Aquaculture
December 2012, Volumes 370–371, Pages 84–89
http://dx.doi.org/10.1016/j.aquaculture.2012.10.008

Gonad volume assessment in the oyster *Crassostrea gigas*: Comparison between a histological method and a magnetic resonance imaging (MRI) method

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Abstract:

Previous studies comparing *Crassostrea gigas* selected lines with different susceptibility to summer mortality concluded that the higher the reproductive effort the lower the survival. But they were lacking individual follow up of this reproductive effort which would be of great interest to help us to understand the physiology of the oysters facing summer mortalities. The most frequently used method for assessing the volume of the gonad is to measure the area it occupies on histological sections. Gonad measurement by magnetic resonance imaging, in contrast, is non-destructive and therefore makes it possible to assess individual evolution as well as to compare individuals. The present study compared the values of gonad volume by this method with the values of gonad surface obtained by the histological method. The two methods were used successively to assess the volume of the gonads of the same individual oysters at different maturation stages. There is a very highly significant linear relation between the results of these two methods. The estimated uncertainty about the volume value is 29%. Causes of this uncertainty are considered and discussed.

Highlights

► How to measure the gonad development of one oyster *Crassostrea gigas*? ► Gonad volume on MRI and gonad surface on histological slides are highly correlated. ► Uncertainty of the measure by use of MRI is estimated.

Abbreviations

ANOVA, Analysis of variance ; MRI, Magnetic resonance imaging ; ROI, Region of interest

Keywords : Oyster ; *Crassostrea gigas* ; Reproduction ; Histology ; Magnetic resonance imaging ; MRI

1. Introduction

Oysters are a major aquaculture species produced along the coasts of France. In 2010, the total French production consisted of 82 800 T Pacific oyster *Crassostrea gigas* and 1300 T European flat oyster *Ostrea edulis* (FranceAgriMer, 2011). However, since 2008, significant mortality has affected *C. gigas* spat in all French oyster production areas. Over the years, these high mortality rates have led to seed-supply problems in most shellfish farming areas. Moreover, both types of spat—hatchery and capture—are affected by the phenomenon. As resistance to summer mortality is a highly heritable character (Dégremont et al., 2010), genetic selection is underway to improve the survival of farmed oysters. A national program has been established that will select improved strains and distribute them to oyster farms. The diagnosis of the causes of these mortalities is based on the analysis of environmental factors, farming practices, occurrences of new pathogens, and oyster physiology (Samain et al., 2007, Huvet et al., 2010, Segarra et al. 2010).

The common practice for studying oyster biology in oyster beds is to sacrifice sampled oysters and make direct observation of their whole soft tissues and microscopic observation of histological sections. Consequently, oysters cannot be followed individually over time, which prevents the measurement of some physiological parameters and, more importantly, prevents the monitoring of individual phenotypes over the maturation period. A new technique has recently been tested that offers a non-destructive alternative to these methods. The first analyses of *C. gigas* by magnetic resonance imaging (MRI)(Davenel et al., 2006, Pouvreau et al., 2006a) concluded that the MRI technique is able to quantify the volume and mass of the whole flesh and of some organs in a non-destructive way, thereby allowing the observer to follow the growth of individual oysters over time. Considering the large investment oysters make in reproduction (Bourles et al., 2009, Deslous-Paoli and Héral, 1988, Pouvreau et al., 2006b) and the observation of selected oyster lines with different gonad average sizes and different susceptibilities to summer mortality (Samain et al., 2007, Huvet et al., 2010), the gonad was considered a priority organ for study. Further research using the MRI method highlighted a positive relationship between the effort devoted to reproduction and subsequent growth of the tissues and showed that growth variability within a group of oysters was overestimated by the more commonly used histological method (Hatt et al., 2009).

The most commonly used method for assessing the volume of the gonad is to measure the area it occupies on histological sections (Fabioux et al., 2005). The present study compared gonad measurement by MRI with gonad measurement by the histological method, applying the two methods successively to assess the volume of the gonads of the same individual oysters.

2. Materials and methods

2.1. Oysters

The 300 oysters used in this study were all members of a single bi-parental full-sib family, from a second laboratory generation (F2 family). Reproduction and larval rearing took place at the Ifremer hatchery in La Tremblade (Charente-Maritime, France) in February 2010. Post-larval rearing was also done at this hatchery. Afterwards, the oysters were grown on at the Ifremer nursery in Bouin (Vendée, France) from April to October 2010. They were then transferred to the Agnas area of the Marennes-Oléron oyster *parcs* (France) where they were reared at 50 cm from the ground in oyster bags, with approximately 150 oysters per bag.

2.2. Magnetic resonance imaging

These oysters were imaged on the PRISM facilities at Irstea (Rennes, Brittany, France) on April 4th, May 5th, and June 7th 2011. They were transported out of water between La Tremblade and Rennes (time of transport between 4 and 14 h) in coolers to limit temperature stress, covered with cotton clothes soaked in seawater. In clear seawater many oysters kept closed and could not expel air bubbles. Therefore, prior to MRI scanning, they were placed in vats containing phytoplankton-enriched seawater from the La Tremblade hatchery. After one hour, most of the oysters had opened their valves and had filtered water from the tray, thus expelling most of the air bubbles that had become trapped between their valves during transport. The few bubbles that remained in the oysters were clearly visible on the MR images and their volume was measured and corrected for when making oyster volume measurements. The epibionts were brushed from the shells and then the shells were dried with tissues to limit parasitic signals, without any other modification.

MRI measurements of oysters were performed with a Siemens Avanto imager operating at 1.5 T (63.86 MHz), equipped with a ~~h~~ead" probe, which allowed the simultaneous examination of 15 oysters placed on five levels. A T1-weighted 3D Gradient Echo sequence provided 52 contiguous images, highlighting animal tissues, particularly gonadic tissues.

The main parameters of the MRI protocol were:

- Dimensions of the observed field = 161.9 × 185 mm^2 (matrix: 280 × 320)

- Dimensions of the pixel on transverse cuts = 0.58 × 0.58 mm^2

- Distance between two successive transverse cuts = 1.5 mm, making the dimensions of the voxel 0.58 × 0.58 × 1.5 mm^3

- Repetition time = 11 ms; echo time = 3.01 ms; flip angle = 20°

- Number of transverse cuts across the volume = 52

- Filters = correction distortion + standard pre-acquisition + elliptical filter

- Time of acquisition for 52 sections = 12 min 56 s (number of excitations = 6)

An algorithm based on a 3D Object Counter plug-in using the ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD and available by ftp at zippy.nimh.nih.gov/ or http://rsbweb.nih.gov/ij/index.html) was used to extract an image stack of each oyster and calculate its grey-level stack histogram.

2.3. Histology

Forty two oysters were sampled the day after MR imaging, 10 on April, 10 on May and 22 on June, They were selected on view of MRI so the sample comprised males and females of various gonad size and development; no fully immature adults were observed. For each sample, histological sections were prepared as described in Fabioux et al. (2005). Briefly, 3-mm cross sections of the visceral mass were excised in front of the pericardial cavity and immediately fixed in Davidson's solution (Shaw and Battle, 1957) for 48 h. After dehydration and paraffin wax embedding, 5-µm sections were cut, mounted on glass sections, and stained with Harris' hematoxylin-Eosin Y (Martoja and Martoja-Pierson, 1967).

2.4. Qualitative analysis of histological sections

All the 42 sampled oysters were analysed. Sex and gamete maturation stages were determined under a light microscope according to the reproductive scale of Steele and Mulcahy (1999). Only the three stages detailed by Huvet et al. (2010) were observed. Briefly, stage 1 was assigned when there were no mature gametes, stage 2 was assigned when there were maturing gametes, and stage 3 was assigned when the gonad was full of mature gametes.

2.5. Analysis of images of histological sections

Histological sections were also scanned with a digital scanner (Hewlett Packard Scanjet 7400c), and images were saved in TIFF format. Tissue areas were measured using IMAQ Vision Builder image analysis software (National Instruments Corp, Austin, Texas, USA). Firstly, each image was transformed to greyscale. Then, a first region of interest (ROI) was drawn using a graphics tablet (Wacom Bamboo Pen and Touch, Wacom, Tokyo, Japan) to remove areas corresponding to gills and other peripheral tissues. The surface of this ROI was considered as the whole flesh section area. Finally, a second ROI was plotted to remove digestive gland cells. Gonadic cells were detected by adjusting the grey-level threshold specific for this tissue, and the gonadic area was automatically calculated in pixels (Heffernan and Walker, 1989).

2.6. Analysis of MRI images

For each of the 42 oysters, an image was chosen from the MRI scan that was as close as possible to the histological section made in front of the pericardial cavity, clearly visible on the images as a dark mass. This selected image was analysed using ImageJ software. Using the "Threshold" macro and the image of the histological section as a reference all pixels that appeared as part of the oyster flesh were selected. The threshold value was an average of 96 for all oysters. The number of pixels thus selected was then multiplied by the surface of the pixel (0.58×0.58 mm^2). This value was taken as the surface of the oyster flesh on the image.

In the "Threshold" macro, the lower and upper threshold values were adjusted to select pixels that could be considered as part of the gonad by reference to the histological section. However, these pixels were selected according to their grey-level, and some were clearly a part of other organs, mainly gills, gut, and digestive gland. A polygon was drawn around the gonad, once again by making reference to the histological section. The pixels within this polygon were considered as belonging to the gonad, and their number was multiplied by the unit surface of the pixel. Preliminary trials, done using the same method in previous years (Davenel et al., 2006, Hatt et al., 2009), concluded that the grey-level for mature gonads on MRI was between 166 and 510. On the grey-level histograms established on whole oyster body soft tissues with the methods described in subsection 2.2, all the voxels above 165 were counted as a part of the mature gonads. The volume of the gonad was then computed by multiplying the number of the selected voxels by the voxel unit volume ($0.58 \times 0.58 \times 1.5$ mm^3).

2.7. Data analysis

To determine relationships between the measurements made on the MR images and those made on histological sections, linear regressions were tested using the "linear model" function of the R software package (available at http://www.r-project.org/). The surface values measured on histological sections were used as explanatory variables, and the values from measurements on the MR images as variables to explain.

The residual values from the linear relation were analysed with a two-way analysis of variance (ANOVA) using the function "ANOVAs" of the R software package (R development core team, 2011) software. This allowed us to assess the contribution of the residual values of some factors such as the sex of the gametes and the maturation stage of the gonad to the variance.

3. Results

The results of the data analysis are given in Table 1; all relationships were established from measurements made on 42 oysters. The gross weights were 9 to 27 g and the total wet flesh volumes measured on MRI from 2.2 to 9.1 c.c.

Histological sections analysis led to the conclusion that, among the forty two oysters, 26 were males, 14 females and 2 undetermined. A fully mature gonad was observed on 28 oysters (16 males and 12 females), a mid-mature on 8 (6 males and 2 males), an incepting on 5 (4 males and one undetermined), and one showed no gonad.

Figures 1 to 3 show selected examples of histological sections and the MR images of oysters for each maturation stage, one without any visible gonad, one stage 1 (undetermined), one male and one female for stage 2 and for stage 3.

The grey-level of the female gonad is higher than that of the male gonad for both maturation stages. On the histological sections, vesicular cells are clearly distinct inside the gamete crown. In the same location on the MRI, the pixels have a grey-level that is higher than that of gamete pixels. The grey-level of the digestive tract is frequently amongst the highest levels on the MRI. This might be due to the phytoplankton that the oysters ingested when they were stored in phytoplankton-rich seawater before being passed through the scanner.

The whole body is clearly visible on the MR images. As a validation test for the whole body, we first tested the relation between the assessed surface on the histological section and that on the closest MR image. Because this relation was very highly significant, we then tested the relation between the gonad surfaces measured on the histological section and those on the closest MR image. Finally, because this relation was also very highly significant, the relation between the gonad surface measured on the histological section and the gonad volume measured on the whole MRI was then assessed.

The value of the Y-intercept of the last relation is significantly higher than zero. The linear relation between the gonad surface from the histological section and the gonad volume from the MRI is shown in Figure 4, with a point for each oyster. From this relation, we estimated that there was an uncertainty of 29% for the volume value measured by MRI ($p < 0.05$) on the scope of sampled sizes.

The gonad volume from the whole MRI corresponded to the number of voxels in which the grey-level was above 165. This included other organs, such as the vesicular cells and the digestive tract, observed on the MR image.

The volume of these organs varied among the maturation stages, and the grey-level of the gonad on the MR image was lower for males than it was for females. From these observations, the influence of maturation stage and sex was tested on the above linear relation. Two-way ANOVA on the residual values from this linear relation showed no difference between males and females or among maturation stages.

4. Discussion and Conclusions

The surface values obtained from transverse sections examine by the two methods were very highly correlated for both the whole body and the gonad alone. The volume of the gonad measured by MRI was very highly correlated with the surface of the gonad measured on the histological sections but, for small oysters in the first stage of gamete maturation, gonad volume measurements by MRI were proportionately higher than for large fully mature oysters: however this is based upon a small sample and needs a larger one for confirmation.

When the gonad was large, and therefore had a greater number of voxels, the contrasts between organs were better and the effects of small volumes added by the partial inclusion of other organs became significantly less important. In addition, the size of a pixel (0.58 × 0.58 mm^2) on the MR image was much larger than the size of a pixel on the image of the histological section (0.042 × 0.042 mm^2). On the MRI, a pixel could consist of mature gametes and somatic cells; as such, its grey-level was higher than the surrounding pixels consisting of only somatic cells, and the pixel was therefore recorded as "mature gonad". However, this approach gave an overestimation of the volume of gametes. On the histological section, it was possible to distinguish mature gametes from other cells. On the MRI, the accuracy of the image did not always make it possible to distinguish the mature gonad from other organs, such as vesicular cells or digestive tract, which frequently had a similar grey-level and were adjacent to the mature gonad.

As indicated above, the gonad volume assessed from the MRI was the number of voxels with a grey-level above 165. But the observation on successive transverse cuts led us to conclude that these measurements had included tissues other than the gonad because they had a similar grey-level (e.g., vesicular cells, sometimes gills or digestive tract). In the linear relationship between the gonad volume from the MRI and the gonad surface from the histological section, the Y-intercept was very high, and the average gonad volume was 20 times that of the average gonad surface. It can therefore be hypothesized that the high values observed were related to additional volume from the inclusion of these somatic organs.

Measurement of the surface of the gonad on histological sections is a reference method for assessing reproductive effort and its evolution over time within a population of oysters. The linear relationship established between the value obtained by this method and the value obtained by MRI demonstrates that this last method is appropriate for computing a volume that is proportionate to the actual gonad volume. It is non-destructive (MRI was conducted with shell intact) and therefore makes it possible to assess individual evolution and to compare individuals.

A first estimation of the uncertainty for this volume value was 29% on the scope of sampled sizes. Further measurements will be used to validate this first estimation and to test a possible difference according to maturation stage and/or sex. However, this method could already be very useful for finer analysis of some phenotypic characteristics of oysters within a maturation and spawning period, namely, the effort devoted to reproduction, and the precocity and duration of gonad maturation. This individual examination will also allow the study of gonad development to be put in its genetic context through attempts to link some of these traits to parts of the oyster genome using a Quantitative Trait Loci approach.

Acknowledgements

This work was funded by a grant from the Agence Nationale de la Recherche ~~«~~Génomique animale" program for the GAMETOGENES project (ANR-08-GENM-041). The authors are grateful to all members of staff of the Ifremer hatchery in La Tremblade and of the nursery in Bouin for producing and rearing the oysters and would particularly like to thank Virgile Quillien for his contribution to making and analysing the histological sections.

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Tables

Table 1 : Parameters of linear regressions between measurements made on the histological sections (explanatory variable) and on the MRI (explained variable) (***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; n.s.: not significant). These relations are based upon a sample of 42 oysters of different sex (2 undetermined, 26 males and 14 females) and different maturation stages (1 immature, 5 at stage 1, 8 at stage 2 and 28 at stage 3).

Parameters	Value	Standard error	Student's ***t***	P (>|t|)
Whole-body surface (mm^2)				
Y-intercept	93	12	7.4	$5e^{-09}$ ***
Regression coefficient	0.57	0.05	10.7	$2e^{-13}$ ***
F Fisher	115***			
Gonad surface (mm^2)				
Y-intercept	11	6	1.9	0.06 n.s.
Regression coefficient	0.65	0.04	14.7	$<2e^{-16}$ ***
F Fisher	216***			
Gonad surface on histological sections (mm^2) and gonad volume on MRI (mm^3)				
Y-intercept	1345	194	6.9	$2e^{-08}$ ***
Regression coefficient	14.3	1.6	9.2	$2e^{-11}$ ***
F Fisher	84***			

Figures

Figure 1 : Histological sections (left) and closest original MR image (right) of an oyster at maturation stage 1 (upper) and an immature oyster (lower).

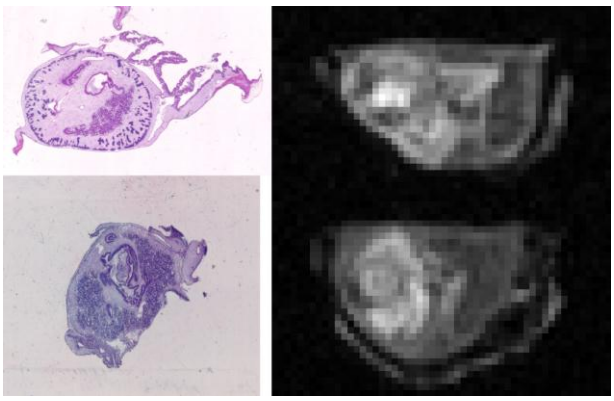

Figure 2: Histological sections (left) and closest original MR image (right) of oysters at maturation stage 2, male (upper) and female (lower).

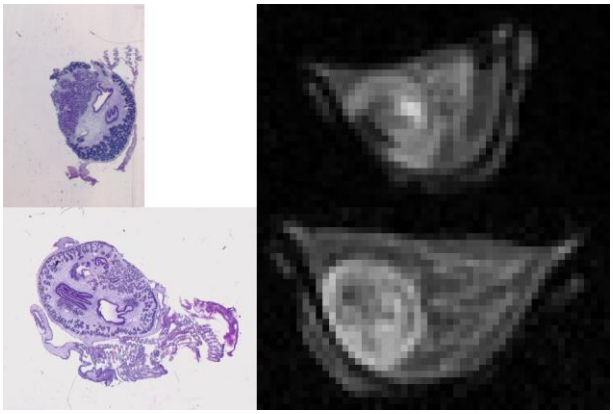

Figure 3 : Histological sections (left) and closest original MR image (right) of oysters at maturation stage 3 (upper) , male (upper) and female (lower).

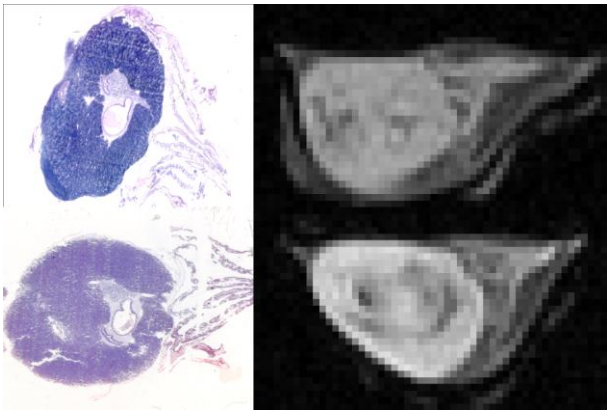

Figure 4: Relation between the gonad surface measured on the histological section and the gonad volume measured on the whole MRI. The different symbols indicate the different gonad maturation stages observed on the histological sections.