A Comparison Of ArcGIS And Image J Software Tools For Calculation Of Gonad Volume Fraction (GVF) From Histological Sections Of Blue Mussel Cultured In Deep And Shallow Sites On The Northeast Coast Of Newfoundland.

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2018

Canadian Technical Report of
Fisheries and Aquatic Sciences 3275

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Canadian Technical Report of Fisheries and Aquatic Sciences

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A Comparison of ArcGIS and Image J Software Tools for Calculation of Gonad Volume Fraction (GVF) From Histological Sections of Blue Mussel Cultured In Deep and Shallow Sites on the Northeast Coast Of Newfoundland

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Cat. Fs97-6/3275E-PDF ISBN 978-0-660-27971-8 ISSN 1488-5379

Correct citation for this publication:

Murray, H.M. and Ollerhead, L.M.N. 2018. A comparison of ArcGIS and Image J software tools for calculation of gonad volume fraction (GVF) from histological sections of blue mussel cultured in deep and shallow sites on the Northeast coast of Newfoundland. Can. Tech. Rep. Fish. Aquat. Sci. 3275: vi + 17 p.

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ABSTRACT

Murray, H.M., and L. M. N. Ollerhead. 2018. A comparison of ArcGIS and Image J software tools for calculation of gonad volume fraction (GVF) from histological sections of blue mussel cultured in deep and shallow sites on the Northeast coast of Newfoundland. Can. Tech. Rep. Fish. Aquat. Sci. 3275: vi + 17 p.

The stereological determination of reproductive indices in bivalve shellfish is accepted as standard and is a reflection of changes in gonadal development and thus can be predictive of spawning activity and industry meat yield. The measurement of gonadal characteristics associated with calculation of the above indices has been traditionally limited to small sample number due to the time and effort involved in making individual measurements. The evaluation of larger sample sizes would be beneficial as it is more representative of the population of interest. In the current paper we have applied tools from ArcGIS software (ArcGIS, 2016) designed for spatial analysis to measure histological features from photomicrographs of bivalve mantle and applied these to the calculation of reproductive indices like gonad volume fraction. These software based determinations allow for an automated rapid analysis of a large number of images. Subsamples of the above images were also analysed manually using commercially available image analysis software (i.e. Image J) in order to quality control the automated analysis. Generally the calculations were comparable, however it was noted that histological features mostly related to post-spawn tissue characteristics created some discrepancies specifically in females. Some of these issues included excessive inter-follicular space, undefined edges, and high contrast background due to large numbers of interstitial adipogranular cells. Male mantle tissue was observed to be more easily analysed then female mostly due to the higher contrast produced by the dense follicles characteristic of male gonad. Additionally, technical adjustments related to lighting and photo contrast need to be considered carefully when capturing images for future analysis. Based on these evaluations, we conclude that the ArcGIS method is usable in this application but with careful attention to initial image quality and random manual checks in questionable samples.

RÉSUMÉ

Murray, H.M., and L. M. N. Ollerhead. 2018. Comparaison des outils logiciels ArcGIS et Image J pour le calcul de la fraction volumique des gonades à partir des coupes histologiques des moules bleues d'élevage dans des sites d'eau profonde et peu profonde de la côte nord-est de Terre-Neuve. Can. Tech. Rep. Fish. Aquat. Sci. 3275: vi + 17 p.

La détermination stéréologique des indices de reproduction des mollusques bivalves est acceptée comme norme et reflète les changements dans le développement gonadique; elle peut donc être prédictive de l'activité reproductrice et du rendement en chair de l'industrie. La mesure des caractéristiques gonadiques associées au calcul des indices ci-dessus a été traditionnellement limitée à un petit nombre d'échantillons en raison du temps et des efforts requis pour effectuer des mesures individuelles. L'évaluation d'échantillons de plus grandes tailles serait bénéfique, car elle est plus représentative de la population d'intérêt. Dans le présent article, nous avons utilisé les outils du logiciel ArcGIS (ArcGIS, 2016) conçus pour l'analyse spatiale afin de mesurer les caractéristiques histologiques des photomicrographies du manteau bivalve, et nous les avons appliquées au calcul des indices de reproduction comme la fraction volumique des gonades. Ces déterminations logicielles permettent une analyse rapide et automatisée d'un grand nombre d'images. Des sous-échantillons des images ci-dessus ont également été analysés manuellement à l'aide d'un logiciel commercial d'analyse d'images (c.-à-d. Image J) afin de contrôler la qualité de l'analyse automatique. En général, les calculs étaient comparables, mais il a été noté que les caractéristiques histologiques principalement liées aux caractéristiques des tissus après le frai créaient certaines divergences, plus précisément chez les femelles. Certains de ces problèmes comprenaient un espace interfolliculaire excessif, des bords indéfinis et un arrière-plan à contraste élevé en raison du grand nombre de cellules adipogranulaires interstitielles. On a observé que le tissu du manteau masculin était plus facile à analyser que celui de la femelle, surtout en raison du contraste plus élevé produit par les follicules denses qui sont caractéristiques des gonades masculines. De plus, les ajustements techniques liés à l'éclairage et au contraste des photos doivent être soigneusement pris en compte lors de la capture d'images pour analyse ultérieure. Sur la base de ces évaluations, nous concluons que la méthode ArcGIS est utilisable dans cette application, mais nécessite qu'on porte une attention particulière à la qualité initiale de l'image et aux contrôles manuels aléatoires dans les échantillons douteux.

INTRODUCTION

Common indices of gonad development and condition in bivalves include gonadosomatic index (GSI) and gonad volume fraction (GVF) (Lowe et al. 1982; Heffernan and Walker, 1989; Newell et al. 1982; Toro et al. 2002). Such indices have also been accepted as good predictors of industry relevant meat yield and similarly can also reflect changes in gonadal development and thus are predictive of spawning activity (Dix and Ferguson, 1984; Okumus and Stirling 1998; Duinker et al 2008; Celik et al 2012; Galvao et al. 2015).

Gonad volume fraction (GVF) provides a measure of the relative maturity of the bivalve gonad and has been measured using a variety of classical histological and stereological methods (Lowe et al. 1982; Heffernan and Walker 1989; Toro et al. 2002; Fabioux et al. 2005) and even recently by non-invasive MRI imaging technology (Davenel et al. 2006; Pouvreau et al. 2006; Holliman et al. 2008; Hatt et al. 2009).

These techniques are typically limited by the number of individuals that can be processed accurately at any point in time. Histological processing and the subsequent fine measurement of tissue or cellular structures still remain as the bottle neck for any stereological based analysis, however once histological sections and slides are produced, access to digital microscopy/photographic techniques using high resolution cameras and microscope lenses can easily produce large libraries of digital images in a short period of time. From a stereological perspective high throughput processing of large numbers of histological images would allow for more individuals from a population to be analysed over a relatively short period. These types of techniques are currently being developed for application in human pathology including in the field of cancer biology and thus have the potential for shortening the time to diagnosis and the evaluation of more patients in a short period of time (Ong et al. 2010; Lahrmann et al. 2011; Eriksen et al. 2017).

Quantitatively describing the reproductive status of cultured bivalve populations is an important step toward understanding its impact on meat yield and hence

production standards, as well as contributing to an improved understanding of reproduction. The present study illustrates the novel application of ArcGIS software (ESRI) to the high through-put analysis of gamete volume in cultured blue mussels using a large library of histological images. A subsample of images will be analysed using a classical manual stereological method and Image J software in order to quality control the automated protocol.

MATERIALS AND METHODS

EXPERIMENTAL SET UP

This study was part of a larger investigation to evaluate the effect of culture depth on the reproductive patterns of *Mytilus edulis* (Murray et al. (in Press)). Briefly, local seed was collected using standard industry techniques during the summer of 2015 and set up in commercially available socking material then deployed in three culture sites within the South Arm region of Notre Dame Bay, NL: Mouse Island (MI); South Arm (SA) and Bulley's Cove (BC). Each site included shallow water (headlines at 5 m and bottom depths < 20 m) and deep water (headlines at 15 m and bottom depths up to 60 m) culture zones. South Arm had one shallow water zone (SAS) and one deep water zone (SAD). Bulley's Cove had two shallow water zones (BCS1and BCS2) and one deep water zone (BCD). Mouse Island was located in a deep channel further off shore and included only a single deep water zone (MID).

SAMPLE COLLECTION

At monthly intervals from May to December 2016, three mussel socks were sampled from each respective site and zone (two from each end and a third from the center of the line) and transferred back to a commercial processing plant where 100 representative mussel samples were removed for evaluation of reproductive status. In order to avoid positional bias on an individual sock, a portion of the samples were randomly removed from the top, middle and bottom of each sock. These samples were then shipped on ice to laboratories located at the Northwest Atlantic Fisheries Centre (Fisheries and Oceans Canada) in St.

John's, NL. At the laboratory, 50 mussels were further subsampled and processed using protocols modified from Toro et al. 2002.

TISSUE PREPARATION

For monthly histological analysis one mantle lobe from each dissected mussel was subsampled by cutting a transverse section midway along the anteroposterior axis according to that described in Toro et al. 2002. Tissue was chemically fixed in 10% neutral buffered formalin for 24 hours and then dehydrated in an ascending alcohol series, cleared in xylene and embedded in paraffin wax. Forty paraffin blocks were chosen randomly from each site for sectioning and slide production. Only one sample of mantle tissue was used for each individual as the mantle of *Mytilus spp*. is known to be homogeneous (Lowe et al. 1982; Bayne, 1985; Toro et al. 2002). Ten female and ten male blocks were further randomly subsampled from each site and sectioned in series at 7 um to produce 5 replicate slides for each individual per gender. Each slide contained 5 sections from each individual and was stained using a standard H & E protocol.

IMAGE CAPTURE

The third section on each slide was captured at 50X magnification with a calibrated scale using a Motic stereoscope equipped with a 5.0 mega pixel Moticam 580 digital camera. Each image was captured using Motic images plus 2.0 software in grey scale on a white background while illuminated with dual LED spotlights set at medium intensity and saved as individual files in TIF format. The images were not post-processed and the images were passed as is into the automated processing software. Six hundred images were captured per sample month for a total of 4800 images for the study period.

GONAD VOLUME FRACTION ANALYSIS (GVF)

AUTOMATED IMAGE ANALYSIS (ArcGIS v10.4.1)

Image classification and spatial analysis techniques within the ArcGIS platform were applied to the captured images from each replicate slide to determine the GVF. The GIS analysis is an automated workflow that delineates the gonadal

tissue areas, and calculates and exports the GVF for each slide. The images are grey scale and have brightness values ranging from 0 to 255 where the dark and light areas correspond to gonadal tissue and interstitial space respectively. The first step in the analysis was to apply the ArcGIS tool, Slice to partition the images into dark and light regions using a Natural Breaks (Jenks) classification. Natural Breaks (Jenks) classification partitions data by minimizing the within group variances and maximize the between group variances to identify the natural groupings within a dataset (de Smith et. al., 2009). The areas identified by the Slice tool as gonadal tissue were extracted and exported to separate polygon layers. To further refine the gonadal regions, the ArcGIS function Delineate Built Up Areas (DBUA) was applied to the gonadal tissue polygon layers created in the previous step. This function is designed to identify boundaries for regions of densely clustered arrangements of polygons in large-scale mapping (ESRI). The DBUA tool was applied to the gonadal tissue polygon layers to better delineate the follicle boundaries and remove some of the nuclei within the follicles that were mis-classified as interstitial space. The GVF was then calculated as a ratio of the area of the gonadal tissue regions to the total image area. Using Python, the native ArcGIS scripting language, the entire workflow was automated to process the image dataset and export the results to a spreadsheet.

MANUAL IMAGE J ANALYSIS

As a direct comparison to the automated ArcGIS technique a subsample of 30 male and 30 female individuals were randomly chosen from those previously analysed and used for the manual measurement of gonadal area with Image J software tools. Briefly, individual images from the above replicate slides were uploaded to the Image J interface where the measurement scale was standardized based on an embedded scale bar of known size. Subsequently, the area of individual gonad follicles (male or female) were manually measured using the image J measurement/analysis function, followed by a calculation of the percentage coverage of gonadal tissue to surrounding interstitial tissue (gonad volume fraction). This data was then uploaded to a Sigmaplot spreadsheet for statistical analysis.

STATISTICAL ANALYSIS

Individual deep and shallow sites were considered replicates and combined into two categories i.e. deep and shallow for technique evaluation. For comparison, the mean GVF of 30 individuals from each culture depth was calculated from each of the respective techniques and plotted against month. Plots were also separated based on gender. One-way analysis of variance (ANOVA) using Tukeys Test for post-hoc testing was used to compare mean GVF from each technique per sample month and culture depth (deep vs shallow). Males and females were analysed separately. Means ± SD were calculated. Significance was set at $\alpha = 0.05$. All statistical analysis was performed using Sigmaplot statistical and graphical software (12.0 and successive versions, Systat Software Inc., San Jose California, USA)

RESULTS

HISTOLOGICAL CHARACTERISTICS OF MUSSEL GONAD

Characteristically, gonadal tissue in the blue mussel is found to be homogeneously distributed through the mantle, which is located adjacent to the inner surface of the shell. Male and female mantle tissues have obvious gender based histological differences that can vary seasonally with reproductive cycle. In the present study, the period between May and July is characterized by ripe follicles containing developing gametes in both males and females (Figure 1AB). Females exhibit many large mature detached oocytes with distinct nuclei and nucleoli (Figure 1B). Similarly, the male mantle during the May to July period was filled with large ripe follicles containing numerous mature sperm and developing spermatids (Figure 1A).

The period following the primary spawn in July/August (August through to December) (Figure 1CD) was characterized by spent or redeveloping gonadal tissue. Follicles from both genders were significantly reduced in size with few mature gametes of either sex. Interstitial tissue is prominent; especially in females where follicle definition is also significantly reduced (Figure 1D). Male

follicles are also comparatively reduced in size during this period but still exhibit good contrast against the interstitial tissue background (Figure 1C).

GONAD VOLUME FRACTION (GVF): ARCGIS PLATFORM VS MANUAL IMAGE J PLATFORM

The mean GVF calculated for male mussels using automated ArcGIS processing was not significantly different from that calculated based on manual measurements of a subset of the same slides using Image J software for any month from either deep or shallow sites (Figure 2AB). The mean GVF calculated for female mantle tissue from deep culture sites was also not significantly different between measurement techniques for the months of May through September and November or December ($p>0.05$) (Figure 3A). However, the mean GVF calculated for females using the ARC-GIS platform (36.81± 7.02) was significantly higher than that for Image J (30.03± 8.01) in deep sites during the month of October ($p <0.05$) (Figure 3A). Also, mean GVF calculated for females from shallow sites was not significantly different between measurement techniques from May to July and during September or November ($p > 0.05$) (Figure 3B). However the same calculated for shallow females was again significantly higher ($p< 0.05$) for ARC-GIS then Image J during August (43.36 ± 7.22 vs 33.41 ± 9.4), October (36.9 ± 6.7 vs 28.16 ± 10.9) and December (47.32 ± 9.7 vs 39.71± 11.7) (Figure 3B).

EVALUATION OF EFFICIENCY AND SOURCES OF ERROR

The time spent on outlining regions of interest for the automated ArcGIS method was negligible whereas the same task using the manual Image J technique took on average 10-20 minutes per slide including setting the scale, measuring the total area of chosen field of view, and then obtaining the individual areas of gonadal tissue. Values were then manually exported to spreadsheet for final GVF calculation. Batch analysis using the ArcGIS method required no significant human input thus analysing 100% of the region of interest with no need for hands on with the exception of initial set up. Software based image analysis of the tissue sections could be run overnight with no observer interaction needed. GVF is calculated automatically and data is exported to a spreadsheet.

While both techniques have advantages and disadvantages, three distinct sources of tissue based error were identified that affected both and included 1) undefined spaces within follicles, 2) undefined edges, and 3) background noise due to infiltration of interstitial cells associated with post-spawn recovery. These sources of error were more common in females then males due to the gender specific histological characteristics of the germinal tissue especially in post-spawn stages.

1. *SPACES WITHIN FOLLICLES*: Post-spawn female gonadal tissue frequently contained follicles having clear central spaces with an outside darker layer of cells containing redeveloping or reabsorbing oocytes (Figure 4A). The presence of empty areas leads to an under or over estimation by the automated software since the low contrast confuses follicular space with background and thus only picks up a fraction of the true follicle or reports an over estimation of follicle area due to inclusion of background in any calculations. With the manual Image J technique, the whole follicle can be visually recognised and thus outlined easily providing a more accurate evaluation.
2. *UNDEFINED EDGES*: Female follicles were also observed to occasionally have edges that were undefined (Figure 4B). Undefined edges can create difficulty in manually outlining follicular tissue but can also again lead to an overestimation of gonadal tissue using the automated platform as it can pick up non gonadal tissue outside of the follicular wall and include this in overall calculations.
3. *BACKGROUND NOISE*: Cells associated with the interstitial space outside of gonadal tissue i.e. adipogranular cells and vesicular connective tissue cells involved in post-spawn tissue remodeling, can also lead to an overestimation of GVF by the automated software platform since it cannot easily distinguish between high contrast background due to cellular material and actual gonadal tissue (Figure 4C). This does not create a problem using the manual platform as a human user can easily distinguish between actual gonadal tissue and interstitial background.

While biological and tissue specific characteristics were a recognized source of error, it was also critical that good and consistent initial image quality is maintained during successive image captures (i.e. lighting, contrast). For example, uneven lighting conditions can create shadows causing contrast confusion for the ArcGIS software platform. Figure 5AB demonstrates how uneven lighting can cause a lighting gradient across an image. Additionally technical issues with tissue sections i.e. folds or contamination can also create dark spots on an image leading to an over or under estimation of GVF while using the software based technique (Figure 5A).

DISCUSSION

The measurement of cellular structures or components in a histological preparation of a tissue is defined as stereology and is basically the extrapolation of measurement data from two-dimensional to three dimensional spaces (Lowe et al. 1982). The accuracy of these measurements is dependent on whether thin sections of a tissue are representative of the components of interest and whether their distribution is uniform (Lowe et al. 1982; Heffernan and Walker 1989; Toro et al. 2002). Bayne (1985) showed that the mantle of *Mytilus edulis* is homogeneous with respect to the distribution of germinal cells. This observation permits the reliable calculation of the gonad volume fraction (GVF) from histological preparations of mantle tissue and thus a measure of the relative maturity of the bivalve gonad.

In this study we compared the calculation of GVF using traditional manual stereology or automated software-based image analysis for high through-put. We found an excellent correlation between the ArcGIS software based calculations and the manual stereological measurements of GVF for individual male mussels. There was no statistical difference between measurements obtained through either technique for any time point in this case and was primarily due to the characteristic high contrast of male gonadal tissue regardless of the stage in the spawning cycle. In contrast, the mean GVF calculated from female mantle tissue was on occasion over-estimated using the ArcGIS software platform when

compared to manual measurements, especially during the post-spawn period. These discrepancies are most likely due to the characteristic histological features of the post spawn female gonad and are related to the specific stage in the spawning cycle. These histological features are important contributors to many of the tissue based sources of error outlined above.

Eriksen et al. (2017) noted in a study comparing automated image analysis and stereology for cancer diagnosis that while stereological methods have evolved over the years to be more efficient they are still labour intensive and time consuming. They observed that the automated analysis required less human resources then the manual stereological technique and thus increased the efficiency of the process. Similarly, Ong et al. (2010) concluded that computer assisted pathological screening was more time effective than conventional manual scoring that did not provide any analytical advantage. In the present study, accuracy of GVF calculation was very much dependent not only on the quality of the original histological slides and the subsequent images but also on aspects of the biology of the mussel including the gender and where in the spawning cycle the sampled individual happened to be.

In summary, the ArcGIS software workflow was capable of rapidly detecting and measuring features of interest from large batches of histological images with results being statistically similar to that obtained using manual data collection techniques and manual assisted measurement software like Image J tools. Detected errors were primarily associated with females during post-spawn and redevelopment stages when follicles are breaking down and immature oocytes are developing along the inner follicular borders. The presence of adipogranular and vesicular connective tissue cells involved in post-spawn tissue remodeling also created background noise resulting in an over estimation of GVF measurements. While the automated batch processing of images does largely produce comparative quality results when compared to traditional stereological manual protocols, the presence of tissue specific sources of error will still require manual verification of a subsample of images. These issues might be alleviated by post-processing the imagery to reduce artefactual problems for the software.

With this in mind, we feel that the processing of thousands of images in a short period of time that produces reliable population estimates of gonad maturity in mussels justifies the minimal effort required for QA/QC. Further work will be necessary to determine whether this technique can be applied more generally to the determination of the reproductive maturity in other species of shellfish.

ACKNOWLEDGEMENTS

This project was funded though the Aquaculture Collaborative Research and Development Program (ACRDP), Fisheries and Oceans Canada. The authors would like acknowledge and thank the staff and boat crew at Norlantic Processors Ltd. for their many hours of boat time during sample collection. Additionally, thanks to K. Stagg and S. Kenny for contribution to the sample processing and histological preparation. S. Kenny and V. Olford are also acknowledged for the Image J analysis.

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FIGURE 1

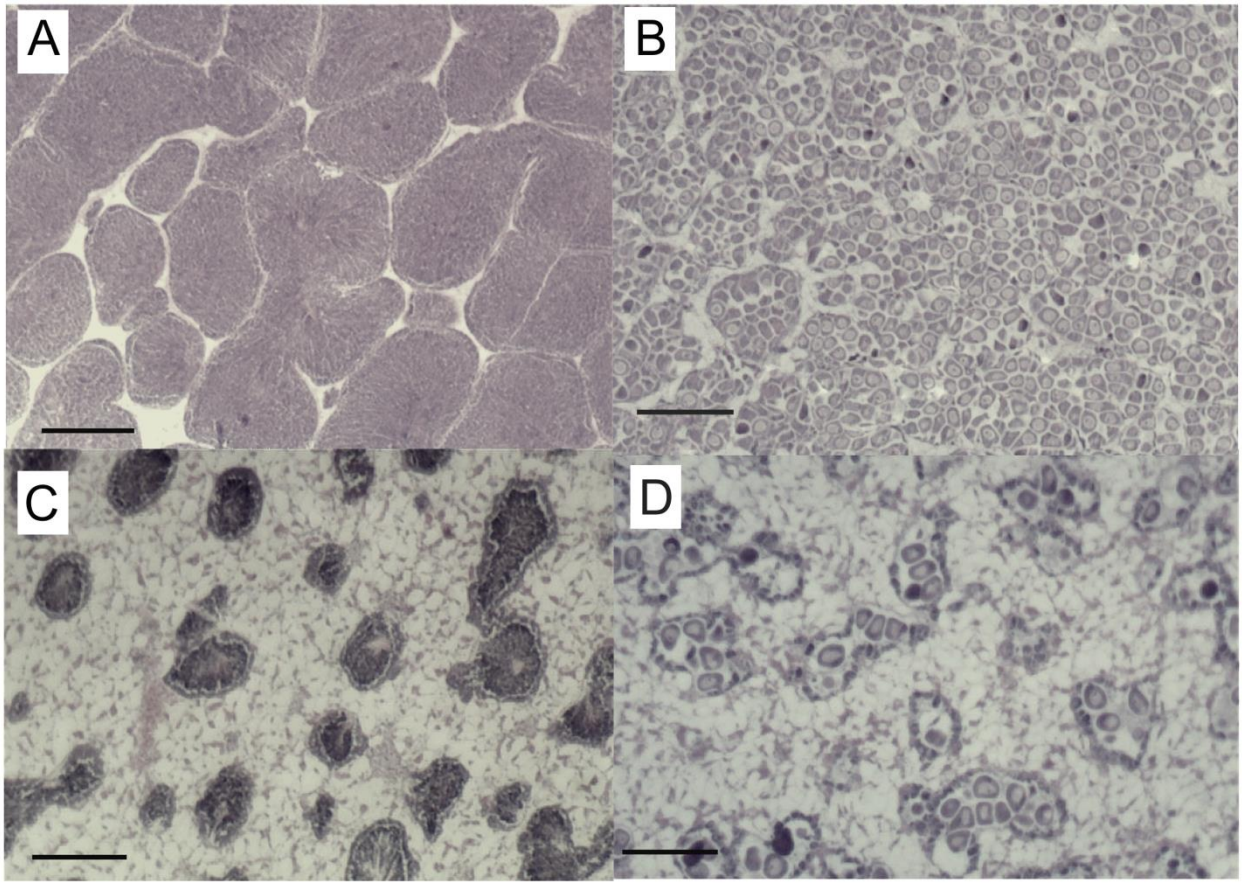
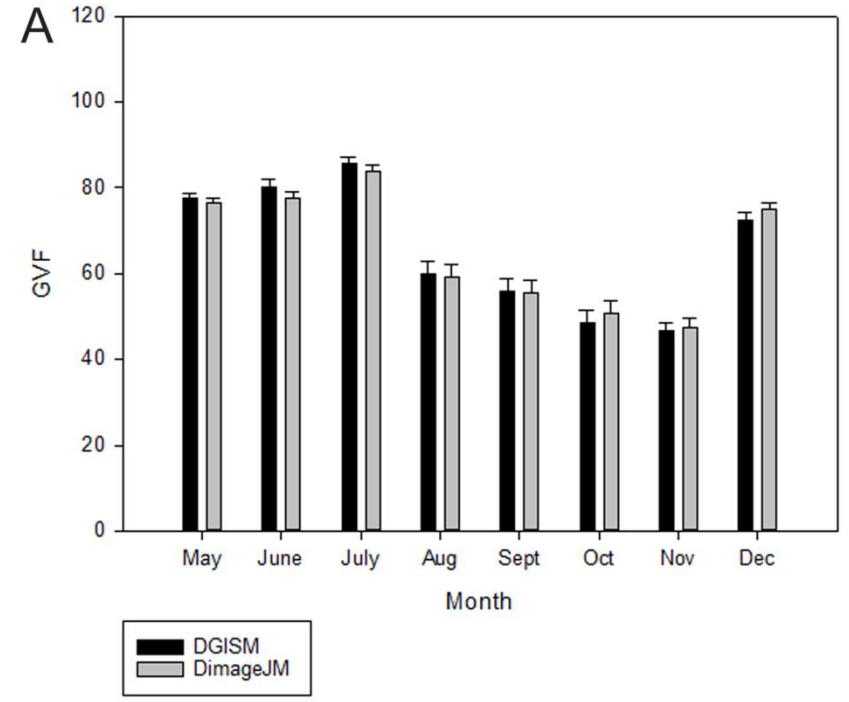


FIGURE 2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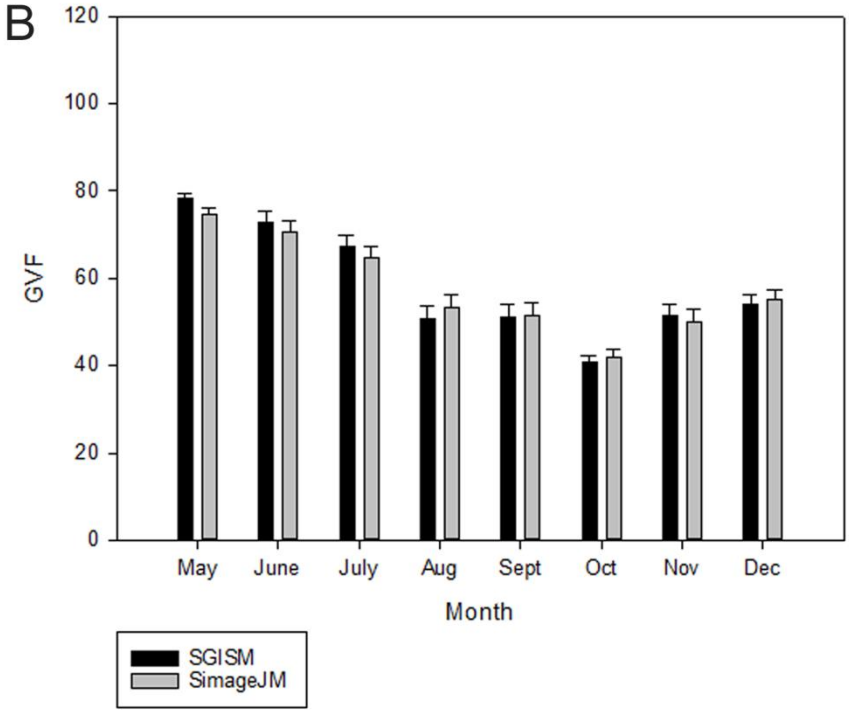
B
120
100
80
60
40
20
0
GVF
May
June
July
Aug
Sept
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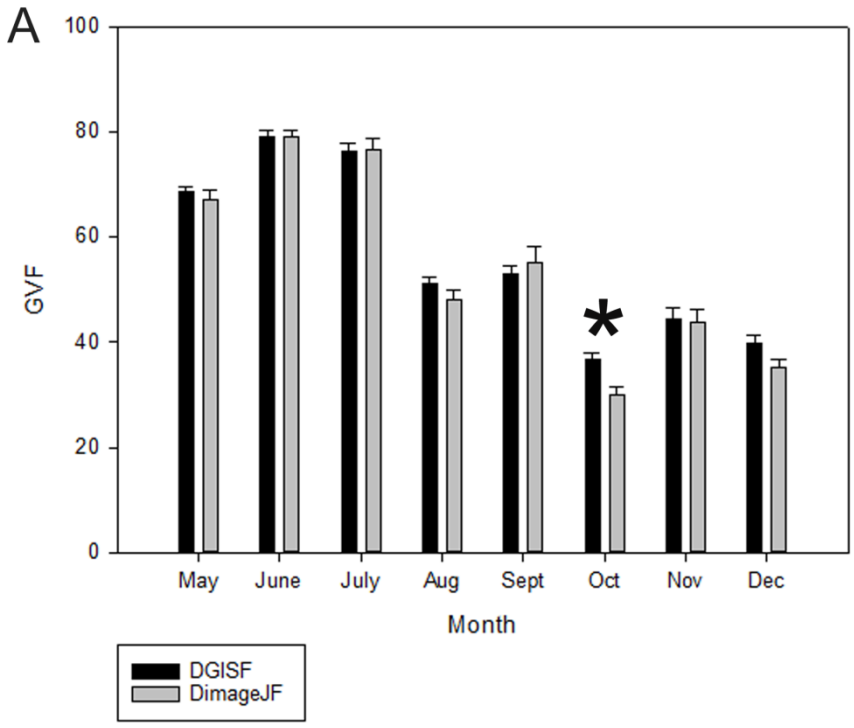
Nov
Dec
Month
SGISM
SimageJM

FIGURE 3


A
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*
May
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DGISF
DimageJF

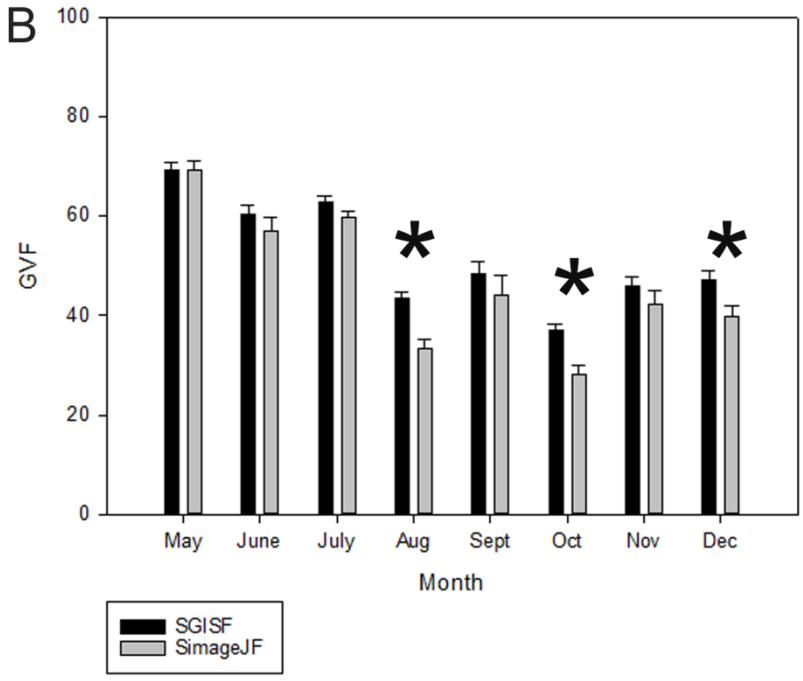
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FIGURE 4

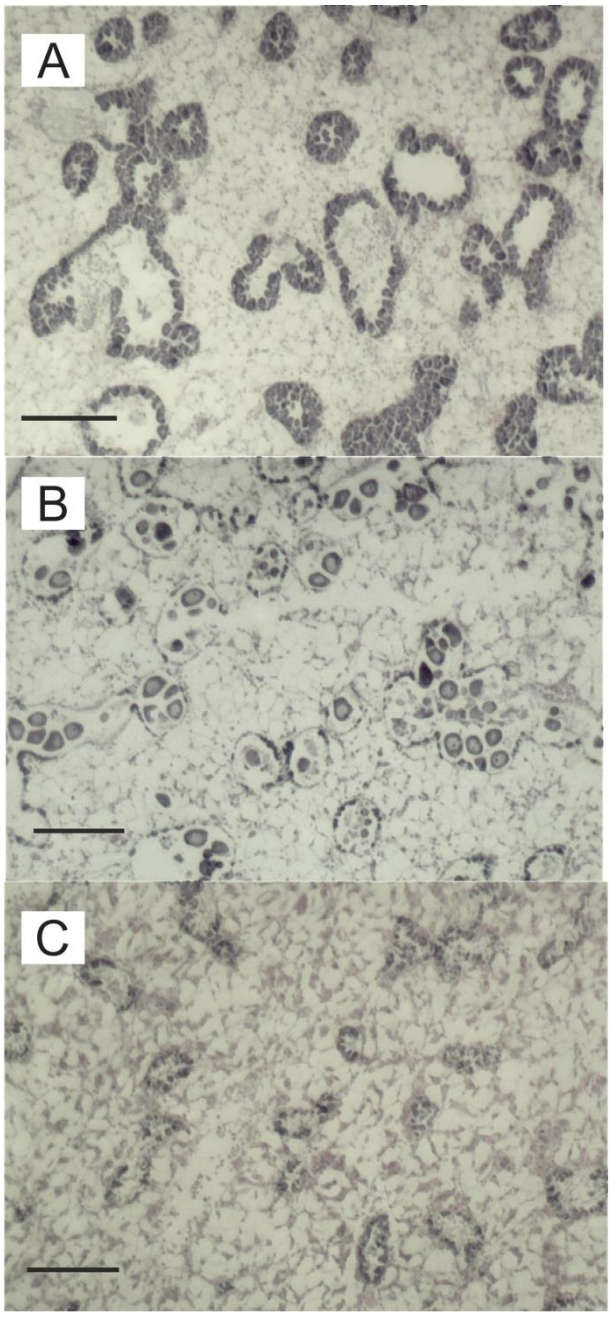

FIGURE 5