



Universidade de São Paulo

Biblioteca Digital da Produção Intelectual - BDPI

Departamento de Ciências de Computação - ICMC/SCC

Comunicações em Eventos - ICMC/SCC

2015-10

Automatic counting and measuring fish oocytes from microscopic images

Workshop de Visão Computacional, XI, 2015, São Carlos.

<http://www.producao.usp.br/handle/BDPI/49483>

Downloaded from: Biblioteca Digital da Produção Intelectual - BDPI, Universidade de São Paulo



Laboratório de Visão Computacional



XI Workshop de Visão Computacional
October 05th - 07th 2015 - São Carlos (SP) - Brazil



**XI Workshop de Visão Computacional
WVC'2015**

October 05th – 07th, 2015

São Carlos – SP - Brazil

Proceedings

University of São Paulo
São Carlos School of Engineering

Automatic counting and measuring fish oocytes from microscopic images

Jonathan S. Ramos*, Agma J. M. Traina*, Carolina Y. V. Watanabe[†]
 Diogo Hungria^{§¶}, Talles Colaço-Fernandes^{‡¶} and Carolina R. C. Doria[¶]

* Instituto de Ciências Matemáticas e Computação - ICMC - USP, São Carlos, São Paulo, 13566-590
 Email: jonathan@usp.br and agma@icmc.usp.br

[†] Departamento de ciências da computação, UNIR, Porto Velho, Rondônia, 76808-659

[‡] Programa de Pós-Graduação em Biologia de Água Doce e Pesca Interior - BADPI, INPA, Manaus, Amazonas, 69060-001

[§] Programa de Pós-Graduação em Zootecnia, UFPR, Curitiba, 80035-050

[¶] Laboratório de Ictiologia e pesca - LIP, UNIR, Porto Velho, Rondônia, 78.900-000

Abstract—Fish oocyte counting and measuring is an important step for estimating fecundity, which is normally done by analyzing oocytes's diameters frequency distribution, taking a long time to be done manually and is a laborious task. Oocytes digital images segmentation can make the process faster and more precise. However, proposed methods to address this problem do not deal with overlapped oocyte or tissues and need image processing techniques knowledge to be used. In this paper, we propose a new pipeline to deal with overlapped oocytes so that no image processing knowledge is required for the user. We have used well-known image processing techniques, such as Otsu's method, Watershed transform, mathematical morphology and regional minimum. Experiments using fish samples from Madeira river show that the proposed pipeline has a huge potential to aid in the task of counting and measuring oocytes. The time spent in this task using the pipeline is twice faster than manual analysis.

Keywords—Fish oocytes, computer vision, image segmentation, Otsu's method, watershed transform.

I. INTRODUCTION

Fish oocyte counting and measuring is a very laborious task, which consists of looking at oocytes in a stereoscopic and measuring them. It is used to estimate natural fisheries stocks, which leads to information about the fecundity [20].

Over hundreds studies use the fish fecundity analysis to describe the reproductive strategies and the assessment of fecundity [7], [11], [20], it can not only elucidate the basic biology of the fishes but also help in their management and conservation [7]. It is a fundamental topic in the study of the biology and population dynamics of fish species [6]. Therefore, it plays a really important role in fishing biology [11].

Fecundity is generally estimated using the gravimeter method, in which fecundity is the product of the weight of ovary and fish oocytes per gram of ovary tissue [18]. Another way is to measure each oocyte diameter and analyze its frequency distribution [11]. If the frequency distribution has two peaks, i.e., two population of oocytes at different development stage, we have a total spawning specie. If there are more than 2 peaks, we have a batch spawning [11], which can be seen as a reproductive strategy that increases the survival probability [9].

In this paper, we have used samples (fishes) acquired from Madeira river (Amazon river biggest tributary), where a Hydro-

electric power station¹ is being built and therefore it is needed to analyze the biology and ecology ichthyofauna before and after the dam implementation to promote conservation actions.

A pipeline to count and measure oocytes in microscopic images is proposed in this paper. We have used well-known techniques, such as Otsu's method, Watershed transform, mathematical morphology and regional minimum to address the issue of overlapped eggs and tissues. It is made in an automatic way so that biologists specialists do not need any image processing knowledge. Moreover, the process is standardized, so that subjective analysis made by specialist are discharged.

The remainder of this paper is organized as follows. In Section II, we describe related works. In Section III, we describe our proposed pipeline. In Section IV, we describe the experimental results. In Section IV we present the conclusion.

II. RELATED WORK

Only a few methods for automatic counting and measuring oocytes have been described previously [2], [19], [18], [4]. Most of them do not deal with overlapped oocyte or tissues in the images. They normally ignore this issue by using the *roundness* value of segmented oocytes.

In 1967 was proposed a machine which only counts oocytes with diameter between 200 – 2500 μ m [2]. An automatic particle counter is proposed in [19], which seems to be precise and accurate. However, a particle counter is expensive, needs extensive calibration and it is not a standard equipment in most biology labs. Moreover, the oocytes have to be perfectly separated, which is laborious and takes a long time [19].

Image processing techniques are used in [18] to measure oocytes. However, they ignore overlapped oocytes using the roundness value [18]. In [4] is used mathematical morphology for segmenting touching oocytes in the image, which in most cases does not segment properly [4]. In [8] is proposed a low-cost laboratory setup. They use ImageJ[®] [1] to automatic count particle (oocytes) in the image. However, they only uses Watershed transform to separate overlapping particles, which does not always gives appropriated results. Besides,

¹Santo Antônio Dam, located in Porto Velho in the state of Rondônia, will contribute to Brazil's growth. <http://www.santoantonioenergia.com.br/en/>

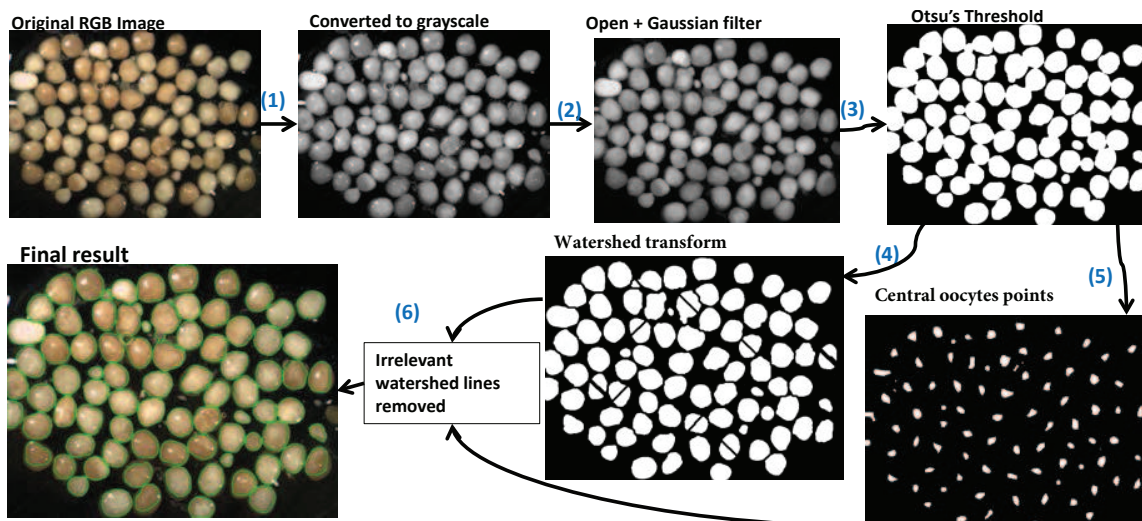


Fig. 1. Proposed pipeline for oocyte counting and measuring in digital images.

to use ImageJ it is necessary some knowledge about image processing, which most biologists do not have.

III. PROPOSED PIPELINE

In Figure 1 is shown the pipeline proposed our work. First, the image is converted to gray-scale (Section III-A), then morphological and Gaussian filters to remove noise (Section III-B). Afterwards, the image is segmented using Otsu's threshold (Section III-C) and Watershed transform is applied to separate overlapping and touching oocyte (Section III-D). Finally, irrelevant Watershed lines are removed based on central oocytes points, which are estimated using regional minimum and local thresholds (Section III-E).

A. Converting original image to gray-scale

The oocyte images are in RGB format, as color components are not relevant in this work, the image is converted to gray-scale using Equation 1 [13]:

$$I_g = 0.2989 * R + 0.5870 * G + 0.1140 * B \quad (1)$$

where R is the red channel, G is the green channel, B is the blue channel and I_g is the resulting image in gray-scale.

B. Morphological opening and Gaussian filter

The oocytes images presents noise introduced by the camera and gonadal tissues [21], which are removed applying an average filter (size 5×5) and a morphological opening (in a shape of a disk with size of 15 pixels). These filters were used for noise removal in images from blood cells [17], [14] and, as shown in Fig. 2, presented suitable results for our work.

C. Otsu's method

Otsu's method exhaustively searches for the threshold that minimizes the intra-class variance, defined as a weighted sum of variances of two classes [12]:

$$\sigma_w^2(t) = \omega_1(t)\sigma_1^2(t) + \omega_2(t)\sigma_2^2(t), \quad (2)$$

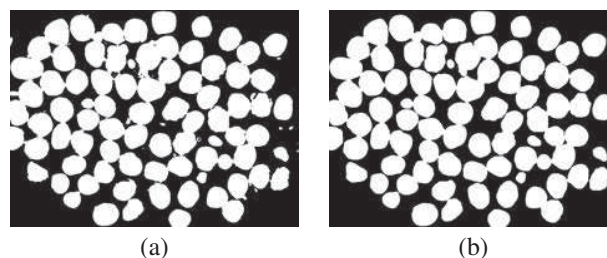


Fig. 2. Otsu method: (a) without applying any previous filter; (b) Gaussian and morphological opening previously applied.

weights ω_i are the probabilities of the two classes separated by a threshold t and σ_i^2 are variances of these classes. In Fig. 2 is shown the results of Equation 2 applied to two images.

D. Watershed transform

Watershed transform correctly separates overlapping oocyte after applying Otsu's method. However, it also over-segments a few oocytes, as is shown in Fig. 3. It is highly sensitive to local noise in Euclidean Distance Map - EDM [16].

To detect Watershed over-segmented lines, central region of oocytes are estimate [16], [15], as presented in Section III-E.

E. Calculating fish oocytes central points

To estimate central oocytes regions, local threshold and regional minimum are used. Local thresholds do not detect all the central oocyte regions, on the other hand, regional minimum can detect all of them, but in smaller size.

Watershed over-segmented lines do not always pass exactly through the center of the oocytes. Therefore, regional minimum works as a complement to the local threshold, giving the central regions that were not detected.

1) *Local threshold*: To get all the regions in the image, an Euclidean Distance Map (EDM) is calculated [3], and then a local threshold is calculated in a each region using Equation 3;

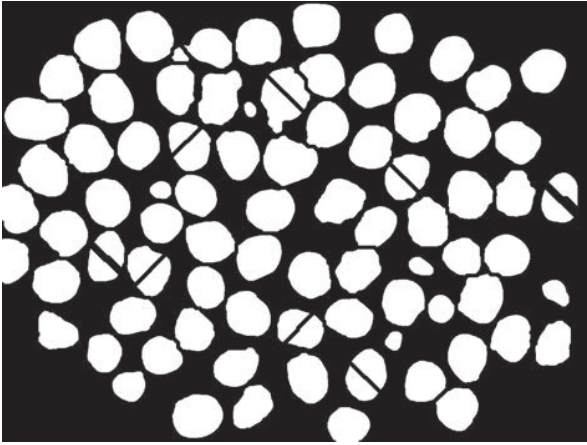


Fig. 3. Watershed transform applied on Fig. 2(b) to separate overlapping oocytes (a morphological erosion was applied for better visualization).

$$t = \mu + x \times \sigma \quad (3)$$

where t is the threshold to be applied on that region, μ and σ are, respectively, the mean and the standard deviation different of zero on that region, and x is a constant.

We have empirically set x as 1.5. In Fig. 4 is shown the results for distinct values of x applied over Fig. 3. In Fig. 5 is shown the results for different images with $x = 1.5$.

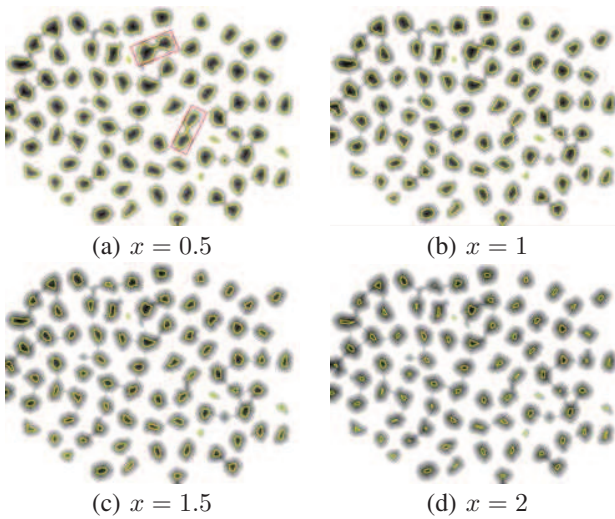


Fig. 4. Result of Equation 3 applied on the same image with distinct x values. The red rectangles show oocytes overlapped.

As shown in Fig. 4, as the value of x increases, the central regions size decrease and vice-versa. However, if the value of x is lower than 1, it is likely that a few oocytes remain overlapped. Therefore, based on these results, the x value in Equation 3 is set to 1.5.

In Fig. 5 is shown Equation 3 applied on different images and with the same x value. None of the fish oocyte is left overlapped. However, the central regions of a few oocytes are not detected. Thus, in order to detect these undetected oocytes, we used the regional minimum technique [5].

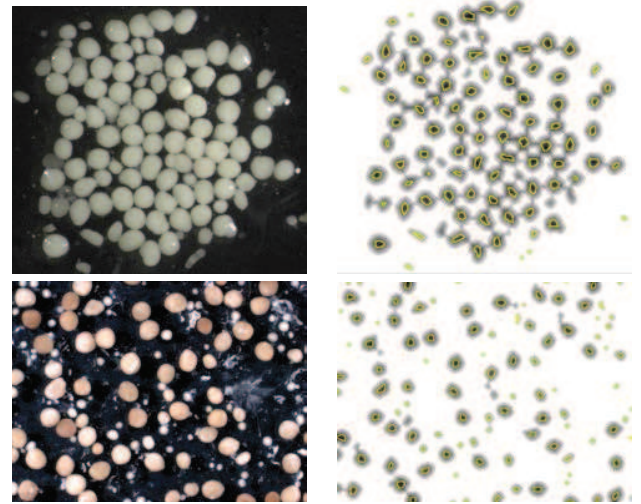


Fig. 5. Result of Equation 3 applied on different images with $x = 1.5$.

2) *Regional minimum*: The regional minimum is also calculated on the EDM (Euclidean Distance Map). In order to get bigger central points, morphological dilation previously is applied to the EDM, as shown in Fig. 6(b). In Fig. 6(a) is shown the results of regional minimum over the EDM without applying the dilation filter.

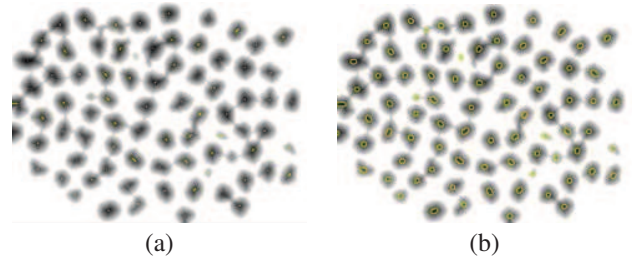


Fig. 6. Central points calculated using regional minimum over the EDM: (a) without applying any filter; (b) morphological dilation previously applied.

F. Removing irrelevant Watershed lines

We join the results from local threshold and regional minimum to remove irrelevant Watershed lines using Equation 4:

$$I_{cp}(x, y) = \begin{cases} 1 & \text{if } A(x, y) = 1 \text{ or } B(x, y) = 1, \\ 0 & \text{otherwise} \end{cases} \quad (4)$$

where A is the resulting binary image of local threshold, B is the resulting image of regional minimum and I_{pc} is the result of a logic OR function between A and B . Therefore, all the central points are detected as is shown in Fig. 7(b).

As shown in Fig. 7(b), local threshold does not detect all the central points; regional minimum detects all central regions. However, a few central regions are too small. The central regions have to be as big as possible, since irrelevant Watershed lines do not always cut the oocytes exactly in the middle. To better illustrate the problem, in Fig. 8(a) is shown the results of the regional minimum and the Watershed lines. In Fig. 8(b) is shown the final result.

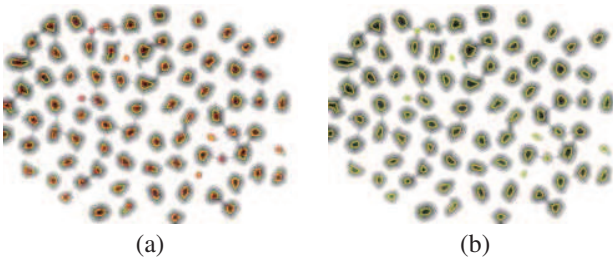


Fig. 7. Logic OR function applied on two binary images: (a) in yellow, central points detected by local threshold; in red, central points detected by regional minimum (b) final result obtained by logic OR.

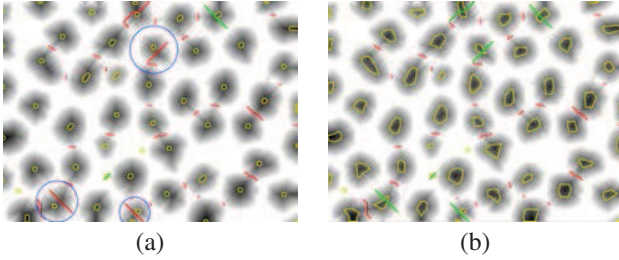


Fig. 8. For both images red lines represents Watershed lines, green lines represent irrelevant lines detected and yellow lines represents central points; (a) Regional minimum: blue circles indicate irrelevant lines not detected. (b) final result using the local threshold and regional minimum, all irrelevant Watershed lines are detected.

When irrelevant Watershed lines are removed, we get the result shown in Fig. 9.

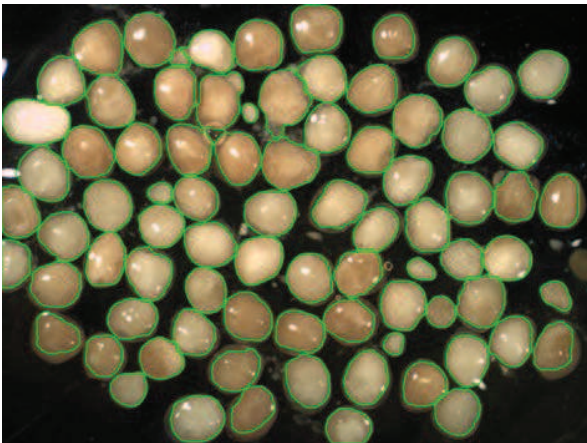


Fig. 9. Final oocyte segmentation result.

IV. EXPERIMENTS

We have used a 1,3 megapixels (AxioCam - ICc1 Zeiss) stereomicroscope for the image acquisition. The magnification varied to suit the range of oocytes in the image. The images are in RGB format and has resolution of 1388×1038 pixels.

The oocytes were taken from Madeira river's fishes. Four different species with different reproductive strategies were analyzed: *H. incognitum* and *P. nattereri* with batch spawning; *P. latior* and *P. nigricans* with total spawning. Total spawning is defined when a specie presents two populations of oocytes

diameters, i.e., when there are two peaks in the oocytes diameter frequency distribution. On the other side, a specie with batch spawning presents three or more well-defined peaks.

In total, 20 samples (fishes) were used and 147 distinct images were taken. In Table I is shown the amount of images per sample taken. For each sample it was taken a representative percentage of its ovary, and then kept for a month in Gilson's solution [10], which breaks the ovarian tissue and make possible to analyze the oocytes, in a Petri dish, through a stereomicroscope.

TABLE I. FOR EACH ONE OF THE FOUR SPECIES WERE USED 5 SAMPLES (FISHES) (A, B, C, D, E), SUMMING UP TO 20 SAMPLES.

Specie	Sample					Sum (images)
	A	B	C	D	E	
<i>H. incognitum</i>	4	7	15	14	10	50
<i>P. latior</i>	6	3	10	13	10	42
<i>P. nattereri</i>	4	7	7	10	7	35
<i>P. nigricans</i>	3	4	6	3	4	20
Total						147

As shown in Table I, there is not a fixed number of images for each sample. The amount of images per sample depends on its gonadal sample size, which is empirically defined by the specialist for each sample.

Counting results are presented in Section IV-A, where we used only the images taken. Measuring results are presented in Section IV-B, where we compare automatic and manual measuring.

A. Counting results

To validate our segmentation pipeline we counted the amount of oocytes in each image (ground-truth). We defined which segmented oocytes may or may not be used for analysis. Bad segmented oocytes, tissues or oocyte cut by the image boundary are not relevant for measuring analysis. In Fig. 10 it is shown the counting results for each specie

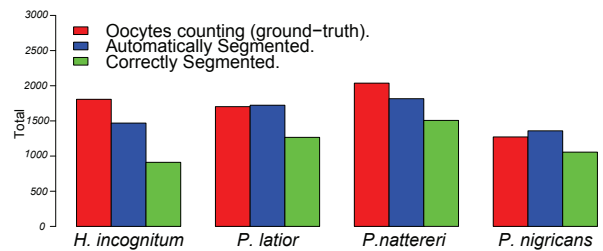


Fig. 10. Oocytes counting for *H. incognitum*, *P. latior*, *P. nattereri* and *P. nigricans* manually counted, i.e., ground-truth (red), automatically segmented (blue) and correctly segmented (green).

As shown in Fig. 10, in species *H. incognitum* and *P. nattereri* it is segmented less oocytes than there really are. This is caused due to diversity of oocytes sizes in the same image (batch spawning), as shown in Fig. 11, too small oocytes are not detected among bigger oocytes. Also, a few oocytes were bad segmented and, consequently, should not be considered for further analysis. On the other hand, for species *P. latior* and *P. nigricans*, slightly more oocytes were segmented than there really were. This is due to tissues in the image (Fig. 12).

For all species, even though a oocyte is correctly segmented, it might not be relevant, since the oocyte is deformed

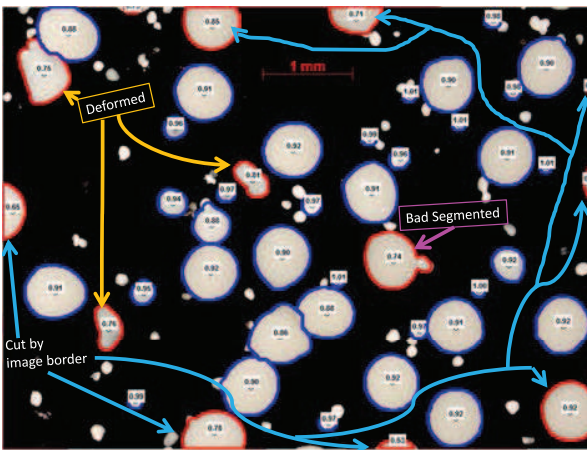


Fig. 11. Considered (blue) and not-considered oocytes (red). Non-considered; deformed, bad segmented or cut by image border. The roundness value are shown in the middle of each oocyte.

from its original shape, which means it is not useful for measuring analysis. Moreover, oocytes cut by the image border are also not relevant, as can be seen in Fig. 11.

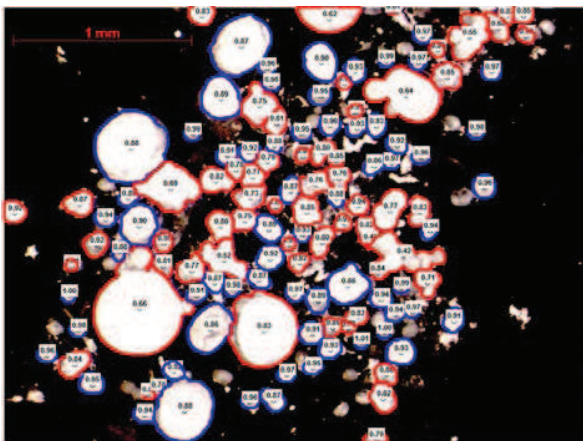


Fig. 12. Image with tissues from specie *P. nigricans*.

B. Measuring results

Manual measuring is done by eye-sight through a stereoscopic using a melimetric rule. Automatic measuring is done on segmented oocytes in previous step (Section IV-A). In Fig. 13 we compare the amount of oocytes measured in each method. Automatic measuring allows to measure, in average, more oocytes, since it is easier and faster. Besides, automatic measuring is more precise. In manual measuring, oocytes sizes are rounded, e.g., 0.1 mm, 0.2 mm, and so on, while automatically it is properly measured.

To compare automatic and manual measuring frequency distributions, samples from the same specie are grouped, since they work as a replica to the analysis. In Fig. 14 are compared the measuring results for species with batch spawning and in Fig. 15 species with total spawning.

In Fig. 14(a), manual measuring has the expected result (batch spawning) for both specie. For automatic measuring,

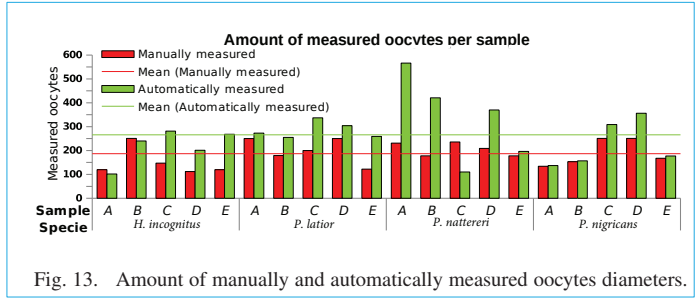


Fig. 13. Amount of manually and automatically measured oocytes diameters.

the frequency distribution does not match the expected result, it shows only 2 peaks. Too little oocytes bigger than 1 mm were automatically measured, leading to different results. In Fig. 14(b) there are three peaks, which matches the expected result. For automatic measuring, too little oocytes bigger than 1.4 were measured, leading to different results.

In Fig. 15 is shown comparison of automatic and manual frequency distributions for total spawning species. In Fig. 15(a) the frequency distribution for automatic measuring does not agree to the expected spawning type (total), which normally presents two peaks in the distribution. However, in Fig. 15(b), the same happens to the manual measuring. Probably, for both methods, ovary tissues fragments were counted as oocytes.

Automatic measuring is estimated to be twice faster than manual measuring.

V. CONCLUSION

In this paper, we introduced a new pipeline for fish oocytes digital images segmentation, in which we have used well-known techniques, such as Otsu’s method, Watershed transform, mathematical morphology and regional minimum. These methods well combined deal with overlapped oocytes or tissues in the image with promising results.

In automatic measuring the error is standardized, meaning that the results is always the same for any specialist who measures. This does not hold for manual measuring, the results is affected, subjectively, by who is measuring.

The proposed pipeline makes the process faster than the manual analysis. Experiments made by biologists specialists, using fish samples from Madeira river, show that the proposed pipeline has got a huge potential to aid in the task of counting and measuring oocytes. The time spent in this task using the pipeline is twice faster than manual approach. Besides, it is a lot more precise than manual measuring.

ACKNOWLEDGMENT

The authors acknowledge CNPq (National Council for Scientific and Technological Development) for providing his grant. #350251/2014-2, and also Professor Dr. Carlos Tenório for making the link between the Biological and Computer Science Departments.

REFERENCES

[1] D. M. D. Abrmoff, D. P. J. Magalhães, and D. S. J. Ram, “Image processing with imagej,” *Biophotonics International*, vol. 11, no. 7, pp. 36–42, July 2004.

[2] H. C. Boyar and R. A. Clifford, “An automatic device for counting dry fish eggs,” *Transactions of the American Fisheries Society*, vol. 96, no. 3, pp. 361–363, 1967.

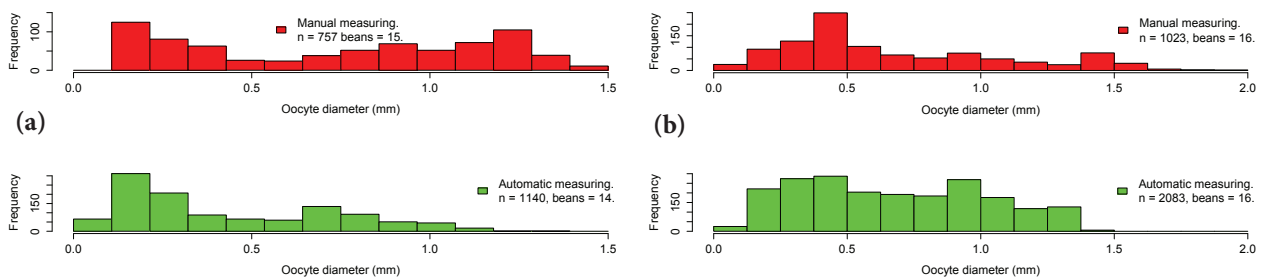


Fig. 14 Oocytes diameters for (a) *H. inopitum* and (b) *P. nattereri* manually measured (red) and automatically (green).

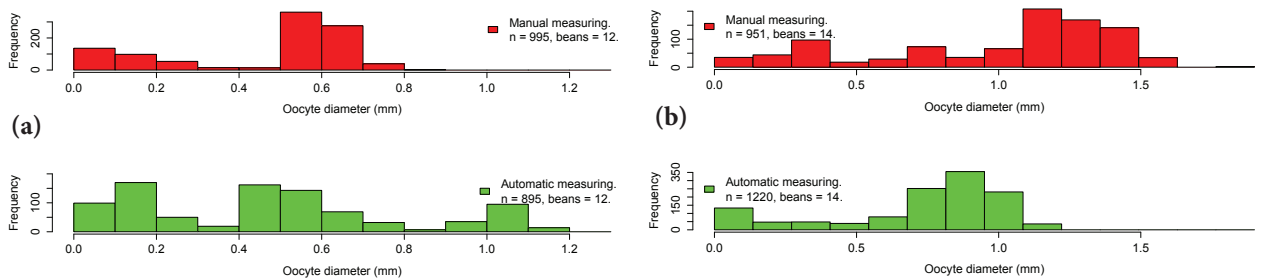


Fig. 15 Oocytes diameters for (a) *P. latior* and (b) *P. nigricans* manually measured (red) and automatically (green).

[3] G. M. Faustino, M. Gattass, S. Rehen, and C. J. P. de Lucena, "Automatic embryonic stem cells detection and counting method in fluorescence microscopy images," in *Proceedings of the Sixth IEEE International Conference on Symposium on Biomedical Imaging: From Nano to Macro*, ser. ISBI'09. Piscataway, NJ, USA: IEEE Press, 2009, pp. 799–802.

[4] K. D. Friedland, D. Ama-Abasi, M. Manning, L. Clarke, G. Kligys, and R. Chambers, "Automated egg counting and sizing from scanned images: Rapid sample processing and large data volumes for fecundity estimates," *Journal of Sea Research*, vol. 54, no. 1, pp. 307–316, 2005.

[5] R. González and R. Woods, *Digital image processing*. Pearson/Prentice Hall, 2008.

[6] J. Hunter, B. Macewicz, N. LO, and C. Kimbrell, "Fecundity, spawning, and maturity of female dover sole, with and evaluation of assumptions and precision," *Fish Bull.*, vol. 90, no. 1, pp. 101–128, 1992.

[7] M. Jan, U. Jan, and G. M. Shah, "Studies on fecundity and gonadosomatic index of schizothorax plagiostomu (cypriniformes: Cyprinidae)," *Journal of Threatened Taxa*, vol. 6, no. 1, pp. 5375–5379, 2014.

[8] N. Klibansky and F. Juanes, "Procedures for efficiently producing high-quality fecundity data on a small budget," *Fisheries Research*, vol. 89, no. 1, pp. 84 – 89, 2008.

[9] T. C. Lambert and D. M. Ware, "Reproductive strategies of demersal and pelagic spawning fish," *Canadian Journal of Fisheries and Aquatic Sciences*, vol. 41, no. 11, pp. 1565–1569, 1984. [Online]. Available: <http://dx.doi.org/10.1139/f84-194>

[10] S. K. Lowerre-Barbieri and L. R. Barbieri, "A new method of oocyte separation and preservation for fish reproduction studies," *Fishery bulletin-national oceanic and atmospheric administration*, vol. 91, pp. 165–165, 1993.

[11] H. Murua, G. Kraus, F. Saborido-Rey, P. R. Witthames, and S. J. A. Thorsen, "Procedures to estimate fecundity of marine fish specie in relation to their reproductive strategy," *Journal of Northwest Fisheries Science*, vol. 33, no. 1, pp. 33–54, Jan. 2003.

[12] N. Otsu, "A threshold selection method from gray-level histograms," *IEEE Transactions on Systems, Man and Cybernetics*, vol. 9, no. 1, pp. 62–66, 1979.

[13] W. K. Pratt, *Digital Image Processing: PIKS Inside*, 3rd ed. New York, NY, USA: John Wiley & Sons, Inc., 2001.

[14] C. D. Ruberto, A. G. Dempster, S. Khan, and B. Jarra, "Analysis of infected blood cell images using morphological operators," *Image Vision Comput.*, vol. 20, no. 2, pp. 133–146, 2002.

[15] J. Russ, *The Image Processing Handbook, Sixth Edition*. Taylor & Francis, 2011.

[16] J. C. Russ, *Image analysis of food microstructure*. CRC Press, 2005.

[17] F. B. Tek, A. G. Dempster, and I. Kale, "Parasite detection and identification for automated thin blood film malaria diagnosis," *Comput. Vis. Image Underst.*, vol. 114, no. 1, pp. 21–32, Jan. 2010.

[18] A. Thosen and O. Kjesby, "A rapid method for estimation of oocyte size and potential fecundity in atlantic cod using a computer-aided particle analysis system," *Journal of Sea Research*, vol. 46, no. 1, pp. 295–308, 2001.

[19] P. R. Witthames and M. G. Walker, "An automated method for counting and sizing fish eggs," *Journal of Fish Biology*, vol. 30, no. 3, pp. 225–235, 1987.

[20] P. J. Wright, "Methodological challenges to examining the causes of variation in stock reproductive potential," *Fisheries Research*, vol. 138, no. 0, pp. 14 – 22, 2013.

[21] Q. Wu, F. Merchant, and K. Castleman, *Microscope Image Processing*. Elsevier Science, 2010.