

# Digital image analysis to estimate the minimum number of *Eurytrema coelomaticum* eggs in the uterus of adult specimens

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## Article info

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## Summary

This study was carried out to estimate the minimal number of eggs present in adult *E. coelomaticum* uterus. Samples were collected during post-mortem inspection and were submitted to light microscopy (bright field). The length, width, the total area of the parasite, uterus, and eggs were measured. The ImageJ software was used to calculate the area of the different parameters analyzed in this study. It was possible to observe that the uterus corresponds on average to 51.9 % of the total area of the parasite (ranging from 45 to 64 %). The number of eggs present in the uterus of parasites ranged from 5,946 to 15,813. To estimate the number of eggs three scenarios were considered, where the first taken into account the number of whole eggs observed in the image. In the second way to estimate the number of eggs, all the structures were considered (whole eggs and fractions that could be delimited) and compared with manual counting. Finally, in the last scenario, was considered an occupancy rate of 100 % of the uterine area per eggs, since there are overlapping eggs and these cannot be correctly delimited and accounted for. This study describes an important tool for quantifying eggs in a nondestructive manner and aggregate information until then is not explained by other works.

**Keywords:** Trematoda; Dicrocoeliidae; ImageJ

## Introduction

The digenetic trematode *Eurytrema coelomaticum* (Giard & Billet, 1892) Looss, 1907 is parasite of pancreatic ducts of ruminants and are endemic in several regions of the world, including countries of South America, Europe and Asia (Sakamoto *et al.*, 1981; Ilha *et al.*, 2005; Sakamoto & Oikawa, 2007; Quevedo *et al.*, 2013). In Brazil, this helminth is widely distributed and commonly found in slaughterhouses in several states (Azevedo *et al.*, 2004; Bassani *et al.*, 2006) The life cycle is heteroxenic, requiring two intermediate hosts and another definitive. The primary intermediate host is the mollusk *Bradybaena similis* (Férussac, 1821) (Gastropoda, Xanthonychidae) (Ilha *et al.*, 2005). In Brazil, the second

intermediate host is a grasshopper of the genus *Conocephalus* (Thunberg, 1815) (Orthoptera, Tettigoniidae) and in some regions of Asia the cricket belongs to the genus *Oecanthus* (Serville, 1831) (Orthoptera, Gryllidae) (Ilha *et al.*, 2005; Pinto & De Melo, 2013, 2016). Finally, the definitive hosts become infected upon ingestion of infected arthropods with metacercariae when feeding on grazing, thus completing the cycle (Bassani *et al.*, 2007).

The eurytrematosis is clinically asymptomatic, but is a silent disease which causes losses in milk and meat production. When it considered in a little number of animals these losses are diluted and not clearly visible. But, when you consider a loss of 1 l of milk or 1 kg of meat production per animal in a cattle with 10,000 animals this is very significant in an economic point of view. Although

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it is commonly found infecting herds in Brazil, there are no calculations about the impact of these organisms on national livestock production, with estimated costs varying from 1 to 3 % of the total amount (Ilha *et al.*, 2005). Is associated with hyperplasia and hypertrophy of the pancreas and bile ducts, as well as fibrosis and partial or total obliteration of the pancreatic ducts when in massive infections (Rachid *et al.*, 2011; Figueira *et al.*, 2014; Schwertz *et al.*, 2016).

The World Association for the Advancement of Veterinary Parasitology (WAAVP) recommends performing count of eggs per gram of faeces (EPG) to assess the intensity of parasitic infection of various trematodes species (Wood *et al.*, 1995). Observation of the EPG levels is largely recommended and is historically used as a diagnostic and monitoring tools in the control of parasites of veterinary importance, supporting in the treatment and mainly in prophylaxis (Bassani *et al.*, 2007; Wood *et al.*, 1995). Although it is an important tool for the evaluation of worm eggs, in general, the technique presents values underestimated and can in some cases compromise the treatment and the prophylaxis, fact that reinforces the importance of the present study. Gassó *et al.* (2015) have shown that depending on the location where the parasite is situated the eggs prevalence has decreased due to the routes through which the eggs must pass until reaching the intestine. They further cite that accuracy has declined over time and therefore it is recommended that samples be processed within 5 days after collection. The *E. coelomaticum* eggs are oval with a coloration ranging from

yellowish to brownish when immature and brown when fertile (Rachid *et al.*, 2011; Pinheiro *et al.*, 2015). Egg size ranges from 39 to 50  $\mu\text{m}$  in length by 20 to 32  $\mu\text{m}$  in width (Yamamura, 1989; Mohanta *et al.*, 2015; Pinheiro *et al.*, 2015).

Yamamura (1989) analyzing the oviposture of *E. coelomaticum* (*in vitro*) observed an average daily of 641 eggs. Brandolini and Amato (2001) testing different physiological media observed that Locke solution presented the best oviposture rate with an average of  $746 \pm 139$  eggs, followed by Earle solution with  $304.6 \pm 154.7$  and finally the saline solution to 0.85 % with  $194.5 \pm 132$  eggs. About this information, there are no records in the literature of estimate number of eggs present in the uterus of *E. coelomaticum*.

The use of techniques such as the analysis of digital images makes possible the estimation of eggs present in an adult animal and thus aggregates information about its fecundity and epidemiology (Rosati *et al.*, 2015; Mains *et al.*, 2008). In this work, we describe methodologies that were used to calculate the mean uterine size, the proportion of the organ in relation to the total size of the animal, as well as to estimate the number of eggs of *E. coelomaticum* according to the occupancy rate of the parasite uterus.

## Materials and Methods

Samples of *E. coelomaticum* were obtained during the post-mortem inspection at the slaughterhouse in the city of Campo Belo, Minas Gerais, Brazil. The samples were processed at the Labo-

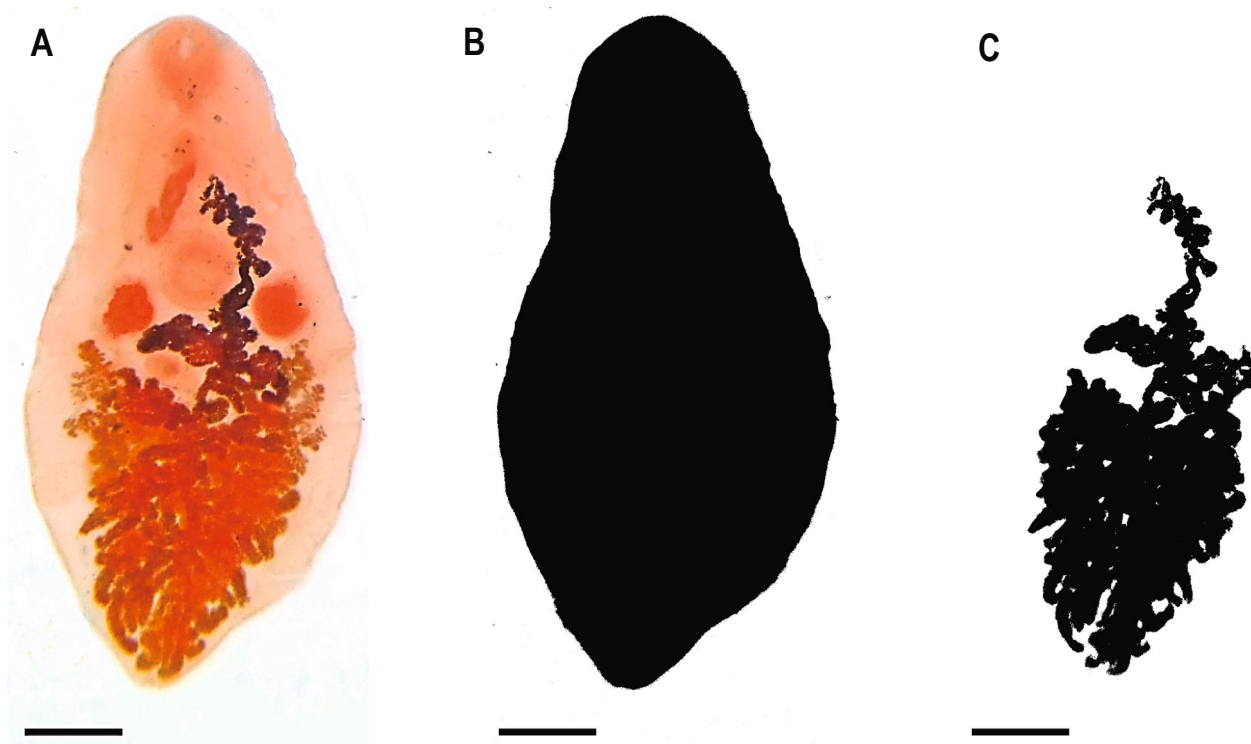


Fig. 1. The binarization process to measure the surface area. (A) general view from *Eurytrema coelomaticum*. (B) binary image after transformation in ImageJ software showing the total area of the parasite. (C) uterus area after the binarization process. Scale bar 1 mm

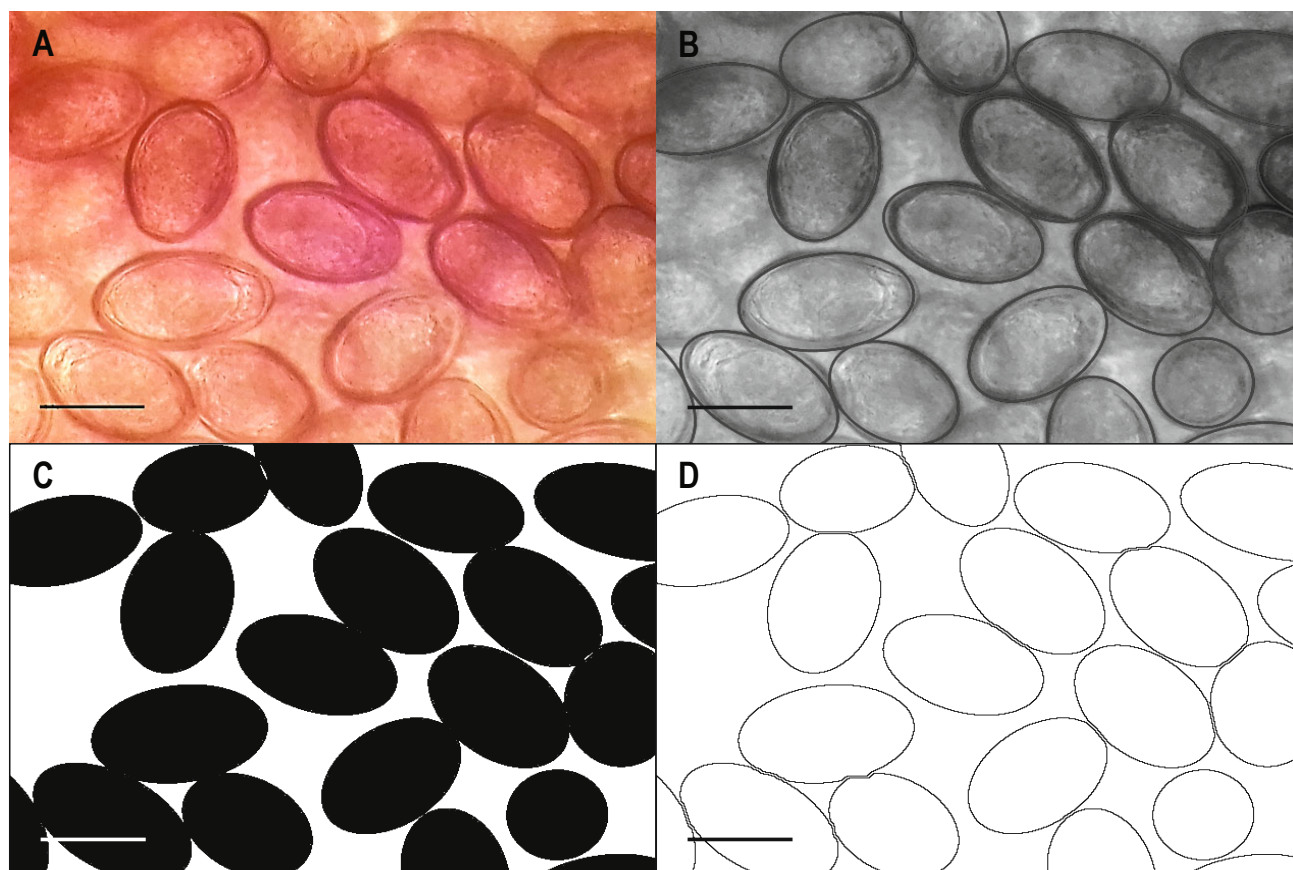


Fig. 2. Automatic counting process of *Eurytrema coelomaticum* eggs. (A) Original image. A micrograph (bright field) of the surface of *E. coelomaticum* eggs. (B) recognition pattern for structures by ImageJ software. It is the first step to the binarization process. (C) a secondary binary image is created. (D) transformation of the binary image to segmented lines with the capacity to measure the total area and perimeter of eggs. Scale bar 30  $\mu$ m.

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The pancreas were washed inside of a bowl, so that, *E. coelomaticum* specimens could be recovered after the passage of water retained in the bowl through nylon sieves with a mesh of 150  $\mu$ m. Subsequently, transverse and longitudinal sections were performed in the bovine pancreas in order to investigate the presence or absence of the parasites. For the light microscopy analysis the specimens were washed in 0.9 % physiological solution and fixed in AFA (Glacial Acetic Acid Solution, 2 parts, 37 % Formalin, 5 parts and 70 % Ethanol, 93 parts) (Corrêa *et al.*, 2016). After fixation were placed between two glass slides and were lightly pressed for maintenance of their original shape. They were then stained with alcoholic chlorhydric carmine and observed under a light microscope (Olympus CX22 Led), coupled with a camera Moticam 5.0 MP and using Images Plus 2.0 software (Motic China Group Co LTD). All images used for comparison were photographed at the same magnification and with the same resolution (amount of pixels in the images)

To measure the total area of the adult animal, uterus and eggs,

ImageJ software (NIH, USA) was used. In order to separate the uterus from the animal in the images was used a tool of creation of vectors in the Gimp 2.8 software (GNU Image Manipulation Program Open Source), and in this way it was possible to calculate the size of the organ through of creation of a secondary image with the same amounts of pixels of the original image. In ImageJ program, all images captured through the microscope and measured with the Images Plus 2.0 software were converted to gray scale images. Subsequently, the threshold function was used to improve the contrast of the structures to be analyzed in relation to the background. After the use of the threshold function, if there was any residual "noise" in the image (material that was not being evaluated), it was removed using the Gimp 2.8 software. For the delimitation between one object and another, the watershed tool was used. The total area of parasite, uterus and eggs was measured using the tools: Measure or Analyze Particles. The process of binarization, measurement and counting of the items evaluated in this work can be visualized in Figs. 1, 2 and 3.

From these data it was possible to estimate the total parasite area, uterine size and number of eggs per  $\text{mm}^2$ , adapting the methodology used by Rosati (2015). In order to verify the minimal number



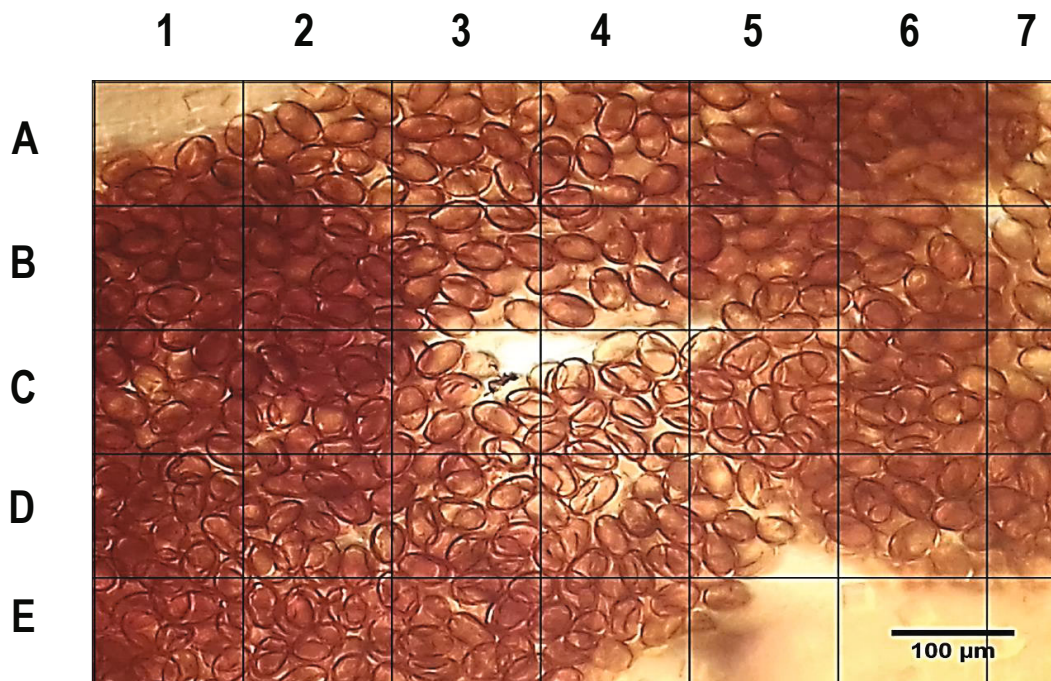


Fig. 3. Manual counting process: represent a picture with grids where was counted the *Eurytrema coelomaticum* eggs.

of eggs of *E. coelomaticum*, three scenarios were considered. In the first scenario, only the whole eggs observed in the field were counted. In the second process to estimate the number of eggs, were considered the areas of all visible structures and with clear delimitation. Manual count of eggs was also performed so that to establish the accuracy of the software. The comparison between the counting performed by the software and manually was performed only in this scenario because they are measures that have the closest characteristics and could be correlated. Finally, in the last scenario, it was considered an occupation of 100 % of the eggs in the image, since that the eggs into the uterus has three-dimensional characteristics, there are overlapping eggs and therefore cannot be correctly visualized and delimited in the image, which has a character two-dimensional. In this way it was calculated the maximum amount of eggs that can be seen in an image with 230 x 170  $\mu\text{m}$ , taking as reference the average area of the eggs.

To measure the mean area of adult individuals, their respective uterus and the average egg size, an arithmetic mean was calculated based on 10 images. The manual counts were performed on images with the same area (in micrometers) and the values were extrapolated to  $\text{mm}^2$ , using a cross multiplication. The area used for counting the eggs by ImageJ software was 230 x 170  $\mu\text{m}$  (totaling 0.0391  $\text{mm}^2$ ) and the images used for manual counting had an area of 790 x 593  $\mu\text{m}$  (equal to 0.468  $\text{mm}^2$ ). To facilitate the manual counting of eggs, the selected images were divided into grids and these were numbered from 1 to 7 in the columns and from "A" to "E" in the lines according to Fig. 3.

#### Statistical Analysis

The paired T-test was applied to compare the number of eggs observed through manual counting and software. Linear regressions were made between the uterus area and total body area, and between the number of eggs observed by manual and software counts. The significance level of 5 % was used for both cases.

#### Ethical Approval and/or Informed Consent

All applicable national and institutional guidelines for the care and use of animals were followed. The study entailed samples of the normal meat inspection process of animals sent to slaughter for human consumption. This study was performed in strict accordance with the recommendations of the Brazilian law n° 11.794/08 (which regulates procedures for the scientific use of animals) and Normative Resolutions of CONCEA (National Council for Control of Animal Experimentation).

#### Results

The adult specimens showed a flat body, fusiform or oval shape with an average length of 7.67 mm ( $\pm 0.34$ ) and 3.66 ( $\pm 0.41$ ) of width. The average area of adult individuals corresponded to 29.4  $\pm 4.26 \text{ mm}^2$ , while the average area of the uterus represented 15.5  $\pm 3.18 \text{ mm}^2$ . The total area of *E. coelomaticum* varied between 21 and 36  $\text{mm}^2$  and the total area of the organ oscillated between 9.5 and 20.6  $\text{mm}^2$ . The eggs showed average values of area equal 980  $\mu\text{m}^2$  and average perimeter it was of 119  $\mu\text{m}$  (n = 200). The

Table 1. Registered values for the total area of *Eurytrema. coelomaticum*, uterus size, proportional percent of the organ in relation to total area of parasite and eggs occupational rate into uterus lumen.

| Sample             | Total area (mm <sup>2</sup> ) |                           | Uterus/Animal | Occupancy rate |
|--------------------|-------------------------------|---------------------------|---------------|----------------|
|                    | <i>E. coelomaticum</i>        | Uterus (mm <sup>2</sup> ) | (%)           | (%)            |
| 1                  | 27.3                          | 14.3                      | 51.98         | 85             |
| 2                  | 29.2                          | 14.2                      | 47.98         | 55.6           |
| 3                  | 30.8                          | 16.9                      | 55.00         | 57             |
| 4                  | 36                            | 18.3                      | 50.00         | 63             |
| 5                  | 21                            | 9.5                       | 45.00         | 55.4           |
| 6                  | 28.4                          | 18.2                      | 63.98         | 65.75          |
| 7                  | 32.2                          | 14.5                      | 45.00         | 66.14          |
| 8                  | 28                            | 15.4                      | 55.00         | 55.4           |
| 9                  | 26.8                          | 12.7                      | 45.00         | 55             |
| 10                 | 34.4                          | 20.6                      | 60.00         | 55.6           |
| Average            | 29.4                          | 15.5                      | 51.9          | 61.38          |
| Standard deviation | 4.26                          | 3.18                      | 6.60          | 9.43           |
| Minimum value      | 21                            | 9.5                       | 45.0          | 55             |
| Maximum value      | 36                            | 20.6                      | 64.0          | 85             |

recorded values corresponding to the animals, uterus, proportion that organ represents in relation to the total size of the parasite and the percentage of occupation of the eggs in the lumen of the uterus are presented in Table 1.

The linear regression equation for the size of the uterus according to the total area of the animal (mm<sup>2</sup>) calculated was  $y = 0.6196x - 2.7614$  and  $R^2 = 0.6875$  which was significant ( $p < 0.05$ ). The prediction of the size of the uterus according to the body area of the parasite can be seen in Fig. 4.

In the first scenario, an average of 15 whole eggs was recorded (ranging from 10 to 29 eggs per image). Considering only the average eggs seen in the images (15 eggs in 0.0391 mm<sup>2</sup>) and using a cross multiplication, the estimated number of eggs was equal 383.63 per mm<sup>2</sup>. Using the estimation of whole eggs visible per mm<sup>2</sup> and considering the average area of the uterus of *E. coelomaticum*, it was estimated that an adult specimen has around 5,946 eggs in its uterus.

In second scenario, the average area occupied by eggs in lumen

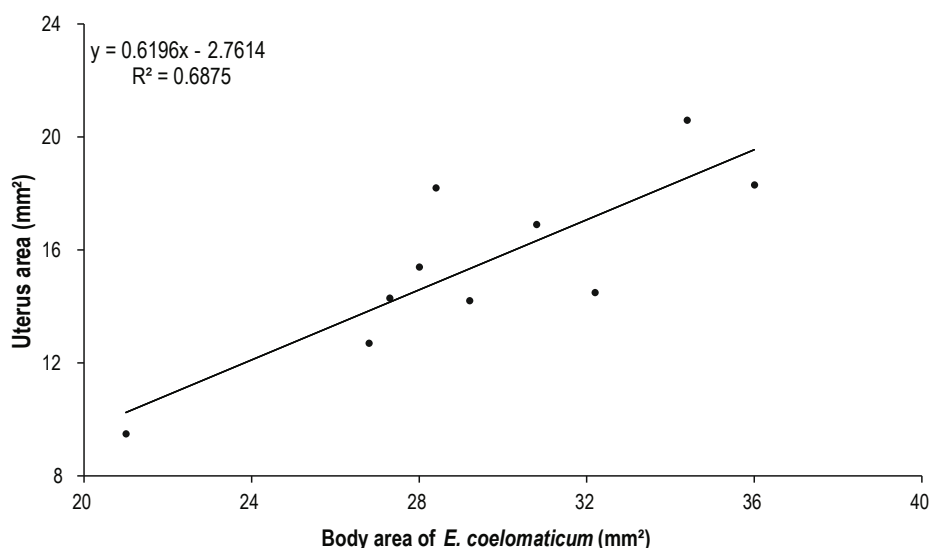


Fig. 4. Correlation between *Eurytrema coelomaticum* total area (mm<sup>2</sup>) and uterus size. Linear regressions equations were determined:  $y = 0.6196x - 2.7614$  where y is estimated size of the organ and x is the body size (total area).  $R^2 = 0.6875$ ,  $p < 0.05$ . The X axis it was changed, initializing in 20.

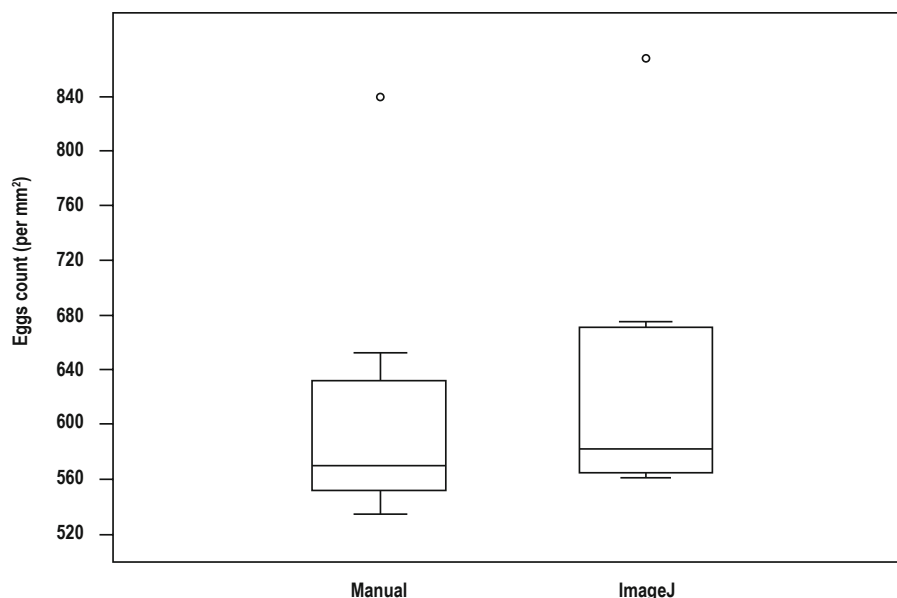


Fig. 5. Box plot of *Eurytrema coelomaticum* eggs per area ( $\text{mm}^2$ ) counted manually and automatized way. No significant difference were observed between counts with p value  $> 0.05$ .

of uterus was considered, i.e, whole eggs and portions that could be delimited and that corresponding to eggs. The area in image filled with eggs represented an average of  $0.024 \text{ mm}^2$ . The average percentage of the area occupied by eggs in the lumen of the uterus was 61.38 % (ranging from 55 % to 85 %). Multiplying the average percentage of eggs occupancy in the uterine lumen by the average area of the organ ( $15.5 \text{ mm}^2$ ) and dividing the result by 100, reached the number of  $9.51 \text{ mm}^2$  (corresponding to the area really used by eggs). Considering that an egg has an average area

of  $980 \mu\text{m}^2$ , in order to obtain the second estimate it was divided the area of occupation of the eggs ( $9.51 \text{ mm}^2$ ) by average area of an egg, resulting at the estimated number of 9,704 eggs. Dividing the estimated number of eggs by the average area of the organ ( $15.5 \text{ mm}^2$ ) reached the value of 626 eggs per  $\text{mm}^2$ . The mean number of eggs counted manually was 9,366, with 604 eggs per  $\text{mm}^2$  in the average. The distributions of the number of eggs and the respective outliers can be observed in Fig. 5.

In the T-test there was no significant difference considering an

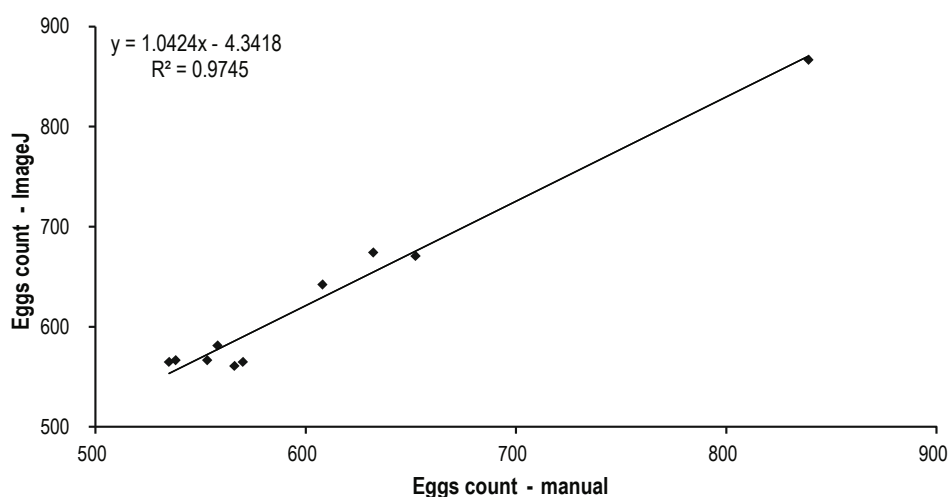


Fig. 6. Estimated amount of *Eurytrema coelomaticum* eggs in the parasite uterus, from cattle slaughtered in southern Minas Gerais, Brazil. Correlation between the number of eggs counted manually and through ImageJ software. Linear regression  $y = 1.0424x - 4.3418$ , where y is the predicted number of eggs estimated by the software and x the variable manual count;  $R^2 = 0.9745$ ,  $p < 0.05$ . The X and Y axes it was changed, initializing in 500.

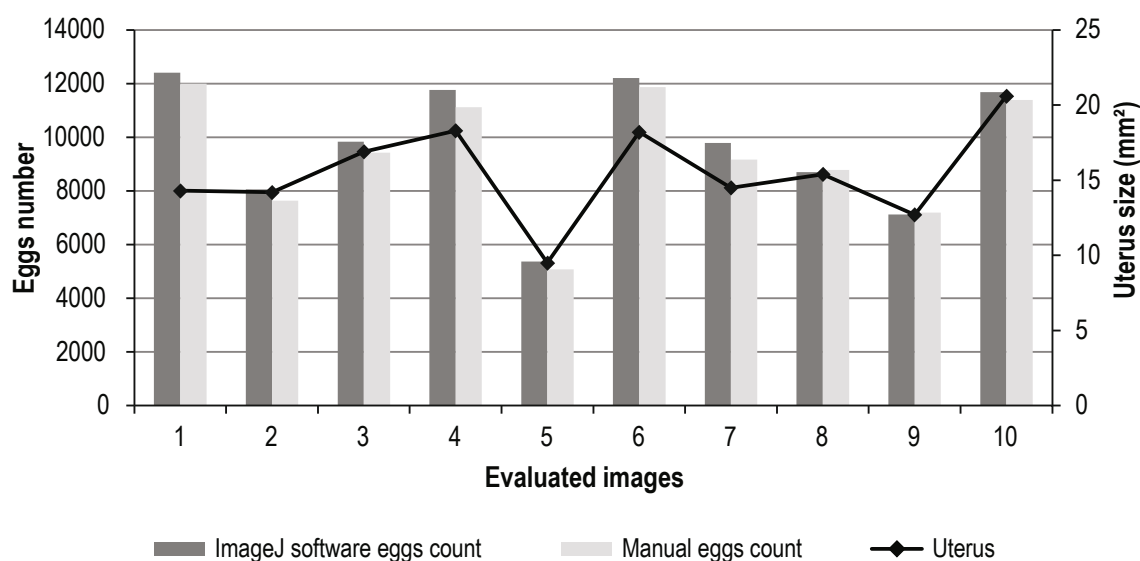


Fig. 7. Comparison of the number of manually counted eggs and the number of *Eurytrema coelomaticum* eggs registered by ImageJ software according to the area occupied by the eggs in lumen of the uterus and according to the size of the organ in square millimeters.

equivalent distribution for the variances of eggs numbers counted manually and estimated by the ImageJ software, with  $p > 0.05$  ( $p = 0.756$ ). A linear regression with a prediction interval of 95 % was performed for the number of eggs counted manually and automatic way, as can be seen in Fig. 6, with  $R^2 = 0.9745$  and  $y = 1.0424x - 4.3418$ . The variation in the number of eggs is directly related to the size of the uterus as well as the occupancy rate in lumen by the eggs, as can be observed in Fig. 7.

Finally, in the third scenario, an occupancy rate of 100 % of the image per eggs was considered. Therefore, it was estimated that in  $0.0391 \text{ mm}^2$  exist 39.89 eggs ( $0.0391 \text{ mm}^2 \div 980 \mu\text{m}^2$ ) and consequently we reached the value of 1,020.20 eggs per  $\text{mm}^2$ . So, if consider the average size of the uterus of an adult animal it possible concludes that have the number estimate of 15,813. eggs in a *E. coelomaticum* specimen. A linear regression was performed and an equation was defined to estimate the number of eggs present in adults according to the area of the uterus. The equation for calculating the number of eggs considering an occupancy rate of 100 % of the uterus is:  $y = 1020.4x + 1.82^{-12}$ , where  $y$  is the predicted number of eggs and  $x$  the variable size of uterus in  $\text{mm}^2$ ;  $R^2 = 1$ ,  $p$  value  $< 0.05$ ).

## Discussion

According to Neuhaus (1978), the length of the uterus of some trematodes correlates with the size of the body. In the present study, it was observed that there is a relation between the size of the animal and the size of the uterus with  $p$ -value  $< 0.05$ . The positive and significant relationship between the body size and uterus indicates that the addition of one is accompanied by proportional

increase of another, without, however, requiring dependence between them, but allowing that from the first one can be estimated the value of the second.

The *E. coelomaticum* uterus has eggs clusters in its lumen, and there is no effective method to quantify the total number of eggs inside the organ without using methods that destroy internal structures of the parasite, causing losses of materials throughout the process of incision and removal of organ, affecting the count and later the results. ImageJ software provides precise measurements of surface area and is, therefore, an exceptional tool to support in counting and consequently creating estimates closer to reality. Mains *et al.* (2008) used successfully a digital image analysis supported with ImageJ program to estimate the number of aedine mosquitoes eggs using similar methodologies applied in this work. In the present study, a simple linear correlation was performed to evaluate the expected size of the uterus of adult animals using the total area of *E. coelomaticum* like parameter to calculate. This procedure was performed since the masses of the specimens were not verified, nor of organs in question, diffculting to estimate by means of an exponential logarithmic equation through of static allometric development. Valero *et al.* (2005) used as reference to evaluate the development of the uterus of *Fasciola hepatica* obtained in cattle from Bolivia, Spain and France an allometric growth model, where the curve shows an exponential growth. Neuhaus (1978) used to calculate the equation of the uterus length of *Dicrocoelium dendriticum*, *Pleurogenoides medians* and *Fasciola hepatica* the corrected body size that is equal to  $\sqrt{\text{width} \times \text{length}}$ . The author observed a sigmoid curve with the middle part presenting a linear section, that he considered an allometric growth.

In the literature, there is no description of estimates of total num-

ber of eggs present in the uterus of *E. coelomaticum*. There are studies that evaluated the oviposition rate of the parasite *in vitro*, and these studies observed a deposition ranging from 194 to 746 eggs per day in different physiological solutions (Yamamura, 1989; Brandolini & Amato, 2001). According to Bassani *et al.* (2007) the proportion of eggs eliminated and observed via EPG is not directly related to number of parasites in the host, even considering those who lay their eggs directly in lumen of intestine.

Our results indicate that simple linear regressions can be established to calculate the burden of eggs based in uterine size, and the equations can be defined by considering the occupancy rate in lumen of uterus or by considering a percentage of 100 %. Rosati *et al.* (2015), used a different design to estimate the number of eggs of blow flies, since they had masses of eggs (aggregates) and, therefore, it was possible to determine the depth of these agglomerates and thus to calculate the volume present in the analyzed samples by created a three-dimensional model to estimate the number of eggs through volume and not only the surface area. Despite the relative success with regard to egg estimation, it is important to note that the methodology adopted in this work has limitations such as the fact that the images analyzed by the software have two-dimensional characteristics, whereas the parasites have three-dimensional conformation. In this way the total number of eggs ends up being underestimated, being calculated in a certain way the minimum number of eggs that can be visualized, since those eggs that are not captured by the image end up not going into calculation of estimate. Therefore, considering the three scenarios established in the present work, it is evident that the minimum numbers of eggs present in *E. coelomaticum* uterus ranges from 5,946. to 15,813, and that is necessary to develop a methodology for extraction of the organ without loss of material and thus accurately determine the total number of eggs present.

## Conclusions

The procedure performed in this study have some advantages like the fact that free software are used (ImageJ and Gimp), and these do not require expensive or specialized hardware. Another positive aspect related to technique is that the scanned images provide permanent records that can be checked by manual counting if this is desired.

Using the technique described in this work it is possible to estimate thousands of eggs in a short time and with a great accuracy. The time required for manual counting can be a complication in experiments that require egg counting, demanding a substantial effort, loss of time and resources. Therefore the method described in this work includes a substantial reduction of time, greater consistency in the obtained data, and decrease of errors besides taking the human bias. This study describes an important tool to quantify the number of eggs in a non-destructive way and aggregates information not explained previously by others works.

## Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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