African Journal of Biotechnology Vol. 11(9), pp. 2333-2339, 31 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3064
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Seasonal growth of the attachment clamps of a *Paradiplozoon* sp. as depicted by statistical shape analysis

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Accepted 15 December, 2011

Geometric morphometric methods using computer software is a more statistically powerful method of assessing changes in the anatomy than are traditional measurements of lengths. The aim of the study was to investigate whether changes in the size and shape *Paradiplozoon* sp. permanent attachment clamps could be used to determine the duration of the organsism's life-cycle *in situ*. A total of 149 adult *Paradiplozoon* sp. ectoparasites were recovered from *Labeobarbus aeneus* and *Labeobarbus kimberlyensis* in the Vaal Dam. The software tool tpsDIG v.2.1 was used on six digitised landmarks placed at the junctures between the sclerites of the attachment clamps from digital micrographs. The tpsSmall v. 2.0 and Morphologika[2] v. 2.5 software tools were used to perform principal component analysis (PCA) on this multivariate dataset. The PCA analysis indicated that the increase in size and linear change in shape of the selected landmarks, were significant predictors of the sampling season. This study suggests that it takes one year for the permanent attachment clamps of a *Paradiplozoon* sp. to grow to their maximum size in the Vaal Dam.

Key words: Diplozoidae, *Labeobarbus aeneus*, *Labeobarbus kimberlyensis*, Vaal Dam, morphometrics.

INTRODUCTION

Statistical shape analysis, more commonly known as geometric morphometrics (GM), compares the size and shape variation in homologous landmark configurations (Bookstein, 1997; Lieberman et al., 2007). GM is useful when studying ontogenic and phylogenetic developmental changes in shape within and among samples of organisms (Free et al. 2001; Dalal and Phadke, 2007). In biology, GM is typically applied to study trends in size and shape variation over time in order to investigate the effect of changes in seasons on growth.

The family Diplozoidae (Palombi, 1949) are all fish ectoparasites with direct life-cycles and are sangiunivores on the gills of their hosts. Some authors consider the life-cycle of diplozoids to be perennial (duration of more than one year) (Zeller, 1872; Sterba, 1957; Bychowsky and Nagbina, 1959; Kamegai, 1968; Khotenovskii, 1977; Kosikivaara et al., 1991; Pečínková et al., 2007), whilst others suggest that it is annual (duration of one year) (Bovet, 1967; Halvorsen, 1969; Kagel and Taraschewski, 1993; Gelnar and Koubková, 1994; Pečínková et al., 2007). There is a lack of clarity in the literature regarding the length of diplozoid life-cycles *in situ*.

The haptoral attachment clamps are permanent structures present from the free-living to parasitic adult stage in the life-cycle of diplozoids. In fact, they are visible through the egg shell in the oncomiracidium (Kotenovskii, 1977). Adult paradiplozoids consist of two hermaphroditic specimens fused in permanent copula, each individual bearing four pairs of bivalve clamps on the haptor (Bovet, 1967). Mature specimens release eggs continuously, and from these an oncomiracidium with two pairs of clamps hatches. Infection occurs when a

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Table 1. The dates and season of sampling efforts in the Vaal Dam.

Sampling effort	Date of sampling	Season of sampling
1	8-11-2005	Spring 2005
2	13-02-2006	Summer 2006
3	31-05-2006	Winter 2006
4	8-08-2006	Winter 2006
5	23-08-2006	Winter 2006
6	27-10-2006	Spring 2006
7	31-01-2007	Summer 2007
8	17-04-2007	Autumn 2007
9	9-06-2007	Winter 2007

potential host swims through a group of oncomiracidia. After attachment on the gills of the host, the oncomiracidium develops into a diporpa with two pairs of clamps and can only attain adulthood after fusion with another diporpa, the complete set of four pairs of clamps develop during this phase of maturation.

Lyons (1966) found that the sclerites in the clamps of some monogenean species grew during their life-cycle. Other hard attachment organs such as marginal hooks, anchors, and anchor bars may move from a peripheral to central position in the stages of development following the free-living one (Malmberg, 1990). In diplozoids, attachment clamps, develop sequentially from a single pair at the terminal end of the opisthaptor to the anterior end of the opisthaptor, until four pairs are present; after the free-living stage. The aim of this study was thus to investigate the duration of adulthood for a *Paradiplozoon* sp. *in situ* by applying GM to the anatomy of attachment clamps. Such information may cast light in finding the duration of diplozoid life-cycle *in situ*.

MATERIALS AND METHODS

Yellow fish and ectoparasitic *Paradiplozoon* sp. were sampled at the Vaal Dam from November 2005 to June 2007, as indicated in Table 1. A total of 125 yellow fish were collected using 70 and 90 mm mesh size gill nets, in the vicinity of University of Johannesburg (U.J.) Island (26° 52′ 29.70″ S 28° 10′ 21.46″ E) situated in the north-eastern reaches of the Vaal Dam. This yellow fish sample obtained in the Vaal Dam comprised 125 Vaal-Orange smallmouth yellow fish [*Labeobarbus aeneus* (Burchell, 1822)], and 46 Vaal-Orange largemouth yellow fish [*Labeobarbus kimberlyensis* (Gilchrist and Thompson, 1913)] specimens. Using a limited mesh size collection of a morphometric similar group of host was allowed, thereby eliminating a possible effect of host size on the parasite's attachment structure.

The yellow fishes were killed by a single cut through the spinal cord and the gills were dissected free from the head and placed into Petri dishes containing Vaal Dam water. Host fish species identification was done visually according to their external anatomy and a guide by Skelton (2001). Examination of the gill filaments was consistently performed at magnifications of 16 and 50 times with a WILD M5 stereomicroscope, using Dumont forceps and a Camel's hair fine paintbrush. Following their recovery, *Paradiplozoon* sp. Were killed in 7 0% ethanol, and fixed in warm aceto-alcohol-formalin. Samples were stored in 10% neutral buffered formalin (NBF). The total sample of adult *Paradiplozoon* sp. collected, suitable for GM, amounted to 149 specimens.

Specimens were stained in Semichon's Acetocarmine before being dehydrated and mounted on glass slides to view the sclerites comprising the attachment clamps. Digital micrographs of one randomly selected haptor on each specimen were acquired at 200 times magnification using a Standard 16 Zeiss light microscope and a Nikon L11 digital camera at a resolution of 300 Dots per Inch (DPI) (2816 pixels by 2112 pixels). The second clamp (penultimate) was measured as these are formed prior to the parasite hatching from the egg and the possible influence of the host on clamp morphology is thereby minimised.

The three landmarks (LM's) selected per specimen, were at the junctures between the terminal ends of the median sclerites of the posterior jaw bone on the most terminal and second most terminal attachment clamps (Milne and Avenant-Oldewage, 2006). In total, six selected homologous LM's, present from the larval to adult stage, were digitized as *X:Y* coordinates from the stored digital micrographs using tpsDIG v.2.0 (Rohlf, 2005). The position of the two clamps used to plot the landmarks is shown in Figure 1. Figure 2 graphically shows the six points which were digitised as landmarks as *X:Y* coordinates for all the specimens. The scale factor used for measurements was equivalent to 1 mm per 384 pixels. The data was pooled for *Paradiplozoon* sp. found on *L. aeneus* and *L. kimberlyensis*, and then stratified according to the sampling season.

The tpsSmall v.1.2 (Rohlf, 2003) software package was used to compute the orthogonal least-squares Procrustes average configuration for the 149 *Paradiplozoon* sp. sclerite LM's using the generalized orthogonal least-squares procedure. This procedure confirmed that the amount of shape variation was small enough (Procrustes distance = 0.22) to proceed with statistical analyses in Kendall's shape space, using Morphologika^2 Tools for Shape Analysis version 2.5 (O'Higgins and Jones, 2006). Generalised procrustes analysis (GPA) was performed in the LM dataset and principal components analysis (PCA) of Kendall's tangent space coordinates was used to examine variation in size and shape.

Multivariate analysis of variance (MANOVA) was applied to determine the effects of independent categorical variables (that is, season and centroid size) on multiple continuous dependent variables (that is, principal components describing size and shape variation). The water temperature of the Vaal Dam was recorded at the time of sampling.

RESULTS

The percentage variance explained by the PC's is a

Figure 1. An unstained adult *Paradiplozoon* sp. specimen was mounted and photographed in lacto-phenol. The arrows indicate the two clamps on the opisthaptor that were selected to be the position where landmark coordinates were placed. Scale bar = 100µm.

function of the sample size, the number and type of landmarks included in the analysis. PC1 accounts for the largest proportion of variation in the data set (that is, 69.23%). This reflects a common direction of ontogenic shape change during ontogeny in statistical shape space. Statistical shape analysis of the six clamp sclerite LM's resulted in nine non-zero eigenvectors.

PC1 explains the variation in the general shape consensus along the longitudinal axis dividing the middle point in the middle of the configuration into two (that is, between the median sclerites of the posterior jaw). The second PC (that is, PC2) explained 14.37% of the remaining variance with the third accounting for 9.98% of the variance. The first three principal components account for 93.49% of sample variance, whilst with the addition of a further six principal components are able cumulatively to explain 100 percent of sample variation.

The lower values for PC1 represent individuals with smaller rounder clamp sclerites. *Paradiplozoon* sp. individuals with more positive PC1 values bear relatively larger median sclerites of the posterior jaw. When PC2 and PC3 are compared with PC1 it can be seen that the shape variation in the sclerites' parallel topographical orientation differs from each other for the first and second clamp. Sclerite conformations clustered to the bottom of the *Y*-axis are more parallel than those LMs plotted in the positive portion of the PC plot along PC2 and PC3.

The change in size and shape was quantified by graphing PC's for each seasonally collected landmark configuration against the season of collection. The ontogenic allometry was quantified using centroid size and PC1. The ontogenic allometry result indicated that each season differed significantly in slope at the intercept. The plot indicated by Figure 3B shows that ontogenic shape change is constant, reflecting a positive correlation for all *Paradiplozoon* sp. specimens.

The dataset clusters (A to D) reflected that winter specimens were the terminally left along the *X*-axis (i.e.

Figure 2. The arrows indicate the six points, between the junctures of the sclerites, where the landmark coordinates were digitised. The specimen was stained with Semichon's acetocarmine. Scale bar = 100µm.

centroid) whereas spring specimens were clustered on the top right of the graph as in Figure 3. It can be shown that the seasonal *Paradiplozoon* sp. clusters increase in size and shape according to the plots and clustering of specimens in the following order from smallest to largest; spring > summer > autumn > winter in Figure 3 B. A MANOVA on PC1 and Centroid size showed a positive and significant correlation correlation between PC1 vs. centroid size ($p < 0.001$, $r^2 = 0.973$; $F_{3,146} = 5240.1$; Wilk's $\lambda = 0.028$, $p < 0.001$, Pillai's Trace = 0.973 Hotellings-Lawley Trace = 35.67, Roy's largest Root = 35.67) as indicated in Figure 4B.

This model suggests that the smallest new generation of immature *Paradiplozoon* sp. specimens attaches to its host mainly in spring, maturing through summer, surviving through autumn until winter when their attachment clamps are the largest size. This indicates that there is a critical inter-season period in the life-cycle of *Paradiplozoon* sp. between winter and spring when specimens of the parent generation die off. This ensures that adult *Paradiplozoon* sp. of the first generation do not directly compete with newly hatched oncomiracidia and diporpa of the second generation in spring. It further suggests that the *in situ* life-cycle duration of *Paradiplozoon* sp. upon yellow fish in the Vaal Dam lasts for a single year (that is, annual).

DISCUSSION

The principle components in this study are shape variables resulting from linear combinations of length measurements of *Paradiplozooon* sp. sclerites junctures. The subsequent PCA in this study provided an ordinal seasonal *Paradiplozoon* sp. rank in size and shape differences in the sample obtained during 2005 to 2007. There is evidence of seasonal size and shape changes in other families of monogeneans (Mo, 1991; Dávidová et al., 2005) and also an indication that environmental aquatic pollution causes structural alterations and changes in the number of attachment clamps in four diplozoid species (Šebelová et al., 2002). This is however

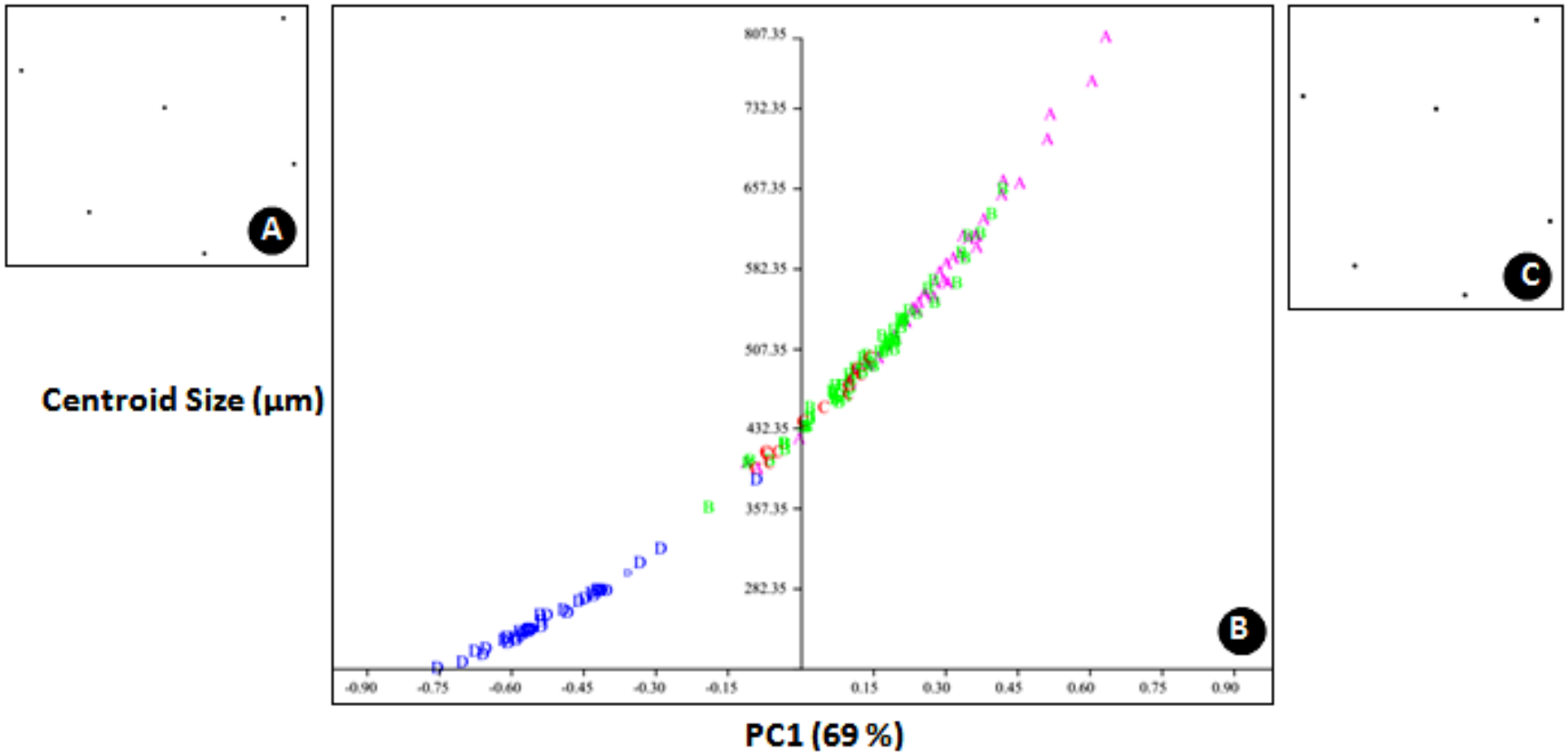

Figure 3. A, Scaled plot showing the form of landmarks for specimens on the extreme bottom left of the PCA plot for specimens collected in winter. B, PCA plot depicting the model between PC1 and LM centroid size (µm). C, Scaled plot showing the form of landmarks for specimens on the extreme top right of the PCA plot for specimens collected in spring. A=Spring, B=Summer, C=Autumn, D=Winter.

the first time that an ordinal seasonal change in size and shape has been recorded in the Diplozoidae.

Differences between the seasonally surveyed *Paradiplozoon* sp. data groups were assessed by Goodall's F-test (that is, Multivariate F-test) using the procrustes fitted data. The result confirms the findings of Kearn (1968), where an increase in the size of the marginal hooks of dactylogyrids, during post-larval development was recorded. More specifically the finding for *Paradiplozoon* sp. aggress with those of Mo (1991) and Dávidová et al. (2005), which found an inverse relationship between the length of the opisthaptoral hard parts in *Gyrodactylus salaris* and *Gyrodactylus rhodei* and water temperature. The trend in size variation for *Paradiplozoon* sp. follows that of *G. salaries* and *G. rhodei*, in that the sclerites are the largest in the winter when the water in the Vaal Dam was cold (17.4 to 18.8°C), and smallest when the water was warm in summer (23.4 to 25.8°C). Winter was also the season when the parasites where at the oldest. The MANOVA used to test the differences between centroid distances and seasonal group relationship was able to signify-cantly demonstrate this.

Some studies have been able to distinguish

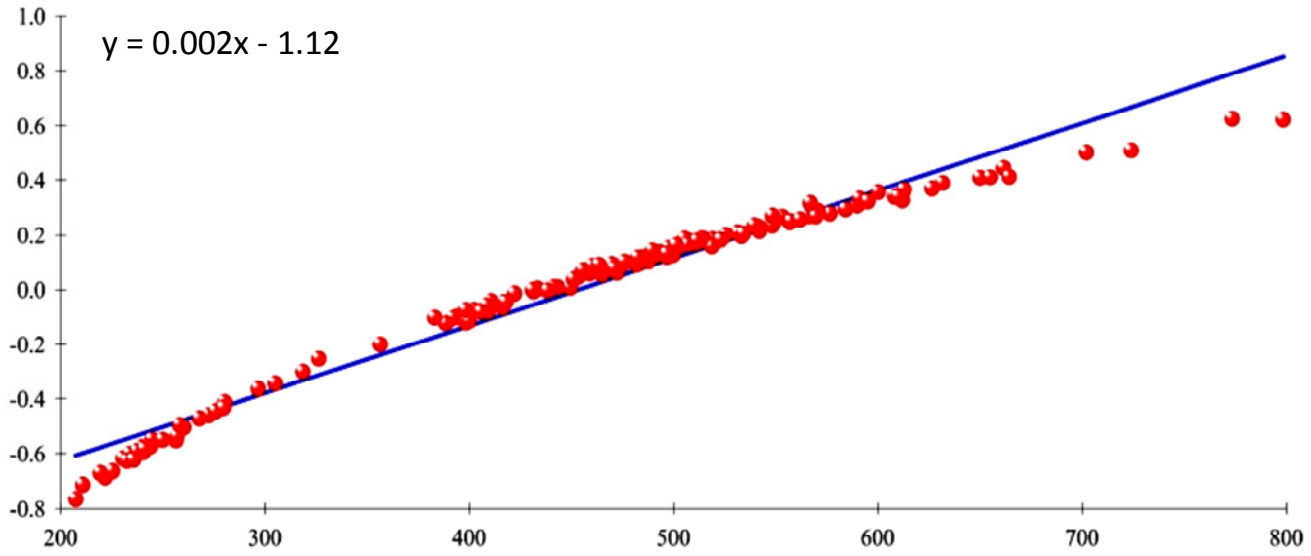

Figure 4. A multivariate regression plot of PC1 against LM centroid size showing a positive correlation between these variables.

between closely related monogenean species based on the metrics of the permanent attachment organs. Unlike other monogeneans, diplozoids lack sclerified genetalia which are used for taxanomic identification. The anatomy of the permanent attachment clamps are used as a morphological criterium for identification of diplozoid species (Khotenovskii, 1985; Le Brun et al., 1988). In this regard, the measurements of the sclerotized attachment organs of gyrodactylid and *Lamellodiscus* monogenean species were useful (Přikrylová et al., 2008; Poisot and Desdevises, 2010). It is suggested that the measured rate of change in the dimensions of the attachment clamps of *Paradiplozoon* sp. may shed light in future to separate it from other diplozoid species.

ACKNOWLEDGEMENTS

The University of Johannesburg and the National Research Foundation is thanked for funding this project.

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