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Noninvasive characterization of gonad maturation and determination of the sex of Pacific oysters by MRI

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Abstract:

The aim of this study was to test the ability of magnetic resonance imaging (MRI) technique to characterize gonad development and to determine the sex of live Pacific oysters through their shells. A preliminary nuclear magnetic resonance (NMR) relaxometry study was conducted to characterize T_1 and T_2 NMR relaxation parameters for the main oyster organs. This showed that T_1-weighted MRI sequences were most appropriate to optimize contrasts between tissues in images. The results showed that gray levels of gonads in images acquired with gradient-echo sequence were variably affected by T_2* weighting effect. However, the ovaries systematically gave a hypersignal in spin-echo T_1-weighted images, and stack histograms of female oysters showed a peak well separated from that of male oysters. An automated method is proposed to quantify the development of oysters and their gonad maturation and to identify their sex.

Keywords: Oyster; Gonad maturation; Sex determination; Gradient echo; Spin echo

Introduction

Marine bivalves, and especially the Pacific Oyster, *Crassostrea gigas*, are economically important in French aquaculture. Their production has now become a major industry with production above 100,000 metric tons in 2002. Nevertheless, the ecology and the physiology of this bivalve are not fully understood, and consequently the control of its growth and reproduction both in the field and in hatcheries still relies on empirical factors.

The investigation of soft tissue in marine mollusks, and especially in marine bivalves, classically relies on destructive methods, since a hermetic shell protects the animal. For example, anatomical structures are generally studied after opening and dissection by means of histological sections with a resolution of 4-5 µm [1]. The evolution of gametogenesis can be quantitatively assessed with the help of image analysis [2, 3]. For the same reasons, sex determination and analysis of physiological and biochemical changes within tissues necessarily involve the sacrifice of numerous specimens and the preparation and analysis of several samples. These standard techniques provide precious information on marine mollusk biology but have two main disadvantages which are limitations for many studies: these methods are very time consuming and they are necessarily destructive. Non-invasive and quantitative procedures have therefore been developed, and after preliminary trials [4], NMR imaging has proved promising.

Non-invasive imaging methods have been investigated to measure anatomic traits and predict body composition in different freshwater and marine species since the beginning of the 1990s. Some of these methods have been developed to assess whole body composition in fish, including X-ray computerized tomography for rainbow trout and salmon in Norway [5,6], and carp in Hungary [7]. Magnetic resonance imaging (MRI) has been tested for the determination of fat content of burbot liver [8] and to characterize water diffusion in carp [9] and trout flesh[10].

Most of these studies have been limited to average body composition, although these techniques can be used to characterize the heterogeneity of lipid distribution in the tissues. X-ray computerized tomography has been used for the measurement of adipose tissue located between the myomeres in salmon [6]. The MRI technique gives a precise picture of the anatomic distribution of lipid deposition in the flesh of brown trout [11] as long as a method for correction of the MRI images for the effects of magnetic field inhomogeneity is applied [12, 13].

However, the MRI technique has rarely been used to observe live marine animals. One of the few studies in this field included the visualizing of water flow in a breathing carp [14]. There has been no MRI study on bivalves (and *a fortiori* on oysters) to date except the promising trial reported by Pouvreau et al. [4]. Oysters constitute a fairly interesting model for MRI study in marine organisms because 1) the oyster is an intertidal animal that encounters emersion periods without any problem and can be analyzed in the imager out of seawater without any stress, 2) its lipid-rich flesh is easily detectable with a MR imager and 3) oysters are easy to immobilize, insensitive to handling stress and internal movements inside the shell are nearly inexistent, allowing long acquisition times for high resolution images without any blurring effects. This last point effectively constitutes a limiting factor when studying the anatomy of small live animals [15], for which anesthetics are often required.

As a complement to this earlier study, the present study aims to test the ability of the MRI technique to characterize gonad development and, if possible, to determine the sex of live Pacific oysters through their shells. A preliminary NMR study was conducted to characterize T1 and T2 NMR relaxation parameters for the main oyster organs and the results of this preliminary study were then used to establish MRI parameters in order to optimize contrasts between tissues on images. MRI images of oysters were then obtained with gradient echo and

spin echo sequences weighted according to the initial NMR results. Both sequences are compared and discussed.

1. Materials and Methods

1.1 Animal origin and preparation

This study was conducted in 2002 and 2003 on oysters originating from a single population collected as spat in the Arcachon Basin (Gironde, France) and reared in the Aber Benoît River (North Brittany, France) for two years. They were 2-3 years old and weighed approx. 100-120g. They were periodically collected each year from February to August in their natural habitat by the IFREMER Shellfish Laboratory located in Argenton near Brest (Brittany, France) and brought to Cemagref located at Rennes (Brittany, France) for NMR and MRI investigations without any specific anesthetization procedure. Measurements were obtained at Cemagref in two stages: first, NMR parameters were measured on sacrificed oyster organs to determine T1 and T2 values and then MR images were acquired on live oysters on several occasions between February and August to determine gonad development according to season (maturity stage) and sex (male, female or undetermined).
For NMR analyses (in February and July 2002), oysters were first dissected just before NMR measurements. Shell length (antero-posterior axis) was first measured for all specimens to the nearest mm prior to dissection. Shells were opened and soft tissues (*i.e.* total flesh) were removed from the shell, drained for 5 minutes on absorbent paper and weighed to the nearest 0.1 g. The flesh was then properly dissected to separate the adductor muscle, the gills and the 'visceral mass'. The gonad of *C. gigas* is fused with the digestive gland to form a complex organ called 'visceral mass'. Nevertheless, since specimens used in our study were large enough, it was possible to separate the gonad from the digestive gland. A sub-sample (biopsy) was then taken from the gonad and analyzed under light microscope to determine sex. The presence of spermatozoa (2-3 µm) or oocytes (30-50 µm) in the sample indicated if oysters were male or female, respectively. For MRI analyses (from February to August 2002 and 2003), dissection and sex determination were also conducted for all individuals after MRI analyses.

1.2 Characterization of tissues of male and female oysters by NMR relaxometry.

NMR measurements of samples of muscles, gills and gonad tissues were performed with a low field NMR spectrometer (0.47 Tesla) operating at 20 MHz for ^{1}H (Minispec PC120, Brucker SA, F-67166 Wissembourg, France). Spin-spin relaxation (T_2) was measured from a Carr Purcell Meiboom Gill curve. The interval between 90° and 180° pulses was 1.5 ms and 845 echo values were acquired. The spin-lattice relaxation (T_1) was measured using a saturation recovery sequence. Fifty points were acquired from 30ms to 5s, and T1 and T2 values were then used in order to optimise the choice of the MRI sequence and its parameters to differentiate gonads and to determine sex.

1.3 MRI acquisition sequences and parameters

The MRI images were acquired with an 0.2 Tesla OPEN SIEMENS system operating at 8.25 MHz for ^{1}H and equipped with a "head" probe. Since the NMR relaxometry study showed that differentiation of tissues on the basis of T2-weighted MRI images was not very relevant, and in order to optimize the signal to noise ratio, a T1-weighted sequence with short echo time TE was chosen with a plane resolution lower than a millimeter.
In 2002, the apparatus was equipped with magnetic field gradients lower than 10 mT/m and a T1-weighted gradient echo sequence with a 9 ms TE value (BW: 78 Hz) was used. The results of the experiments undertaken in 2002 revealed a notable attenuation effect in T2* and the

intensity of this effect was variable from one animal to another. Following the upgrading of the field gradients from 10 to 15 mT/m at the beginning of 2003, a T1-weighted spin echo sequence with an 8 ms TE value (BW: 130 Hz) was tested in the summer of 2003.
The image matrix size was 128 x 128 voxels with a field of view (FOV) of 120 x 120 mm. Four oysters were scanned simultaneously. On the basis of the results obtained in NMR relaxometry and following optimization on some oysters while varying the repetition time TR parameter. A sequence with a TR of 400ms was systematically applied thereafter in order to obtain 18 and 23 consecutive transverse slices of 4 mm thickness per animal, with gradient echo and spin echo sequences, respectively. To improve the signal to noise ratio, the number of accumulations was 15. The acquisition time was 12.5 minutes for four oysters.

1.3.1 Image preprocessing and analysis

The interpretation of raw images obtained from a MRI scanner can lead to erroneous interpretations of the gray level variations in the image. Several sources of spatial in-homogeneities in the apparatus, related to the inhomogeneity of the magnetic field and the geometry of the transmitting and receiving probes, led to undesirable variations in intensity in the images. Moreover, heterogeneity in permanent magnetic field and the antennas was corrected before any post processing by dividing the images of oysters by those of a homogeneous oil phantom obtained with the same MRI sequence pixel by pixel. The 128x128 pixel images were then segmented automatically in four sub-images of 64x64 pixels to separate the four oysters.
For each oyster, a gray level histogram (stack-histogram) was then calculated from all the pixels of the stack of 18 images (TR: 400 ms). Some stack-histograms of oyster images acquired with longer TR were also studied to confirm and illustrate the effects of T1-weighting on contrast between tissues.
The correction of the raw images to eliminate the effects of spatial inhomogeneity of the MRI scanner and the calculation of the histograms were carried out with SCILAB software developed by INRIA with contributions from CEMAGREF for the analysis of images. The images presented in this paper were formatted with ImageJ software (1.34g Version) developed by the National Institute of Health (USA).

2. Results and discussion

2.1 Optimization of MRI acquisition sequences

2.1.1 Preliminary results of the characterization of oyster tissues by NMR relaxometry and first previews of T1- weighted MRI images

The results of characterization of various tissues by NMR relaxometry showed that the T1 relaxation times of the gonad and the muscle were notably shorter than those of the heart, other viscera and gills bathed in seawater. The same results were also illustrated on MRI images (Fig. 1).

More precisely, NMR results obtained on samples of the different tissues in February 2002 suggested the possibility of differentiating testes, ovaries and muscle on the basis of T1 longitudinal relaxation times (Table 1). However, the low number of samples and the particular behavior of two individuals (one male and one female) justified the further experimentation in July 2002 that was also likely to reveal a potential seasonal effect. This new experimentation checked that the NMR technique made it possible to differentiate the ovaries (T1 of about 207 ms ± 21 ms) from testes (T1 of about 456 ms ± 50 ms) and of muscle (T1 of about 461 ms ± 13 ms). However the NMR technique did not make it possible to differentiate testes and muscle on the basis of the T1 value. Moisture values seemed to indicate that these differences in T1 value between ovaries and muscle and testes were mainly attributable to lower moisture content of the ovaries. Reporting on the biochemical composition of oyster organs, Li [15] showed that the glycogen content of ovaries decreased steadily during maturation from 33.5% in May to less than 10% in July and August. The glycogen content of testes showed a similar pattern. The difference in moisture content is probably related to a higher protein concentration in mature ovaries than in testes (55 mg/g and 20 mg/g, respectively, in July and August according to Li [16]). Moreover, Li [16] showed that the same ovaries have clearly higher amounts of triglycerides and phospholipids in the mature state (approximately 30 mg/g and 12 mg/l, respectively) than testes (approximately 5 mg/g and 7 mg/g, respectively). Triglycerides are known to have shorter longitudinal relaxation times than water in interaction with hydrophilic molecules and this would contribute to increased gray-level intensity of ovary voxels in T1-weighted MRI images.

It was on the basis of these NMR results that the choice of a repetition time (TR) was made for the acquisition of MRI images. The effects of TR are clearly illustrated on the T1-weighted MRI image (Fig. 2 and 3). It appeared from these first MR images that the signal to noise ratio was satisfactory and allowed image analysis.

2.1.2 Comparison of the characterization of oyster tissues with a T1-weighted gradient echo sequence (July/August 2002) and spin echo sequence (August 2003).

The NMR measurements presented above made it possible to consider differentiation of sex on the basis of average gray level of the large peak corresponding to the higher gray level values in the histograms allotted to the gonads and muscles. The average T1 values measured by NMR, notably lower for the ovaries, predicted an average gray level higher than that of the testes or muscles in the images.

Processing of stack-histograms of the corrected gradient echo images of oysters scanned in August 2002 showed an opposite trend: the mean gray level of the peaks attributed to testes (Fig. 4) tended to be higher than that assigned to the ovaries. Moreover this trend was not systematic but was observed for males with a well-developed testis. Certain males at a earlier

stage of sexual development presented a peak that did not allow differentiation from female oysters. This tendency was very often contrary with that expected from the NMR results.
The choice that was made to use a T1-weighted sequence in order to increase the contrast between gonads and other tissues thus seemed appropriate. However the signals collected with such a sequence can also be affected by a T2* weighting effect, especially because of the effects of magnetic susceptibility and/or dephasing between water and fat in the voxels. With a TE time of 9 ms, lipids and water spins in the same voxel are dephased by about 90°C in gradient echo images. This phenomenon could partially explain a decrease in the signal of ovary voxels with greater triglyceride content than testis voxels. This attenuation of the ovary signal might be amplified by a magnetic susceptibility effect between the contents of oocytes that are higher in lipids than spermatocytes, and the matrix of vesicular connective tissue surrounding them. The MRI scans with spin echo sequence are not sensitive to such effects , and this might be significant for the gradient echo sequence according to the type of tissue and even (for gonads) dependent on sex and the stage of maturity.
MRI scanning of mature oysters in August 2003 verified that it was possible to reproduce the behavior expected from the NMR results on tissue samples taken in 2002 when a spin echo MRI sequence was used and not a gradient echo sequence. The spin echo sequence provided images free from the effects of chemical shift and (or) magnetic susceptibility that would affected the signal from the ovaries in varying degrees according to the maturation level. Figure 5 shows very clearly on all the stack histograms of 18 female oysters a peak around level 200 that is clearly separated from the peak of 18 male oysters around level 150.

2.2 Automated treatment to quantify gonad development and identify sex from stack-histograms of T1-weighted spin echo images

Although earlier results showed that it was possible to make a reliable assessment of the development of the gonads and to differentiate sex by visualizing the images and/or the histogram for an animal, we tried to reduce the influence of the human evaluation by processing the overall histograms by setting thresholds to separate tissues while counting.
Firstly, to estimate flesh occupation, and consequently gonad development, we choose to analyze pixels with gray levels higher than 114 to eliminate image background and seawater. The results obtained from the experiments conducted in August 2003 on the basis of this first thresholding are presented in figure 6 and show clearly that there was a strong correlation between flesh weight and the number of pixels with gray-levels higher than 114 ($R^2 = 0.92$; n= 40, standard error = 1.36 g). This strong correlation demonstrated that the MRI sequence chosen provided exhaustive scanning of the animal and allowed assessment of the flesh weight and consequently of gonad development.
Secondly, by setting the upper threshold at 172, we isolated the ovary from the muscle, the digestive gland and the testis. The results obtained on the basis of this second threshold are shown in Figure 7. This figure demonstrates clearly that we could separate males from females without any ambiguity. On the basis of the biochemical content of testes and ovaries in oysters [15], the hypothesis that MRI would constitute a tool to discriminate male from female oysters was put forward by Pouvreau et al. [4]. The present study confirms this hypothesis.
Furthermore, on the basis of the intensity on each axis (horizontal axis for the females and vertical axis for males) it becomes possible to estimate the development of the gonads for both sexes. Nevertheless, because of the impossibility of distinguishing testis from muscle on the basis of the gray level intensity, it remains difficult to differentiate an immature animal from a male oyster whose gonad is still not fully developed.

3. Conclusion

In conclusion, as suggesting by Pouvreau et al. [4], application of MR imaging in oysters is able to determine sex and to follow their flesh and gonad development without opening them. To our knowledge, this is the first non-invasive method that can provide such results in oysters.

Applications in aquaculture will depend on the costs of such instruments. Although too expensive to date, the price of an MR imager is beginning to decrease because of the development of smaller MR instruments dedicated to small animals. For example, in mollusk hatcheries, and especially with the development of genetic strains, the potential applications of such results are numerous. They can be used to identify the flesh quality of brood stock on entry to the hatchery, follow the evolution of gametogenesis during brood stock development very quickly, identify the sex of brood stock oysters without invasive methods before fecundation and crossbreeding and provide quality indicators of oocytes and spermatozoa without opening the oyster parents [17]. The results of this study have opened up many perspectives that would constitute starting points for further studies.

4. References

[1] Chavez-Villalba J, Pommier J, Andriamiseza J, Pouvreau S, Barret, J, Cochard JC, Le Pennec M. Broodstock conditionning of the oyster Crassostrea gigas: origin and temperature effect. Aquaculture 2002;214:115-130.

[2] Enriquez-Diaz M, Pouvreau S, Fabioux C, Le Coguic Y, Cochard JC, Le Pennec M. Reproductive strategy: Variability of reproductive pattern in two populations of genetically determined *Crassostrea gigas*. J Shellfish Res 2003;22:328

[3] Fabioux C, Huvet A, Le Souchu P, Le Pennec M, Pouvreau S. Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. Aquaculture 2005; *In Press*, Corrected Proof, Available online 16 March 2005.

[4] Pouvreau S, Rambeau M, Cochard JC, Robert R. Investigation of marine bivalves morphology by *in vivo* MR imaging: first anatomical results of a promising technique. Submitted to Aquaculture in August 2005.

[5] Gjerde B. Predicting carcass composition of rainbow trout by computerized tomography. J Anim Breed Genet 1987;104:121-136.

[6] Rye M. Prediction of carcass composition in Atlantic salmon by computerized tomography. Aquaculture 1991;99,35-48.

[7] Hancz C, Romvari R, Horn P. Prediction of carcass quality traits of common carp by X-ray tomography. Isr J Aquacult-Bamid 2003;51(1):61-68.

[8] Alanen A, Komu M, Bondestam S, Toikkanen S. Determination of fat content of burbot (Lota lota) liver with low field MR imaging (0.04T). Phys Med Biol 1991;36:953-961.

[9] De Boeck G, Vanaudenhove M, Verhoye M, Van Audekerke J, De Wachter B, Blust R, Van der Linden A. Water household of the common carp, Cyprinus carpio, when submitted to an osmotic challenge, as determined by diffusion-weighted MRI at 7T. Magma 1997;5:13-19.

[10] Foucat L, Taylor RG, Labas R., Renou J.P. Characterization of frozen fish by NMR imaging and histology. Am Lab 2001;33:38-43.

[11] Toussaint C, Fauconneau B, Medale F, Collewet G, Akoka S, Haffray P, Davenel A. Description of the heterogeneity of lipid distribution in the flesh of brown trout (Salmo trutta) by MR imaging . Aquaculture 2005;243:255–267.

[12] Collewet G, Toussaint C, Davenel A., Akoka S, Médale F, Fauconneau B, Haffray P. Magnetic Resonance Imaging as a Tool to Quantify the Adiposity Distribution in Fish. In: Webb, G.A., Belton, P.S., Gil, A.M., Degadillo, I. (Eds.), Magnetic in Food Science: a View to the Future. Cambridge : Royal Society of Chemistry, 2001. p. 252-258.

[13] Collewet G., Davenel A., Toussaint C., Akoka S. Correction of intensity nonuniformity in T1-weighed images in a low-field imager. Application to fat-muscle quantification. Magn Reson Imaging 2002;20:365-373.

[14] Van Den Berg C, Van Dusschoten D, Van As H, Terlouw A, Schaafsma TJ, Osse JWM. Visualising the water flow in a breathing carp using NMRI. Neth J Zool 1995;45:338-346.

[15] Goodman BA, Gordon SC, Chudek JA, Hunter G, Woodford JAT. Nuclear Magnetic Resonance Microscopy as a non-invasive tool to study the development of lepidopteran pupae. J Insect Physiol 1995;41:419-424.

[16] Li Q, Osada M, Mori K. Seasonal biochemical variations in Pacific oyster gonadal tissue during sexual maturation. Fisheries science 2000;66:502-508.

[17] Hagedorn M, Hsu E, Kleinhans FW, Wildt DE. New approaches for studying the permeability of fish embryos: Toward successful cryopreservation. Cryobiology 1997;34:335-347.

Table 1. Characterisation of longitudinal (T1) and transverse (T2) NMR relaxation times by NMR relaxometry and moisture of oyster tissues sampled in February and July 2002. T1 and T2 values are expressed as mean ± standard deviation (number of individuals)

		muscle	gonads		sea water
			testes	ovaries	
February 2002	T1 (ms)	493 ± 25 (12)	381 ± 59 (4) 355 ± 25 (3)*	251 ± 95 (8) 219 ± 25 (7)*	1636
July 2002	T1 (ms)	461 ± 13 (11)	456 ± 50 (5)	207 ± 22 (16)	
	T2 (ms)	52.8±4 (11)	63.8± 2.8 (5)	57 ± 4.2 (16)	
	moisture (%)	73.4 ± 0,7 (11)	73.9 ± 0,8 (5)	70.2 ± 1 (16)	

* one suspect individual was eliminated

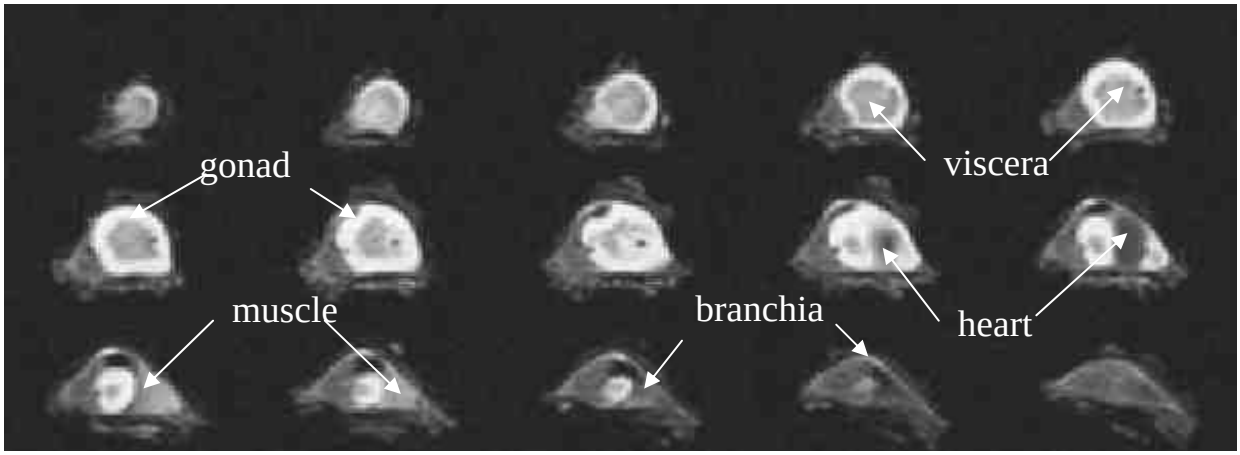
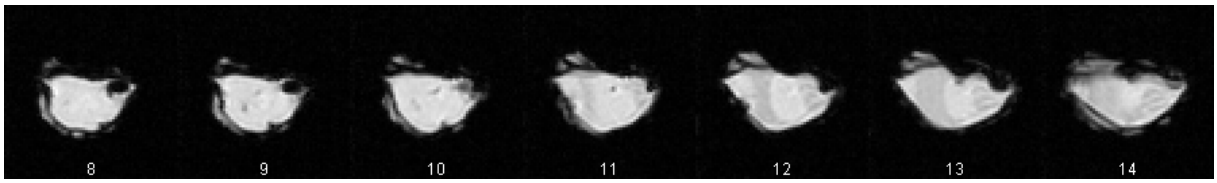
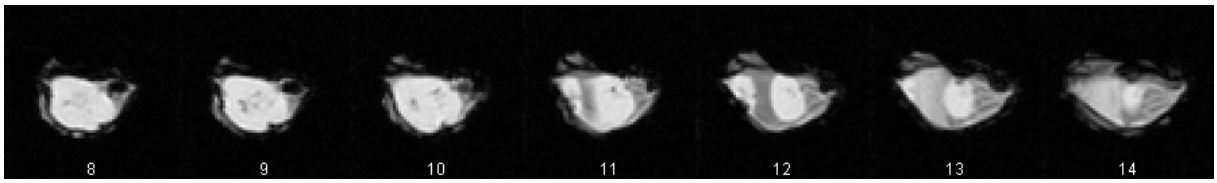


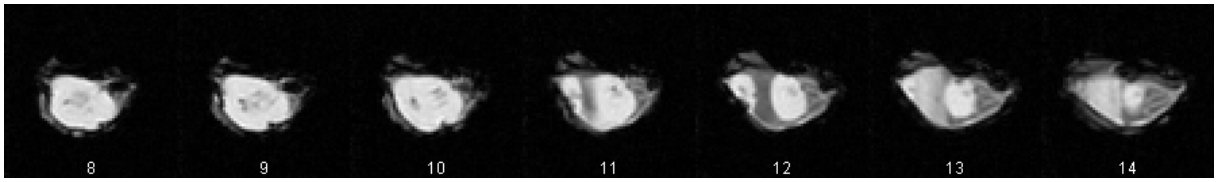
Fig. 1. Stack of 15 MRI images of same female oyster (spin echo sequence; TR : 400ms)



TR: 2000 ms



TR: 800ms



TR: 400 ms

Fig. 2. Effect of the T1 weighting on the improvement in contrast between ovary, muscle, heart and other organs bathed in sea water for TR of 2000ms, 800ms and 400 ms.

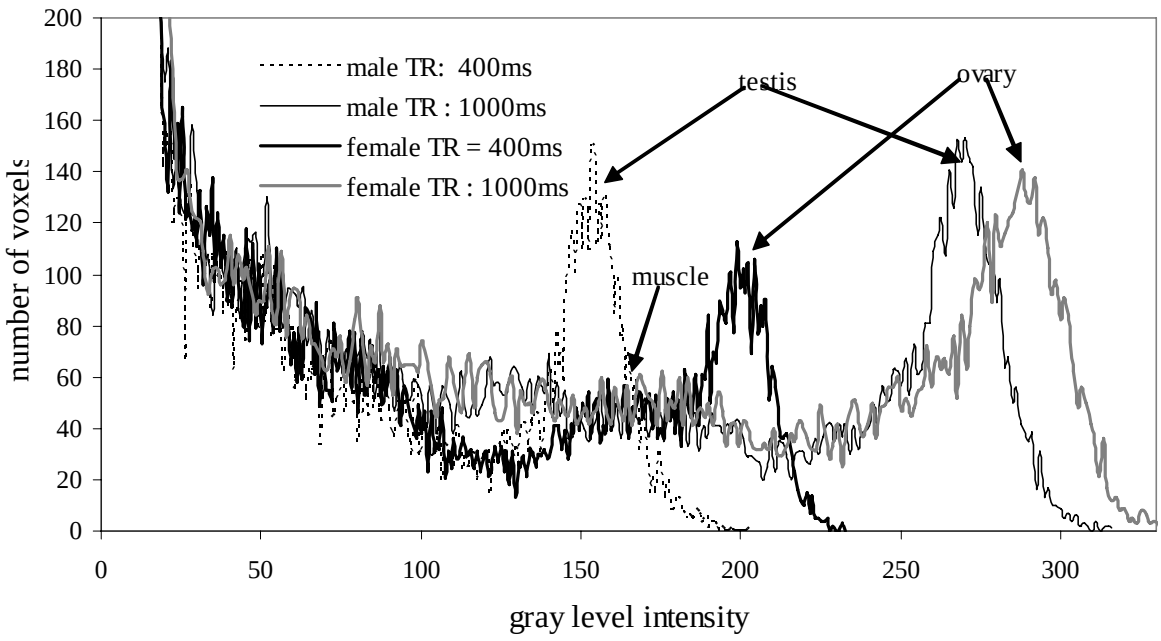


Fig. 3. Improvement in contrast between muscle and ovary by decreasing the TR value from 2000ms to 400ms.

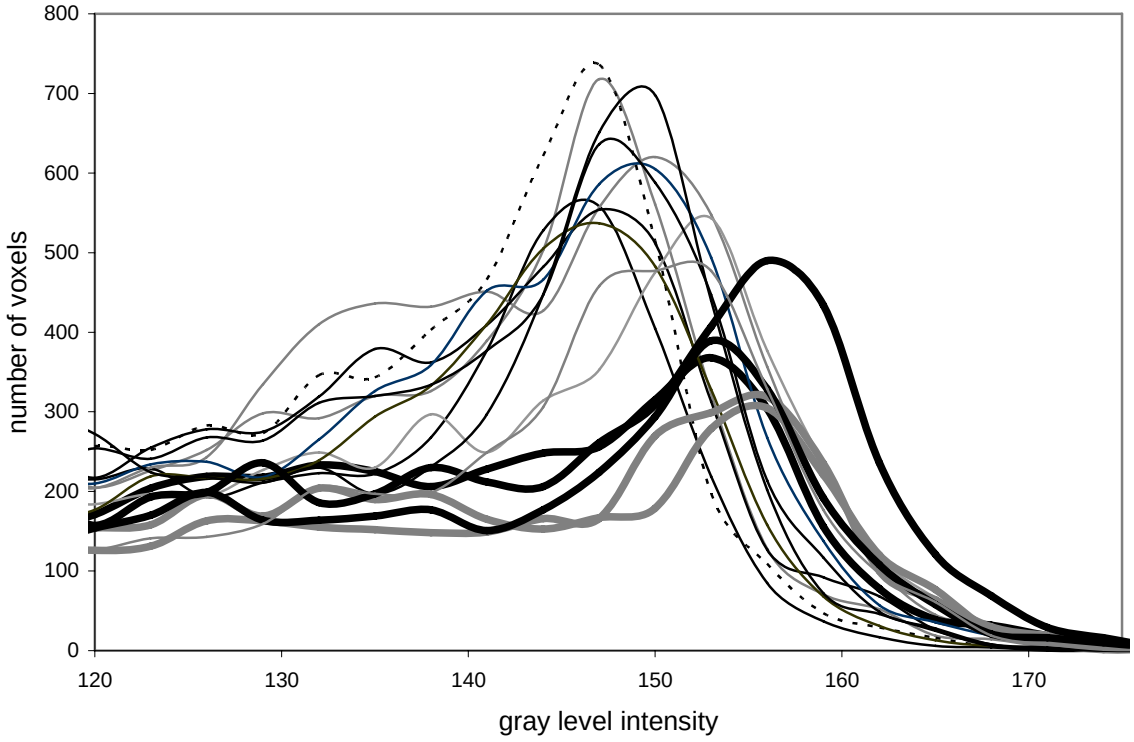


Fig. 4. Gray level histograms of gonad tissues and muscle from stack of 18 MRI T1-weighted (TR : 400ms) gradient echo images. Thick lines represent testis and muscle of five male oysters and thin lines represent ovary and muscle of eleven female oysters.

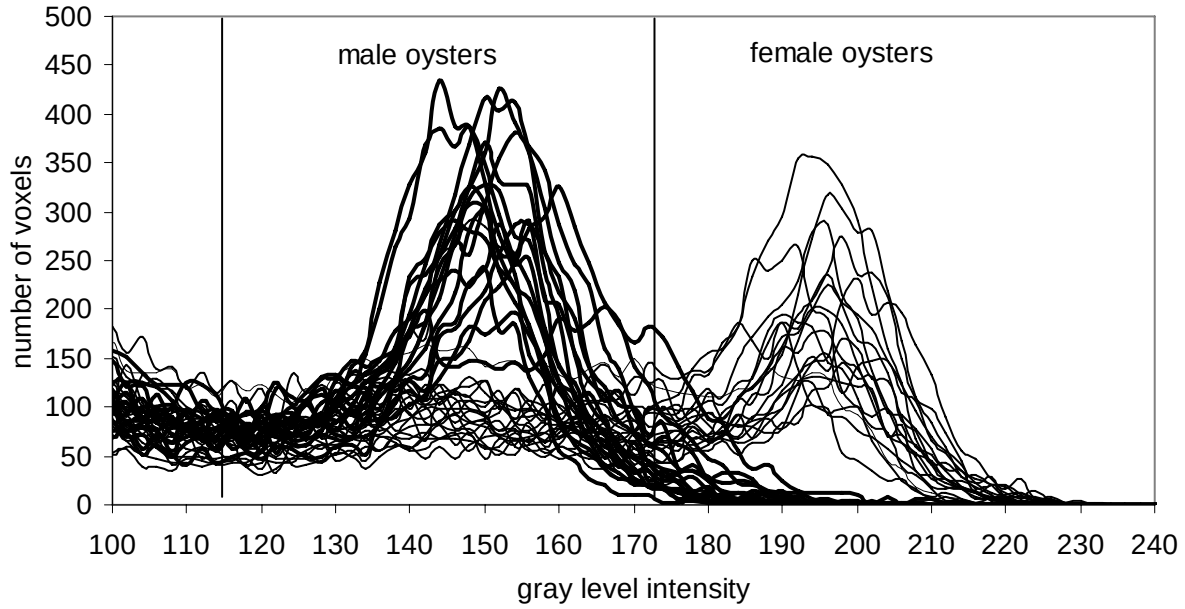


Fig. 5. Gray level histograms of gonad tissue and muscle from stack of 18 MRI T1-weighted (TR : 400ms) spin echo images. Thick lines represent testis and muscle of 18 male oysters and thin lines represent ovary and muscle of 18 female oysters.

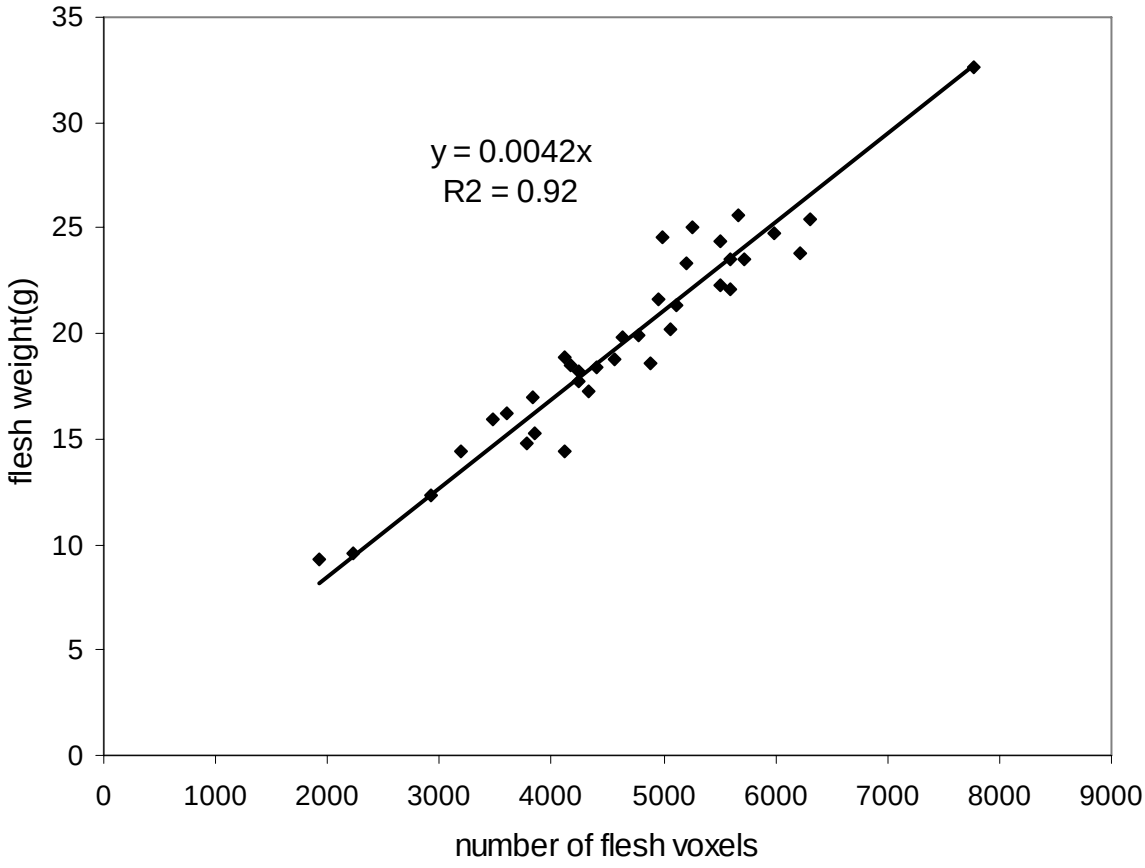


Fig. 6. Flesh weight versus number of flesh voxels measured by MRI (gray levels >114)

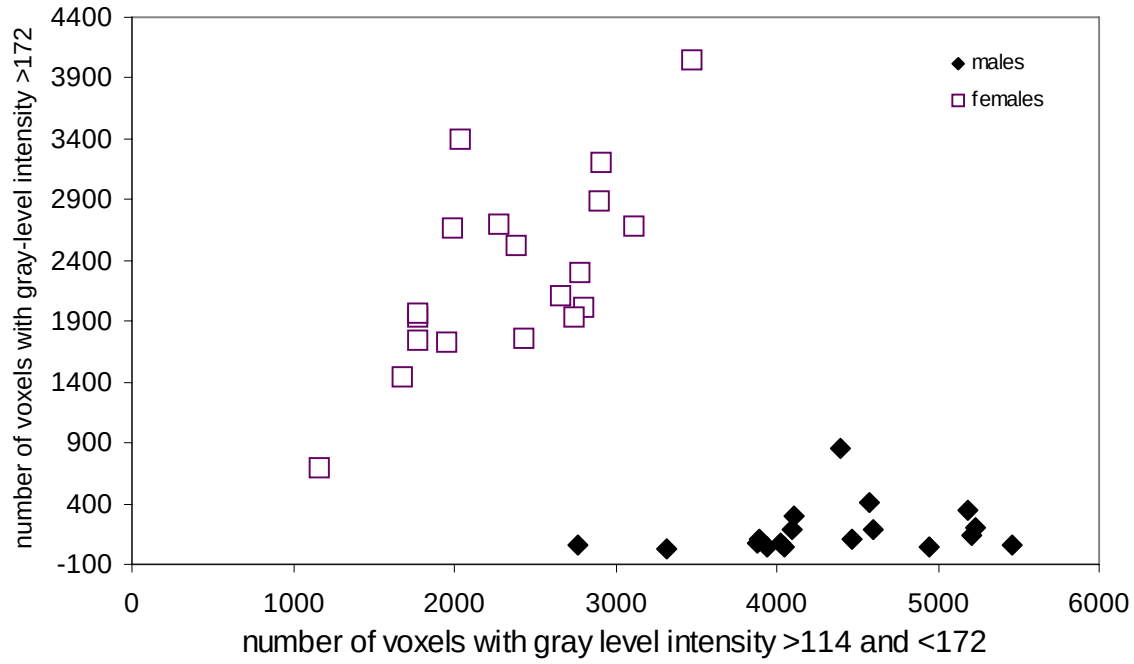


Fig. 7. Number of voxels with gray level intensity greater than 172 versus number of voxels with gray-level intensity greater than 114 and inferior to 172