
228 larger sample groups are required to validate the method. LDA performed on individuals with
229 dry tissues weighing over 0.7g provided a success rate of 100%. This clearly shows that the
230 precision of the NMR method would be lower for small individuals. The diameter of the NMR
231 probe used in this study did not allow scanning of larger oysters, particularly oysters in their
232 second or third year of maturity. Further studies are required to design an NMR probe that is
233 appropriate for bigger oysters and to improve sex discrimination and prediction of gonad
234 development with larger groups. The method to determine soft tissue weight is rapid and
235 probably sufficiently precise to investigate even small animals. The determination of internal
236 shell cavity, sex and gonad development requires the acquisition of a complementary NMR
237 signal based on a CMPG sequence with a long RD parameter. At the present time, this increases
238 the acquisition time by 7 minutes. It would probably be advisable to reduce the overall

239 acquisition time by half and it should be possible to replace the non-T1-weighted CPMG-15000
240 sequence by a slightly T1 weighted sequence with RD between 5000 and 8000ms to achieve this.
241 Another possibility would be to reduce the accumulation number (16 in the present study),
242 particularly for the largest oysters which deliver the strongest signal originated from soft tissues.
243

244 **5. References**

245 Abbe, G. R., Albright, B. W., 2003. An improvement to the determination of meat condition
246 index for the eastern oyster *Crassostrea virginica* (Gmelin 1791). J Shellfish Res 22:747-
247 752.
248 Davenel, A., Quellec, S., Pouvreau, S. 2006. Noninvasive characterization of gonad maturation
249 and determination of the sex of Pacific oysters by MRI. Magnetic Resonance Imaging 24
250 (8), 1103-1110.
251 Dridi, S., Salah Romdhane, M., Elcafsi, M. 2007. Seasonal variation in weight and biochemical
252 composition of the Pacific oyster, *Crassostrea gigas* in relation to the gametogenic cycle
253 and environmental conditions of the Bizert lagoon, Tunisia. Aquaculture 263 (1-4), 238-
254 248.
255 Chavez-Villalba, J., Pommier, J., Andriamiseza, J., Pouvreau, S., Barret, J., Cochard, J.C., Le
256 Pennec, M., 2000. Broodstock conditioning of the oyster *Crassostrea gigas*: origin and
257 temperature effect. Aquaculture 214 (1-4), 115-130.
258 Enriquez-Diaz, M., Pouvreau S., Fabioux, C., Le Coguic, Y., Cochard, J.C., Le Pennec, M.,
259 2003. Reproductive strategy: Variability of reproductive pattern in two populations of
260 genetically determined *Crassostrea gigas*. J Shellfish Res 22:328.
261 Fabioux, C., Huvet, A., Le Souchu, P., Le Pennec, M., Pouvreau, S., 2005. Temperature and
262 photoperiod drive *Crassostrea gigas* reproductive internal clock. Aquaculture 250 (1-2),
263 458-470.
264 Lawrence, D. R., Scott, G.I., 1982. The determination and use of condition index in oysters.
265 Estuaries 5:23-27.
266 Matus de la Parra, A., Garcia, O., San Juan, F., 2005. Seasonal variations on the biochemical
267 composition and lipid classes of the gonad and storage tissues of *Crassostrea gigas*
268 (Thuberg, 1794) in relation to the gametogenic cycle. J Shellfish Res 24 (2):457-467.
269

270 Namba K., Kobayashi M., Aida S., Uematsu K., Yoshida M., Kondo Y., Miyata Y., 1995.

271 Persistent relaxation of the adductor muscle of oyster *Crassostrea gigas* induced by
272 magnesium ion. Fish. Sci. 61, 241-244.

273 Pouvreau, S., Rambeau, M., Cochard, J.C., Robert, R., 2006. Investigation of marine bivalves
274 morphology by *in vivo* MR imaging: first anatomical results of a promising technique.
275 Aquaculture 259 (1-4), 415-423.

276

277

278 **Captions for figures**

279 **Fig.1.** Prediction of dry flesh weight by subtraction of the intensity of the echo at 64 ms from the
280 beginning of the CPMG-400 relaxation curve from the intensity of the first echo. Effects of
281 gonad development stages (0, 1, 2, and 3). 3N individuals were classified as stage 0.

282 **Fig.2.** Prediction of sex (3N triploid asexual oysters, 2N males and females) by discriminant
283 functions originating from chemometric LDA method.

284

285
286

Table 1

Table 1. Parameters of **NMR** sequences

NMR CPMG sequence	RD	TE	Accumulation number	Acquisition time
CPMG-400	15000 ms	2 ms	16	6 min 51 s
CPMG-15000	400 ms	1 ms	32	47 s

Table(s)

Table 2. Prediction of quantitative phenotype characteristics, dry flesh weight (DW), fresh flesh weight (FW) and internal shell cavity (ISC) using simple or bilinear relationship with NMR data.

NMR data	Echo and Echo combination	DW		FW		ISC	
		R^2	RMSEP	R^2	RMSEP	R^2	RMSEP
CPMG-400	echo1	0.78		0.83		0.89	0.92g
	echo1-echo64	0.94	0.100g	0.91	0.54		
CPMG-15000	echo1	0.44		0.53		0.95	0.62g
	echo1-echo34	0.91	0.116	0.89	0.56		

RMSEP means Root Mean Square Error of Prediction

Table(s)

Table 3. Prediction of quantitative phenotype characteristics, dry flesh weight (DW), fresh flesh weight (FW) and internal shell cavity (ISC) using latent variables (LV) originating from chemometric PLS method

NMR data	Latent Variables	DW		FW		ISC	
		R^2	RMSEP	R^2	RMSEP	R^2	RMSEP
CPMG-400	3 LV	0.96	0.093g	0.94	0.52g		
	2 LV					0.93	0,78g
CPMG-15000	6 LV	0.95	0.116g	0.92	0.58g		
	5 LV					0.98	0.67g
CPMG-400-15000	5 LV	0.96	0.110g	0.94	0.53g		
	5 LV					0.99	0.65g

RMSEP means Root Mean Square Error of Prediction

Table 4

Table 4. Prediction of sex (3N, 2N males and females) using discriminant functions originating from chemometric LDA method.

« Sex » observed	n	« Sex » predicted		
		3N	F	M
3N	25	**25** (100%)	0	0
F	15	0	**14** (93%)	1
M	16	1	2	**13**(87%)

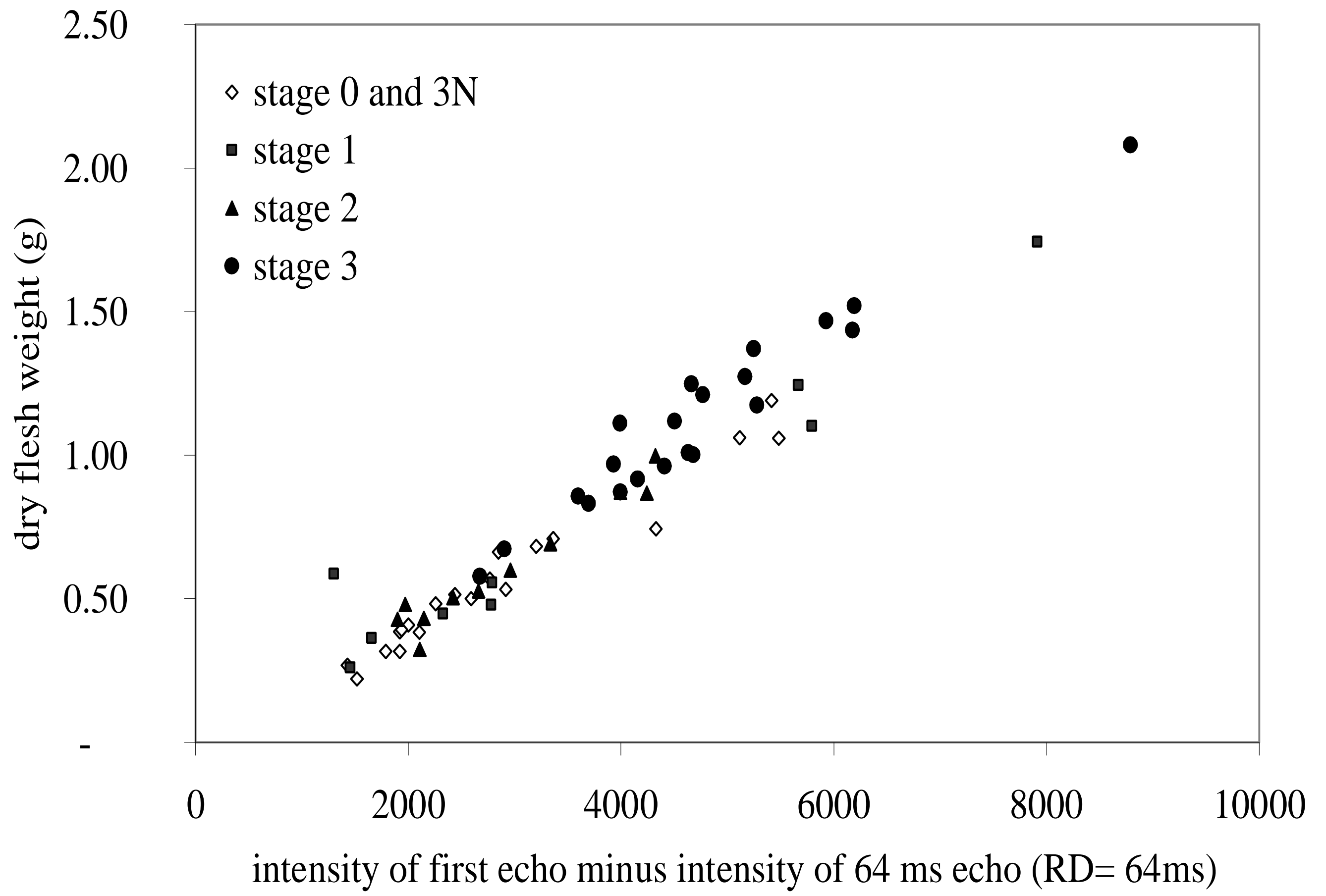
Table 5

Table 5. Prediction of gonad development (GD) stages (1, 2, 3) of 2N males and females using discriminant functions originating from chemometric LDA method

GD observed	n	GD predicted		
		1	2	3
1	2	**1** (50%)	1 (50%)	0
2	10	0	**7** (70%)	3 (30%)
3	19	0	4 (20%)	**18** (79%)

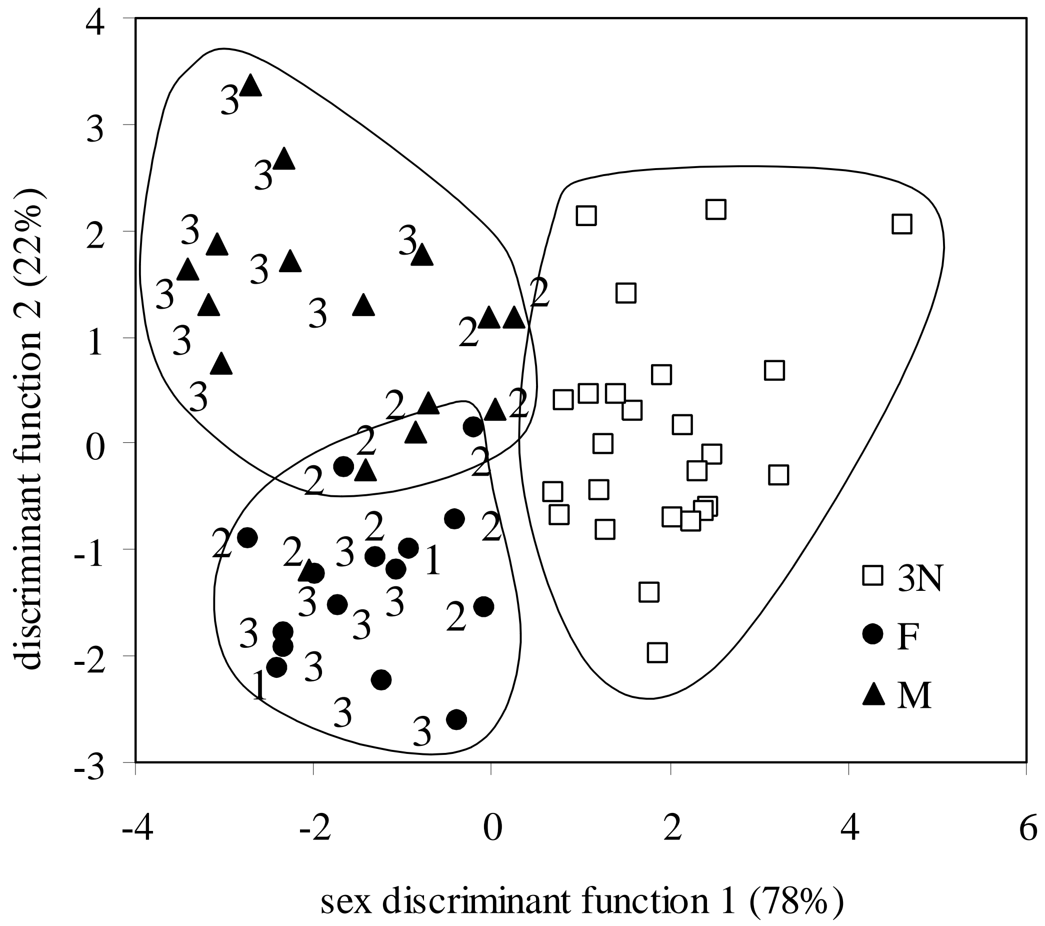
Figure 1

Fig.1.



Figure(s)

Fig.2.



*** Response to Reviewers**

Manuscript : NMR relaxometry as a potential non-invasive routine sensor for characterization of phenotype in Crassostrea gigas, A. Davenel…	
Reviewers' Comments	Corrections or authors' comments
Reviewer 1	
These authors may not mentioned in their publications related to NMR or MRI methods, if these non destructive techniques can have some impact, even very small, on different fecundity parameters (success of the spawning, quality of gametes, number of gametes and larvae) compared to animals in the same batches that have not been investigated in vivo MR imaging.	At the present time, we have not already realized such comparison to evaluate the impact of these non destructive methods on some fecundity parameters. However we have realized individual tracking of group of oysters until five temporal MRI scannings without mortality. We will take into account its valuable suggestion in the future.
Reviewer 2	
Line 35. Gigas, comma is underline.	Comma have been deleted in the text
Line 51.I will propose to add Magnetic resonance imaging "Magnetic Resonance (NMR) and Magnetic resonance imaging (MRI)" to avoid the confusion between the two techniques. Moreover, the authors have published before on MRI and not on NMR.	The proposal has been integrated in the text
Line 92: Do the authors can precise where is localised this NMR equipment?	Location has been added in the text
Lines 152-155. I was little confused with the first paragraph of the results. The authors must precise here, that these relationships concern only data obtained using either standard methods or NMR measurement?	These relationships concern only data obtained using standard methods: further details have been added in the text.
Line 211. Why the authors do not insert the animal in agar gel for ensuring that the shell valves remaining closed during NMR scanning.	In future experiments, we will take into account the interesting suggestion of the reviewer.
I especially recommend the authors to precise quantitative parameters concerning the following progenies obtained with the animals tested by NRM techniques. I will appreciate that these data could be included either in this manuscript if the authors have already some informations or in their future studies.	We have no information concerning the following progenies. We will include these data in our future experiments.
Fig. 2. I will suggest to encircle the three prediction of sex to be more in evidence	We have adopted this suggestion.
Table 2 and 3. Why some data are highlighted in bold type? RMSEP must be explain in the legend of the table.	Bold type has been deleted. RMSEP has been defined in the legend
Reviewer 2	
	No corrections asked