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Diagnostic Morphometry: Identification of Helminth Eggs by Discriminant Analysis of Morphometric Data

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ABSTRACT: The perimeters of strongylid eggs were digitized electronically, the resulting coordinate point data converted into 8 geometric parameters, and these parameters subjected to stepwise discriminant analysis. This procedure distinguished 92% of Ancylostoma caninum eggs from those of Uncinaria stenocephala and 100% of U. stenocephala eggs from those of A. caninum. When applied to strongylid species infecting sheep, eggs of the following species were correctly classified as follows: Bunostomum trigonocephalum (60%), Chabertia ovina (58%), Cooperia curticei (60%), Haemonchus contortus (67%), Nematodirus battus (98%), N. filicollis (100%), N. spathiger (96%), Oesophagostomum columbianum (72%), Oe. venulosum (100%), Teladorsagia circumcincta (49.5%), Trichostrongylus axei (76%), and T. colubriformis (73%). Comparable accuracy of classification was achieved for equine parasites only after pairs of species having morphometrically identical eggs were pooled before analysis and all of the cyathostomin categories except Gyalocephalus capitatus further pooled after analysis. The final diagnostic categories, "large strongyle", Triodontophorus serratus, T. tenuicollis, "small strongyle", G. capitatus, and Trichostrongylus axei, were correctly classified in 76.6, 74.3, 83.0, 79.1, 100, and 80.0% of the cases, respectively. Haemonchus contortus and T. colubriformis eggs recovered from fresh feces were significantly wider than eggs dissected from the uteri of females worms collected from the alimentary tracts of the same sheep. Thorough mixing of sheep feces and 10% formalin (1 gm/30 ml) yielded fixed

H. contortus and T. colubriformis eggs that were morphometrically indistinguishable from fresh ones.

KEY WORDS: Diagnostic morphometry, quantitative microscopy, digitizing tablet, discriminant analysis, coprology, fecal examination, helminth eggs, hookworm eggs, strongylid eggs, Strongyloidea, Ancylostomatoidea, horse, sheep, dog.

Antemortem diagnosis of helminth infections is based primarily on identification of eggs in fecal preparations. In many cases, specific or generic diagnosis can be reached by an experienced diagnostician on the basis of qualitative characteristics alone. When qualitative differences fail, micrometry may provide sufficient additional information to allow differentiation. Unfortunately, in many very important cases, the dimensions are not sufficiently distinctive to permit differentiation by comparison with tabled values of ranges even if simple statistical analysis is brought to bear on the problem. Under such circumstances, micrometry falls short of providing sufficient information to yield a positive diagnosis.

The eggs of the important nematode superfamilies Strongyloidea, Trichostrongyloidea, and Ancylostomatoidea are thin-walled, ovoid or ellipsoid in shape, and contain an embryo in the morula stage of development when laid. With few exceptions exemplified by the genera Nematodirus in ruminants and Gyalocephalus in horses, both of which are exceptionally large, the generic or specific identity of individual strongylid eggs usually cannot be established by inspection or even by micrometry, so similar are they in appearance and size (Shorb, 1939). Most of the serious effort to differentiate helminth eggs by mathematical analysis of their dimensions has been expended on the eggs of strongylids (Tetley, 1941; Krug and Mayhew, 1949; Cunliffe and Crofton, 1953; Christie and Jackson, 1982). However, there remain many diagnostic dilemmas to be resolved beyond the

order Strongylida and we hope that the technique described here will find much broader application in coprology.

Differential diagnosis based on identification of strongylid infective larvae was pioneered in sheep by Dickmans and Andrews (1933) and Andrews (1935) and later applied to cattle by Keith (1953). This approach affords reliable qualitative differentiation of strongylid larvae at the generic level. Russell (1948), for example, made brilliant use of larval identification in her study of the development of nematode parasite burdens in thoroughbred foals.

The principal disadvantages of diagnosis based on larval culture and identification are that it requires the attentions of a highly skilled diagnostician and is very time consuming and tedious to perform routinely on any considerable scale. Other drawbacks of diagnostic larval culture include a delay of at least a week before cultures can be read and the inherently non-quantitative nature of the results. The non-quantitative nature of diagnostic culture methods stems from the differential response of the several species of developing larvae to the ambient cultural conditions which are difficult to control precisely. Therefore, differential counts of the species of infective larvae present in a culture do not provide an accurate estimate of the relative abundances of species of strongylid eggs in the original sample (Cunliffe and Crofton, 1953).

Identification of preinfective larvae (Whitlock, 1959; Ogbourne, 1971) reduces the delay inherent in methods based on

identification of infective larvae but the other disadvantages remain.

The morphologic and geometric characters of the egg afford the most direct approach to differential diagnosis of strongylid infections. The first detailed and comprehensive effort to identify the eggs of ruminant parasites appears to have been that of Shorb (1939) who published a key to the genera. This was a pioneer effort and included detailed investigation of the composition and dimensions of eggs of many species of parasites. Unfortunately, Shorb's key required accurate measurement of shell thickness in the range 1 to 2 um, with distinctions drawn as precisely as 0.1 um; certainly a difficult undertaking with the best of optical equipment. Shorb's key also required counts of the number of cells in the embryo of the freshly passed egg. For example, eggs with combined thickness of the second and third layers of the egg shell equal to 1.5 um and with 24 cells or less were identified as Bunostomum whereas like eggs with 24 or more cells were identified as Haemonchus. The identity of eggs with exactly 24 cells apparently remained problematical. Shorb's key also required the user to reach subjective decisions regarding nuances of shape such as "Eggs tapering toward one or both ends", etc., a process that leaves even the experienced diagnostician with an uncomfortable feeling. Although Shorb's key may have enjoyed very limited if any adoption by parasitologists working with ruminant parasites, it certainly marked the starting point of all serious efforts in direction of differentiating strongylid

nematode eggs.

Tetley (1949) showed, by bivariate graphs, how certain individual strongylid eggs belonging to a specified set of 10 species could be identified. However, eggs with length and width coordinates lying in areas of overlap between species predominated and were unidentifiable by this means. Therefore, in effect, many eggs had to be measured in order to identify a few and the procedure was generally inapplicable to mixed infections.

Cunliffe and Crofton (1953) developed a procedure by which large samples of eggs of 8 species of sheep nematodes could be differentiated on the basis of "the statistical chances of eggs of different species falling into particular size classes". "The accuracy of differentiation increases with the number of eggs classified, but the results can only be regarded as an estimate which is more accurate for the more numerous species in any sample." Again, the onerous nature of the actual microscopic work and the uncertainty of the results have led few to adopt the technique of Cunliffe and Crofton.

The most recent published account of and effort to identify strongylid eggs is that of Christie and Jackson (1982). By combining egg dimensions and information regarding the state of their embryonic development after specified conditions of incubation, these authors claimed to be able to identify more that 90% of Ostertagia spp. and Trichostrongylus vitrinus eggs but admitted that, "For other species supporting information from larval cultures may be needed but this need will vary according

to the composition of the sample".

Measurements of nematode eggs are presented in almost every relevant taxonomic work but the above 4 reports represent the major efforts that have been applied to the problem of differential diagnosis of ruminant strongylid infections. Unfortunately, none of these has provided an accurate, efficient, and practical solution.

The electronic digitizing tablet affords a convenient means of collecting measurements of nematode eggs and the digital computer and multivariate statistical analysis enable us to draw considerably more detailed conclusions from those data than we have been able to do in the past. We report here the results of our initial investigations of multivariate analysis (specifically, stepwise discriminant analysis) of the geometric parameters length, width, area, perimeter, and areas and arc lengths of specified polar areas of eggs, these having been derived from complete sets of coordinate points of the perimeters of eggs collected by a digitizing tablet. Inasmuch as our information consists basically of the contour of the egg, we have accepted the term morphometry, the "measurement of external form" (Webster's New International Dictionary, 2nd Ed., 1935) to denote the general process involved.

Materials and Methods

Morphometric Procedures

Instruments used in collecting coordinate point data included a compound microscope (Zeiss Standard or Zeiss Photoscope I) fitted with a drawing tube (Zeiss Camera Lucida for Standard microscope or Zeiss Camera Lucida for Photoscope I), a digitizing tablet with cursor (IBM 5083 or Zidas), a lamp to illuminate the surface of the digitizing tablet, and a microcomputer of 640 kilobyte random access memory and 10-30 megabyte hard disk memory (AT&T PC6300 or IBM PCXT). With the drawing tube focused on the surface of the digitizing tablet and illumination of the latter suitably balanced with illumination of the microscopic field, the image of an egg under the objective lens of the microscope was made to appear to lie at the surface of the digitizing tablet. As the contour of this image was traced with the cursor, all coordinate points were transmitted to the microcomputer and recorded in memory as a point capture file consisting of several hundred pairs of coordinates, the ordinal number of the measurement, and an end marker.

All measurements were conducted at X1000 magnification except when Nematodirus eggs were the subject, in which case magnification was reduced to X640 to accommodate the image in the microscopic field. Magnification was carefully calibrated before every series of measurements by comparing the scale of an objective micrometer with that of a millimeter rule lying on the

surface of the digitizing tablet. Scales were compared at both top and bottom of the microscopic field so that any distortion due to misalignment of the optical axis of the drawing tube could be detected and corrected.

Conversion of Coordinates into Geometric Parameters

Coordinate point data were converted into geometric parameters (length, width, area, perimeter, areas and arc lengths of poles) with a microcomputer program (PARAM) developed by Miles McCreadie. PARAM first uses the distance formula based on the Pythagorean Theorem to determine the distance between every pair of points on the contour (Fig. 1A). The greatest of these distances is the length or major axis which is inclined at the angle (theta) whose tangent is equal to the ratio of the difference in ordinates to the difference in abscissas of the end points, i.e., the two points of intersection of the major axis with the perimeter (Fig. 1B). The inclinations (alpha) of all line segments drawn from the end point with the smaller value of X to all points on the contour and represented by "d" of Fig. 1C are also determined. PARAM then rotates the egg to the horizontal by translating each point on the perimeter by means of the trigonometric equations of Fig. 1C. Width is then measured as the greatest difference between ordinates of all points lying on the contour of the rotated egg (Fig. 1D). Area is estimated as the sum of the areas of all trapezoids inscribed between the contour and the major axis and perimeter is estimated as the sum of all distances between adjacent points on the contour (Fig. 1E). Areas

and arc lengths of each pole are calculated in the same manner, each "pole" defined to include one-twentieth of the major axis (Fig. 1F). PARAM is written in Fortran and is available from the senior author as the source code or as an IBM-compatible program on double sided, double density diskettes at the cost of reproduction and mailing. The senior author will also be happy to assist those seriously interested in developing a diagnostic morphometry work station with operational details and morphometric data.

PARAM works well for objects with convex contours but does not deal effectively with concavities because the geometric analysis used is appropriate only to shapes made up of convexities and straight lines. Even the gentle concavity of a heat-relaxed strongylid larva is not measured accurately by PARAM. Circles and triangles are measured accurately, but PARAM estimates the diagonals of squares, parallelograms and certain trapezoids instead of the length of the sides. The reason becomes apparent when the above sequence of steps are thought out in relationship to these geometric forms.

Stepwise Discriminant Analysis.

In the normal operation of the discriminant analysis program (P7M, available from BMDP Statistical Software, 1964 Westwood Blvd., Suite 202, Los Angeles, CA 90025), the variables that lend the greatest separation of the species groups are automatically chosen for inclusion in the discriminant function. For some comparisons, one variable will prove sufficient, while for other

comparisons, nearly all the variables will be included in the analysis. Once the variables that are to be included have been selected, the discriminant functions are calculated as the combinations of the selected variables that best separate the species groups. Specifically, linear combinations of the selected variables are considered and the coefficients in the linear combinations are chosen to maximize the ratio of the between group to within group variation (Johnson and Wichern, 1988, Sec. 11.8). The discriminant functions calculated in such a manner can then be used to sort the eggs into groups representing the species under consideration.

A note on terminology is appropriate here. Statisticians refer to the process of sorting cases into groups on the basis of discriminant analysis as "classification". As long as the analysis is restricted to groups of known identity, this use of the term is entirely appropriate in a zoological sense, inasmuch as it is an inductive process similar to the classification of known taxa. However, when we present discriminant analysis with an "unknown" (statistically, any data set not included in the calculation of canonical coefficients employed to sort its cases) the process becomes deductive rather than inductive and is properly termed "identification" instead of "classification". We observe the distinction between identification and classification in both statistical and zoological senses.

Specimens

Strongylid eggs of known identity were obtained for

morphometry from the uteri of adult female specimens and from the feces of hosts with purported single species infections of strongylid worms. Dissection of adult female worms was carried out on both fresh and fixed specimens. The usual and more satisfactory preservative was 10% formalin, but the only available specimens of Cooperia curticei were in 70% ethanol. Certain small clear specimens such as fresh Trichostrongylus colubriformis, eggs were measured in situ.

Artificial Infections. One lamb was infected with 3000 infective larvae of Haemonchus contortus and another with 6000 infective larvae of Trichostrongylus colubriformis; both infections became patent 21 days later. Samples of rectal feces were collected on the first day of patency and at intervals of 12, 14, 20, and 31 days thereafter. On day 31, the lambs were slaughtered, the purity of infection established by examination of their alimentary tracts, and worm specimens collected so that dimensions of uterine and fecal eggs could be compared.

Formalin Fixation of Fecal Specimens. As a standard procedure, 10 g feces were suspended in 75 ml water and the suspension strained through a sieve with 1 mm apertures. The solid material retained by the sieve was compressed, washed with an additional 75 ml water, and compressed again. The resulting 150 ml of egg suspension free of coarse debris was mixed with 150 ml 20% formalin solution (20 ml stock 37% formaldehyde solution + 80 ml water) to yield a final concentration of 10% formalin. Fecal specimens from other laboratories usually contained intact

fecal pellets and so were not preserved in the manner specified above. Eggs from fresh fecal specimens were examined when the opportunity arose.

Sets of Strongylid Egg Data. Morphometry data representing the following 12 species of ovine strongylids was analyzed as a set to evaluate discriminant analysis in multidimensional sample space; abbreviations are those used in tables, the number of eggs representing each species is indicated: Bunostomum trigonocephalum (BUTG, 25), Chabertia ovina (CBOV, 50), Cooperia curticei (CPCR, 50), Haemonchus contortus (HMCT, 100), Nematodirus battus (NDBT, 100), N. filicollis (NDFL, 20), N. spathiger (NDSP, 100), Oesophagostomum columbianum (OGCN, 25), Oe. venulosum (OGVN, 50), Teladorsagia circumcincta (OSCC, 99), Trichostrongylus axei (TRAX, 25), and T. colubriformis (TRCL, 100).

Morphometry data representing the following 13 species of equine strongylids was analyzed as a set to evaluate discriminant analysis in multidimensional sample space; abbreviations are those used in tables, the number of eggs representing each species is indicated: Strongylus edentatus (SGED, 55), S. vulgaris (SGVU, 99), Triodontophorus serratus (TDSR, 70), T. tenuicollis (TDTC, 100), Cylicostephanus calicatus (CSCL, 37), C. longibursatus (CSLG, 52), C. minutus (CSMN, 100), Cylicocyclus nassatus (CCNS, 100), C. leptostomus (CCLP, 85), Cyathostomum catinatum (CYCT, 100), C. coronatum (CYCN, 100), Gyalocephalus capitatus (GYCP, 20), and Trichostrongylus axei (TRAX, 47).

The nomenclature of Lichtenfels (1975) is followed here with all due respect to the excellent monograph of Hartwich (1986) on the subject of cyathostomin systematics.

Results

Analysis in One Dimension; Differentiation of Eggs of *Haemonchus contortus* and *Trichostrongylus colubriformis*

Distribution of Lengths. One hundred *Haemonchus contortus* eggs differed from 100 *Trichostrongylus colubriformis* in mean length ($P<0.001$, $t_{198df}=13.8$) but not in mean width. A frequency distribution of lengths of eggs of these two species is presented in Fig. 2. A line drawn perpendicular to the X-axis between 81 and 82, which is midway between the mean of 77 for *H. contortus* and the mean of 86 for *T. colubriformis*, divided each population of eggs into 2 subpopulations, one lying on the same side as its population mean and the other lying on the side of the opposite population mean. Eighty-five percent of the *H. contortus* eggs and 86% of the *T. colubriformis* eggs lay on the same side as their respective population means. Stepwise discriminant analysis of the same data yielded an identical result, i.e., 85% correct classification of *H. contortus* eggs and 86% correct classification of *T. colubriformis* eggs.

Analysis in Two Dimensions; Differentiation of Eggs of *Ancylostoma caninum*, *Uncinaria stenocephala*, and *Haemonchus contortus*

In Fig. 3A, widths versus lengths of 100 eggs of each of the three species *Ancylostoma caninum* (ANCN), *Uncinaria stenocephala* (UNST), and *Haemonchus contortus* and ellipses bounding 95% of the data points are presented for each species. The populations of *A. caninum* and *U. stenocephala* are seen to overlap each other and to

completely engulf H. contortus. A plot of the canonical variables based on the two geometric parameters selected by discriminant analysis (i.e., perimeter vs. length, Fig. 3B) also conveys the impression that the classification of A. caninum eggs must be completely confounded with the other two species, but in fact, discriminant analysis classified the majority of each species correctly (Table 1). In Table 1, we report the jackknifed classification matrix. A simple classification matrix tends to be overly optimistic in estimating the correct classification rate because the eggs being classified are included in the set used to determine the discriminant functions. In jackknifing, each egg in turn is left out of the calculations and classified using a discriminant function based on all the other eggs. This gives a truer estimate of the correct classification rate. Discriminant analysis estimated correct classification rates of 90% for A. caninum, 73% for U. stenocephala, and 75% for H. contortus (Table 1).

When A. caninum and U. stenocephala were compared with H. contortus absent, correct classifications based on length rose to 92% and 100%, respectively. The difference in correctness of classification was due to the presence of 8 "long" eggs in the A. caninum set misclassified as U. stenocephala; the U. stenocephala set contained no "short" ones to be misclassified as A. caninum.

Multidimensional Analysis; Differentiation of 12 Species of Ovine Strongylids and 13 Species of Equine Strongylids

Ellipses including 95% of the points representing width

vs.length of 12 species of ovine strongylid eggs are plotted in Fig. 4A; the 744 data points have been deleted to avoid cluttering the figure. Ellipses including 95% of the points representing canonical variables 1 and 2 based on 7 geometric parameters (area, length, width, area pole 1, perimeter, arc length pole 1, arc length pole 2) selected by discriminant analysis are presented in Fig. 4B and the corresponding jackknifed classification matrix is presented in Table 2. The overall correctness of this classification was 75.8% and varied from 49.5% for Teladorsagia circumcincta to 100% for Nematodirus filicollis and Oesophagostomum venulosum. The compactness of the distribution of Oe. venulosum data points and the relatively wide dispersion of those of T. circumcincta are largely responsible for the contrasting accuracy of classification of eggs of these 2 species.

Ellipses including 50% of the points representing width vs.length of 13 species of equine strongylid eggs are plotted in Fig. 4C, ellipses including 50% of the points representing canonical variables 1 and 2 based on 4 geometric parameters (area, length, width, perimeter) selected by discriminant analysis is presented in Fig. 4D, and the corresponding jackknifed classification matrix is presented in Table 3. The 965 data points have been deleted from Figs. 8 and 9 and the domain of the ellipses reduced from 95% to 50%; otherwise, the high degree of overlap in dimensions of equine strongylid eggs would have rendered these figures illegible.

Pooling of Nearly Identical Groups. Using the data set of the previous paragraph, those species having nearly identical eggs were pooled as follows: Strongylus edentatus + S. vulgaris (EDVU), Cylicostephanus calicatus + Cylicocyclus nassatus (CLNS), Cylicostephanus minutus + Cylicocyclus leptostomus (MNLP), and Cyathostomum catinatum + C. coronatum (CTCN). These pooled categories were again subjected to discriminant analysis along with TDSR, TDTC, CSLG, GYCP, and TRAX. The resulting classification matrix is presented in Table 4. Classification remained 100% in the case of Gyalocephalus capitatus and was substantially improved for Triodontophorus serratus, T. tenuicollis, Cylicostephanus longibursatus, and Trichostrongylus axei. Classification of the "large strongyles" (EDVU) was raised to a level of possible practical utility (76.6%) but classification the 3 "small strongyle" groups (CLNS, MNLP, and CTCN) remained unsatisfactory. Therefore, the number of cases classified as C. longibursatus (CSLG) plus these 3 groups (see boxes, Table 4) were added together to form a pooled "small strongyle" group (Gyalocephalus capitatus not included) to which 454 out of a total of 574 (79%) were correctly assigned.

Identification of "Unknown" Eggs

Data from a fecal specimen containing an unknown assemblage of nematodes are entered into the program for identification only, i.e., the "unknown" data do not enter into calculation of the discriminant functions but are merely identified on the basis provided by analysis of the reference set of eggs. The "unknown"

eggs referred to in this section were recovered from the feces of animals with purported single species artificial infections or natural infections of known composition but were entered into the program in exactly the same manner as true unknowns would have been. The identification of these "unknown" eggs was therefore governed by the geometric information supplied by the reference set and the percentage correctly identified therefore provided an unbiased estimate of the diagnostic accuracy of the procedure.

Sheep Parasites. One hundred eggs each of Haemonchus contortus (Beltsville, MD, U.S.A.), Teladorsagia circumcincta (Armidale, N.S.W., Australia), and Trichostrongylus colubriformis (West Chester, PA, U.S.A.) were identified 66%, 53%, and 80% correctly when subjected as "unknowns" to discriminant analysis based on the reference set of Table 2. When all diagnostic categories except the 3 species presented as "unknowns" were eliminated from the reference set, classification of the reference set and identification of the "unknowns" both improved substantially (Table 5, Fig. 5). Note that "unknown" HMCT and OSCC were identified more precisely than were the corresponding groups in the reference set were classified (Table 5). This is because more of their data values fell closer to the reference set means than did data values of the reference set itself; i.e., these 2 "unknown" sets happened to be more compact and to display less overlap of neighboring reference sets.

Horse Parasites. Four "unknown" samples were subjected to discriminant analysis using the pooled reference set of Table 4;

they consisted of 100 eggs from a pure Strongylus edentatus infection (Baton Rouge, LA), 100 eggs from a pure S. vulgaris infection (Baton Rouge, LA), 100 eggs from a naturally acquired mixed cyathostome infection (Cylicostephanus longibursatus, C. minutus, Cylicocyclus nassatus, and Cyathostomum catinatum, Ithaca, NY), and 100 eggs from a naturally acquired mixed cyathosome infection (same 4 species + C. calicatus, C. goldi, and C. insigne) plus large numbers of Trichostrongylus axei (Ithaca, New York). The nature of the last two samples was determined at necropsy. Identification of these "unknowns" accorded reasonably well with the type of the infection (Table 4).

Procedural Sources of Morphometric Variation

Worm Eggs vs. Fecal Eggs. One hundred eggs from feces of a lamb with the pure Haemonchus contortus infection were compared with 100 eggs dissected from the uteri of 20 H. contortus worms (5 eggs from each of 10 linguiform and 10 smooth morph females). recovered from the abomasum of the same lamb. In addition, 100 eggs from the feces of a lamb with pure Trichostrongylus colubriformis infection were compared with 100 eggs in the uteri of T. colubriformis worms recovered from the small intestine of the same lamb. Fecal eggs of both H. contortus and T. colubriformis were significantly greater in width than uterine eggs of the same species. The degree of difference was greater for T. colubriformis (46.1 vs. 41.8 um, $P<0.01$, $t_{198df}=11.7$) than for H. contortus (44.7 vs. 43.8 um, $P<0.01$, $t_{198df}=3.19$).

Fresh Eggs vs. Formalin-Fixed Eggs. There was no statistically significant difference between the dimensions of fresh Haemonchus contortus eggs measured promptly and eggs from the same fecal sample subjected to fixation in 10% formalin according to the procedure described in Materials and Methods followed by storage at room temperature for several days. However, when 5% formalin was substituted, the formalin-treated eggs were significantly wider (49.6 vs. 44.7 um, $P<0.01$, $t_{198df}=17.6$) and had advanced as far as the "tadpole" stage of development.

Discussion

Ideally, we would like be able to identify each egg and estimate the relative abundance of each species in a sample of feces. Unfortunately, great similarity exists in the shape and dimensions of many important species of eggs, appreciable variation may occur among different isolates of the same species, and changes in dimensions may be induced by differences in handling, fixation, and storage of specimens, all leading to greater overlap and decreased diagnostic resolution. The most realistic relative abundances are obtained when the reference set contains the same species as the unknown. This is sometimes possible as, for example, when the species composition involved in a natural or experimental epidemic is known.

Differentiation of Strongylid Eggs

Comparison of Haemonchus contortus and Trichostrongylus colubriformis. In the simple case provided by the distribution of lengths of Haemonchus contortus and Trichostrongylus colubriformis eggs, the differences between the length of a particular egg and the population mean for each species provided a criterion that allowed correct classification of 85 of 100 H. contortus and 86 of 100 T. colubriformis eggs. Application of stepwise discriminant analysis to this simple case yielded an identical result automatically but by essentially the same process.

Hookworm Eggs. The eggs of Ancylostoma caninum and Uncinaria stenocephala are sufficiently distinctive that a qualitative

diagnosis of either infection or of a mixed infection can be based on simple length measurements of a few eggs. However, morphometry and discriminant analysis makes it possible to estimate conveniently and accurately the relative abundances of eggs of these two species in a mixed infection and, at least in certain cases, to detect spurious parasites. In the example presented, U. stenocephala eggs resemble those of Haemonchus contortus more closely than those of A. caninum, yet all 3 species could be distinguished morphometrically with reasonable accuracy. Detecting spurious parasites requires a suitable hypothesis, e.g., that Haemonchus contortus eggs might reasonably be found contaminating the feces of a dog with access to sheep feces or offal during summer, so that a set of H. contortus eggs can logically be included in the reference set under such circumstances.

The classification of equine strongylids proved a formidable task. As Figs. 8 and 9 and Table 3 reveal, only Gyalocephalus capitatus was classified 100% correctly. Triodontophorus serratus, T. tenuicollis, Cylicostephanus longibursatus, and Trichostrongylus axei were also classified reasonably well, but the classification of the other species was disappointing. Classification of Cylicostephanus calicatus and Cyathostomum catinatum was at or below the a priori level; i.e., one could do better by drawing numbers out of a hat. The reason for the particularly poor classification of these 2 species is once again the great dispersion and intersection of their data sets with

those of several other species; too many of the data points of C. calicatus and C. catinatum lay closer to the population means of other species than to their own. Correct classification of 80.8% of Cylicostephanus longibursatus might not be anticipated considering its degree of overlap with neighboring species but its eggs were more uniform in size than the others and that is what accounts for the precision of classification in this case.

Advantages of Pooling Diagnostic Categories. Stepwise discriminant analysis deals effectively with overlap in dimensions of different species but it cannot deal with identity of dimensions. It is tempting to combine species into groups that represent practical diagnostic categories. For example, "large strongyles", "small strongyles", "Triodontophorus", "Gyalocephalus", and Trichostrongylus" would satisfy most clinical diagnostic needs. For the purpose of analysis, however, it is important to pool only those data sets that are virtually indistinguishable morphometrically. When this is done, a general improvement in classification is achieved as can be appreciated by comparing Tables 3 and 4. Classification of species left standing as independent data groups is also uniformly improved. On the other hand, when species with morphometrically dissimilar eggs are pooled, the dispersion of data points results in increased misclassification in all groups and a general decrease in precision.

In any case, there is no harm in combining species into desired diagnostic categories after analysis is completed. Adding

all cases classified as Cylicostephanus longibursatus and the 3 pooled cyathostome groups (i.e., all data at intersections of CSLG, CLNS, MNLP, and CTCN in Table 4) and dividing by the 574 total cases representing these groups, resulted in 79% correct classification as "small strongyle".

"Unknowns". In general, the "unknown" samples tended to be identified with about the same degree of precision with which species (individual and pooled) of the reference set were classified (Tables 2 and 4). The relative performance of reference and "unknown" sets of the same 3 species of sheep parasites (Table 5) can probably explained on the basis of the slight differences in the degree of dispersion of their respective distributions (Fig. 5); the more compact the distribution, the greater the precision of identification.

The more categories available for classification and identification, the greater the potential for misclassification and misidentification. Optimum results are achieved when the reference set contains exactly the same species or species groups as the unknown samples to be identified. Usually, this ideal would only obtain in experimental situations in which the composition of artificial infections is under the control of the investigator, at least at the outset. In such a system, diagnostic morphometry would provide an estimate of relative abundance/fecundity of the species present with unprecedented accuracy. At the very least, knowledge of the life histories of the parasites involved should be applied where possible to delete

irrelevant categories from the reference set. For example, removing Oesophagostomum columbianum from the reference set when analyzing data from samples collected outside the geographic range of this parasite effectively removes the most serious pitfall to the correct identification of Haemonchus contortus eggs (Table 2). For another example, the only advantage of including the EDVU category in analysis of a fecal sample from a foal under 6 months of age might be to assess its tendency to practice coprophagy (Russell, 1948). In principle, any species or category may be removed from the reference set for any sound reason. The result will usually be a substantial improvement in precision of classification and identification.

The pattern of classification of each species provides important clues which can be used to enhance interpretation of the identification pattern of the unknown set. For example, in Table 5, 97% HMCT and 99% OSCC in the identification patterns of "unknowns" clearly represent relatively pure infections, but we might question the purity of 81% TRCL with 17% of its eggs identified as HMCT and 2% as OSCC. However, classification of the reference set is seen to follow an almost identical pattern and reassures us as to the purity of the TRCL "unknown" set.

A high percentage value (e.g., above 80%) in a particular diagnostic category represents a condensed clustering of data values within the boundaries of that category; a situation that is very unlikely to arise through scatter from other categories. In priciple, therefore, the highest percentage value in any

identification matrix represents a virtual certainty that the corresponding species of strongylid is actually present in the host and that its eggs are the most numerous in the fecal specimen examined.

On the other hand, any species present in the unknown set but not represented in the reference set will be misidentified as whatever species it most resembles. If a sample of dog feces containing only spurious H. contortus eggs is analyzed morphometrically using only A. caninum and U. stenocephala in the reference set, an incorrect diagnosis of mixed hookworm infection with a preponderance of U. stenocephala will result. The reference set must be as small as possible but must include all possibilities.

Fecal vs. Worm Eggs. Tetley (1941) reported that swelling of Haemonchus contortus eggs manifested principally as an increase in width took place in the interval between being laid and appearing in the feces. Our observations concur with Tetley's with respect to H. contortus and were even more marked in the case of Trichostrongylus colubriformis. Species whose egg dimensions and shapes differ least require the greatest precision of measurement in establishing reference data. These should be based on fresh or properly formalized fecal eggs from single species infections of the parasite in question. Unfortunately, "pure infections" represent considerable labor and expense and a few contaminant eggs from an unwanted species ruin the resulting data set. However, if a practical need exists for the

differentiation of a given set of closely similar species of eggs, the most satisfactory results will be achieved by careful work with pure infections. On the other hand, where distinctions are relatively easily drawn, it is a waste of time and resources to base reference data on any other than eggs from worm uteri; the small changes in dimensions experienced from worm uterus to host's feces do not interfere provided the interspecific differences are sufficiently large in the first place.

Fresh vs. Formalin-Fixed Eggs. Fecal samples carefully preserved in 10% formalin yielded Haemonchus contortus eggs which, after storage for a few days at room temperature, remained dimensionally indistinguishable from eggs measured promptly after being passed. Five percent formalin, on the other hand, was inadequate to prevent partial development and concomitant increase in width. Care must be exercised to obtain thorough mixing of fecal material and formalin. If fecal pellets are simply dropped into 10% formalin, a mix of developmental stages from morula to first stage larva results, the degree of development of a particular egg depending on its distance from the surface of the fecal mass and consequent time required for the formalin to reach it. Such a sample is of limited value for morphometric analysis.

Appraisal and Prospects for Improvement. We consider our technique in its present stage of development to be ready for the hands of competent parasitologists. The optical and electronic equipment and software are relatively inexpensive and present no

serious obstacle to a modest research budget.

As it now stands, diagnostic morphometry is better suited to systems in which repeated measurements and supplementary observations permit suitable reduction of reference sets than it is to the differential diagnosis of casual specimens. However, as the process of measurement is facilitated, the amount of information gathered increased, and the analysis of that information refined, diagnostic morphometry can be expected to cope with more difficult problems and to find more general application.

Highly sophisticated videographic image analysis systems are potentially capable of accomplishing the same ends as the system described herein and perhaps demand less time and effort on the part of the observer. Image analysis systems are also capable of measuring infinitessimal differences in color and optical density and thus provide information that the digitizing tablet cannot. However, in our experience, any color observed in strongylid eggs represents spurious and inconstant optical effects. Whether or not optical density will provide a reliable diagnostic criterion remains to be determined. High cost is the principal disadvantage of videographic image analysis systems.

The value of classification patterns to the interpretation of identification patterns was pointed out above. An objective mathematical method of analyzing these patterns would be a distinct improvement over their subjective interpretation, but remains to be developed.

Applications. Effective application of morphometric analysis in resolving diagnostic problems that have no easier solution requires detailed knowledge of taxonomy and biology of the parasitic organisms under study, skill in the operation of microscope and computer, and meticulous attention to detail at every step of the operation. The serious student of strongylid life history and epidemiology stands to gain most from adopting these procedures. In order to obtain an accurate list of the strongylid species infecting a group of hosts in a natural setting, the contemporary investigator must resort to post mortem examination of those hosts, to exposure of "sentinal" parasite-free hosts to the same environment and subsequent post mortem examination of these "sentinals", or to larval cultures with the limitations of that technique discussed above. By contrast, diagnostic morphometry is completely non-destructive and requires only correctly identified fecal samples to determine the more abundant species or species groups present and to supply an estimate their relative abundances. When applied in a longitudinal study with repeated sampling of the same individual hosts, the temporal pattern of growth and decline of populations of different species can be quantified with unprecedented accuracy.

We believe that diagnostic morphometry can profitably be applied to other parasite groups presenting diagnostic dilemmas (e.g., coccidia, taeniid eggs) and may eventually find application in the routine diagnostic laboratory. Of course, it

would be a simple matter to develop programs capable of differentiating those taxa that are easily identified on sight by a competent parasitologist and indeed such programs might enjoy the greatest commercial potential. Diagnosis based on egg micromorphometry could then be accomplished by any intelligent and painstaking technician after a few hours' instruction and would allow the parasitologist time to study molecular biology and maybe keep his job.

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Table 1. Jackknifed classification matrix of *Ancylostoma caninum*, *Uncinaria stenocephala*, and *Haemonchus contortus* based on stepwise discriminant analysis.

Group	Percent correct	Number of cases classified into group: ANCN	UNST	HMCT
ANCN	90.0	90	7	3
UNST	73.0	0	73	27
HMCT	75.0	6	19	75

Table 2. Jackknifed classification matrix of a 12 species of ovine strongylid eggs based on stepwise discriminant analysis and identification of 3 "unknown" samples from pure infections with the indicated species of strongylid.

Group	Percent Correct	Number of cases classified into group -										
		Reference Set										
		BUTG	CBOV	CPCR	HMCT	NDBT	NDFL	NDSP	OGCN	OGVN	OSCC	TRAX	TRCL
BUTG	60.0	15	7	0	0	0	0	0	1	0	2	0	0
CBOV	58.0	9	29	0	0	0	0	0	2	4	6	0	0
CPCR	60.0	0	0	30	8	0	0	0	5	0	0	4	3
HMCT	67.0	0	0	9	67	0	0	0	16	0	0	1	7
NDBT	98.0	0	0	0	0	98	2	0	0	0	0	0	0
NDFL	100.0	0	0	0	0	0	20	0	0	0	0	0	0
NDSP	96.0	0	0	0	0	0	4	96	0	0	0	0	0
OGCN	72.0	1	0	0	5	0	0	0	18	0	0	0	1
OGVN	100.0	0	0	0	0	0	0	0	0	50	0	0	0
OSCC	49.5	6	14	0	0	0	0	0	3	13	49	0	14
TRAX	76.0	0	0	3	0	0	0	0	0	0	0	19	3
TRCL	73.0	1	0	9	3	0	0	0	8	0	2	4	73
		"Unknowns"											
HMCT	66.0	0	0	3	66	0	0	0	28	0	0	0	3
OSCC	53.0	5	20	1	0	0	0	0	0	20	53	0	1
TRCL	80.0	0	0	1	15	0	0	0	2	0	1	1	80

Table 3. Jackknifed classification matrix of 13 species of equine strongylid eggs based on stepwise discriminant analysis.

Group	Percent Correct	Number of cases classified into group -												
		SGED	SGVU	TDSR	TDTC	CSCL	CSLG	CSMN	CCNS	CCLP	CYCT	CYCN	GYCP	TRAX
SGED	61.8	34	10	0	0	1	0	3	0	4	0	1	0	2
SGVU	56.6	18	56	1	0	0	0	4	9	11	0	0	0	0
TDSR	72.9	0	0	51	10	0	0	0	6	0	1	2	0	0
TDTC	83.0	0	0	5	83	0	0	0	0	0	10	2	0	0
CSCL	5.4	3	2	0	2	2	8	2	5	3	6	4	0	0
CSLG	80.8	0	0	0	0	3	42	2	2	2	0	1	0	0
CSMN	38.0	8	3	0	0	5	6	38	2	24	3	2	0	9
CCNS	36.0	2	16	3	0	9	6	0	36	6	9	13	0	0
CCLP	21.2	6	7	0	0	8	5	28	2	18	1	4	0	6
CYCT	8.0	0	4	6	21	11	7	6	17	6	8	14	0	0
CYCN	28.0	4	0	17	5	9	11	0	11	0	15	28	0	0
GYCP	100.0	0	0	0	0	0	0	0	0	0	0	0	20	0
TRAX	76.6	3	1	0	0	0	0	5	0	2	0	0	0	36
Total	46.8	78	99	83	121	48	85	88	90	76	53	71	20	53

Table 4. Jackknifed classification matrix of 5 species and 4 species pairs of equine strongylid eggs (data of similar species pooled prior to analysis). Boxes enclose data pooled after analysis, forming a 7 species group of "small strongyles" to which a total of 79% of the 674 cyathostome eggs were correctly assigned. Four "unknowns" include pure infections of Strongylus edentatus (SGED), S. vulgaris (SGVU), a natural mixed cyathostome infection, and a natural mixed cyathostome infection with large numbers of Trichostrongylus axei (TRAX). Percentage correct values for these 4 "unknowns" are enclosed in single quotation marks because they involve composite groups instead of single species.

Group	Percent correct	Number of cases classified into group -								
		Reference Set								
		EDVU	TDSR	TDTC	CLNS	CSLG	MNLP	CTCN	GYCP	TRAX
EDVU	76.6	118	1	0	13	0	22	0	0	0
TDSR	74.3	1	52	11	3	0	0	3	0	0
TDTC	83.0	0	9	83	0	0	0	3	0	0
CLNS	38.0	20	5	3	52	15	13	29	0	0
CSLG	86.5	0	0	0	2	45	4	1	0	0
MNLP	62.2	18	0	0	9	15	115	12	0	16
CTCN	32.5	6	24	28	36	27	14	65	0	0
GYCP	100.0	0	0	0	0	0	0	0	20	0
TRAX	80.9	2	0	0	0	0	7	0	0	38
		"Unknowns"								
PURE SGED	'61.0'	61	3	0	1	1	25	4	0	5
PURE SGVU	'67.0'	67	12	0	7	0	8	5	0	1
MIXED CYATH -OSTOMES	'80.0'	1	2	17	15	18	5	42	0	0
MIXED CYATH -OSTOMES + TRAX	'86.0'	10	1	3	0	4	35	6	0	41

Table 5. Jackknifed classification matrix of 3 species of ovine strongylid eggs and identification of 3 "unknown" samples representing purported pure infections of the same 3 species.

Group	Percent correct	Number of cases classified into group -		
		Reference Set		
		HMCT	OSCC	TRCL
HMCT	85.0	85	0	15
OSCC	85.9	1	85	13
TRCL	84.0	13	3	84
		"Unknowns"		
HMCT	97.0	97	0	3
OSCC	99.0	0	99	1
TRCL	81.0	17	2	81

FIGURE CAPTIONS

Figure 1. Graphic representation of conversion of coordinate point data into geometric parameters by PARAM, the computer program written by Miles McCreadie. For each egg entered, the computed length, width, area, perimeter, and areas and arc lengths of each pole are stored in a computer file in form suitable for stepwise discriminant analysis.

Figure 2. Frequency distribution of lengths of 100 Haemonchus contortus eggs and 100 Trichostrongylus colubriformis eggs. The arrows indicate the mean lengths and the vertical line the midpoint between these means. Eighty-five percent of the H. contortus and 86% of the T. colubriformis egg lengths fell on the same side of this vertical line as their respective means. Therefore, classifying each egg as H. contortus or T. colubriformis according to whether its length was closest to one mean or the other, resulted in 85.5% correct classification, on the average.

Figure 3A. Distribution of lengths and widths of 100 eggs each of Ancylostoma caninum (ANCN), Uncinaria stenocephala (UNST), and Haemonchus contortus (HMCT). Ellipses include 95% of the data points.

Figure 3B. Distribution of points representing the first two canonical variables derived by discriminant analysis of the same data set represented in Fig. 3A. The canonical variables are based on length and perimeter, the parameters chosen by the discriminant analysis program. Despite the considerable degree of

overlap, discriminant analysis correctly classified 90% of the A. caninum, 73% of the U. stenocephala, and 75% of the H. contortus eggs (Table 1). Ellipses include 95% of the data points.

Figure 4A. Ellipses including 95% of the points representing width vs. length of 12 species of ovine strongylid eggs. Data points deleted for clarity.

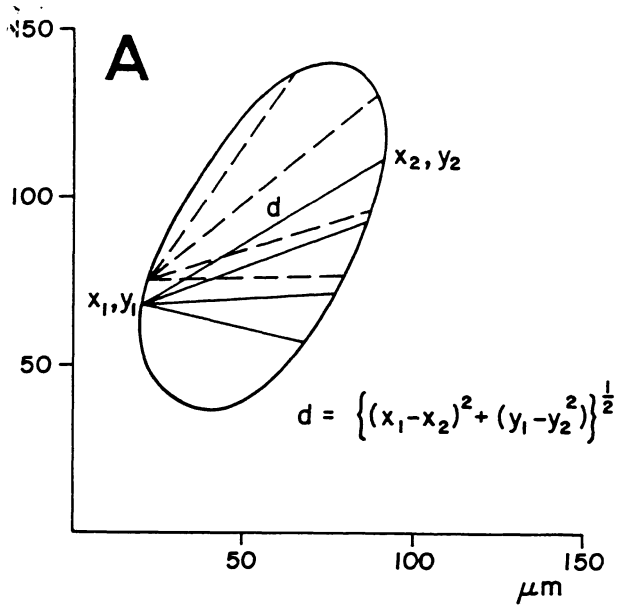
Figure 4B. Ellipses including 95% of the points representing canonical variables 1 and 2 based on based on 7 geometric parameters (area, length, width, area pole 1, perimeter, arc length pole 1, arc length pole 2) of the same data set as that represented in Fig. 4A. Stepwise discriminant analysis correctly identified 75.8% of these 12 species, on average (Table 2). Data points deleted for clarity.

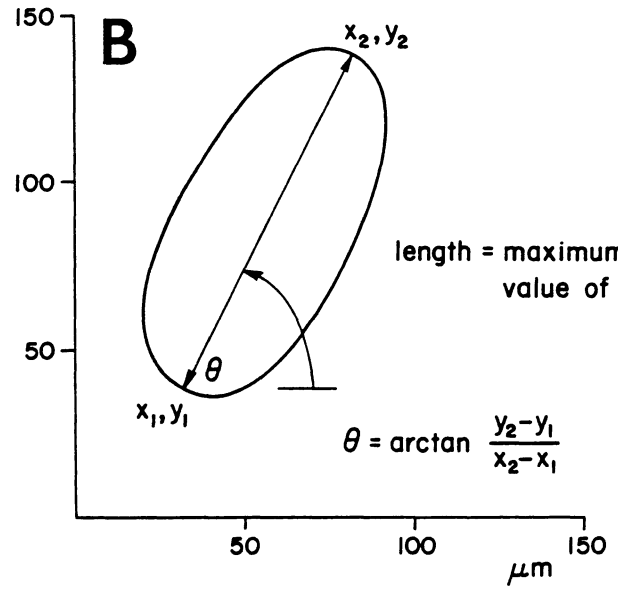
Figure 4C. Ellipses including 50% of the points representing width vs. length of 13 species of equine strongylid eggs. Data points deleted for clarity.

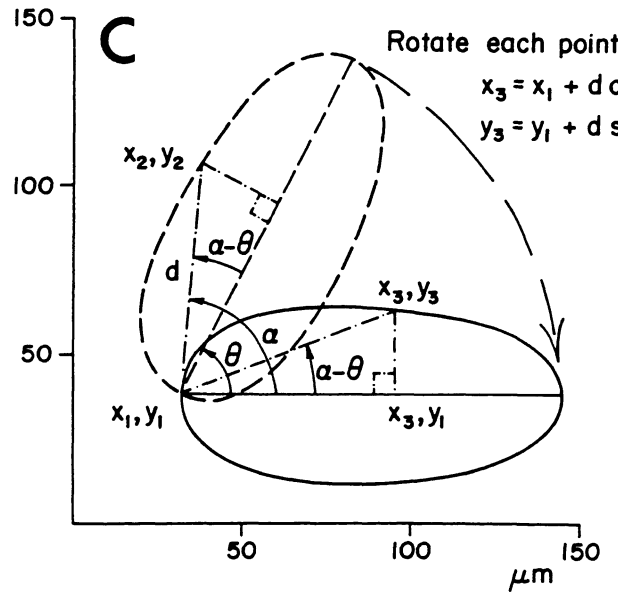
Figure 4D. Ellipses including 50% of the points representing canonical variables 1 and 2 based on 4 geometric parameters (area, length, width, perimeter) of the same data set as that represented in Fig. 4C. Discriminant analysis correctly identified only 46.8% of these 13 species, on average (Table 3). Data points deleted for clarity.

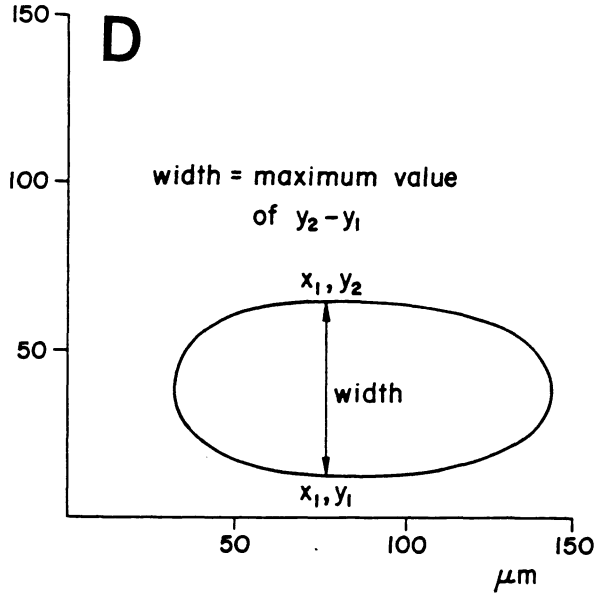
Figure 5. Ellipses including 95% of the points representing canonical variables 1 and 2 based on length and perimeter. Haemonchus contortus, Ostertagia circumcincta, and Trichostrongylus colubriformis reference sets (unbroken contours)

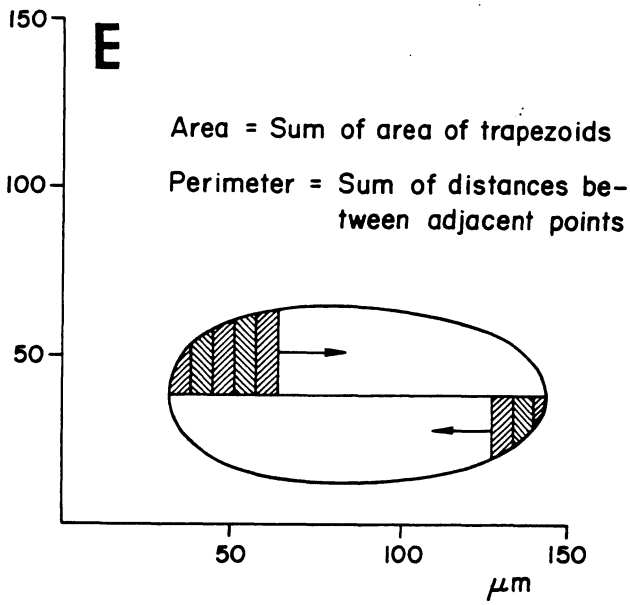
are compared with independent sets of data representing the same species and presented to the computer as "unknowns" (broken contours). Data points deleted for clarity.

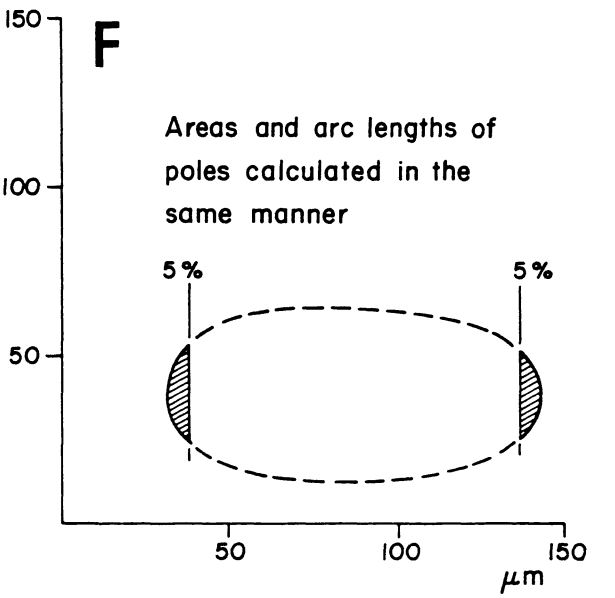

A
150
100
50
d
x_2, y_2
x_1, y_1
$d = \{(x_1-x_2)^2 + (y_1-y_2^2)\}^{\frac{1}{2}}$
50
100
150
μm

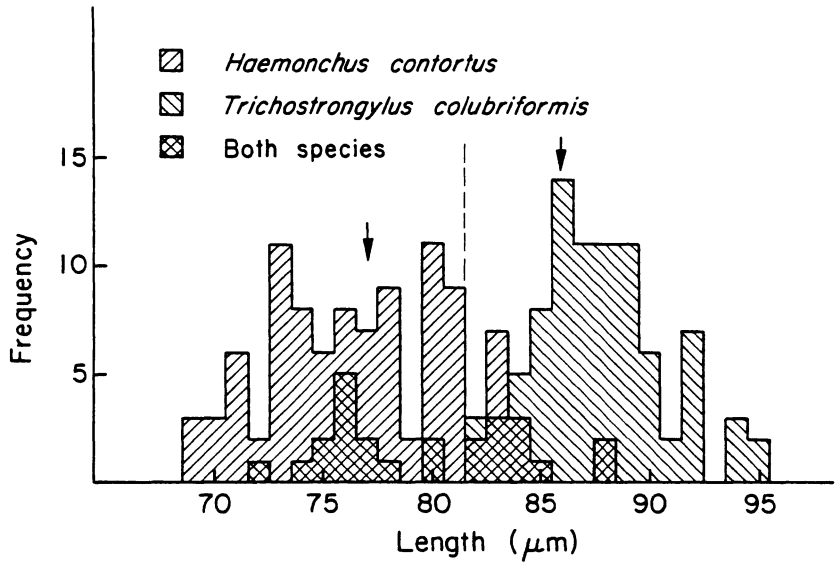

B
150
100
50
x_2, y_2
length = maximum value of d
θ
x_1, y_1
$\theta = \arctan \frac{y_2-y_1}{x_2-x_1}$
50
100
150
μm

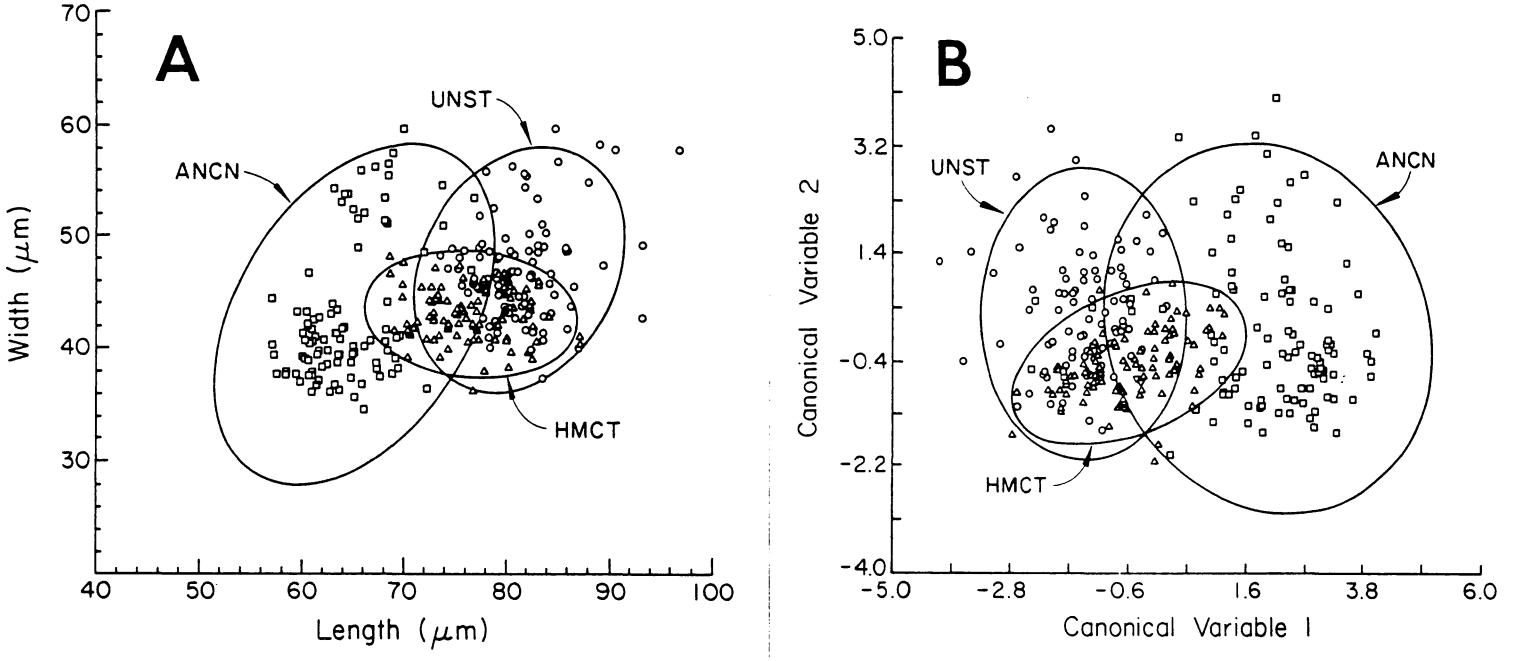

C
150
100
50
Rotate each point
$x_3 = x_1 + d\cos(\alpha-\theta)$
$y_3 = y_1 + d\sin(\alpha-\theta)$
x_2, y_2
α-θ
d
x_3, y_3
θ
α
α-θ
x_1, y_1
x_3, y_1
50
100
150
μm

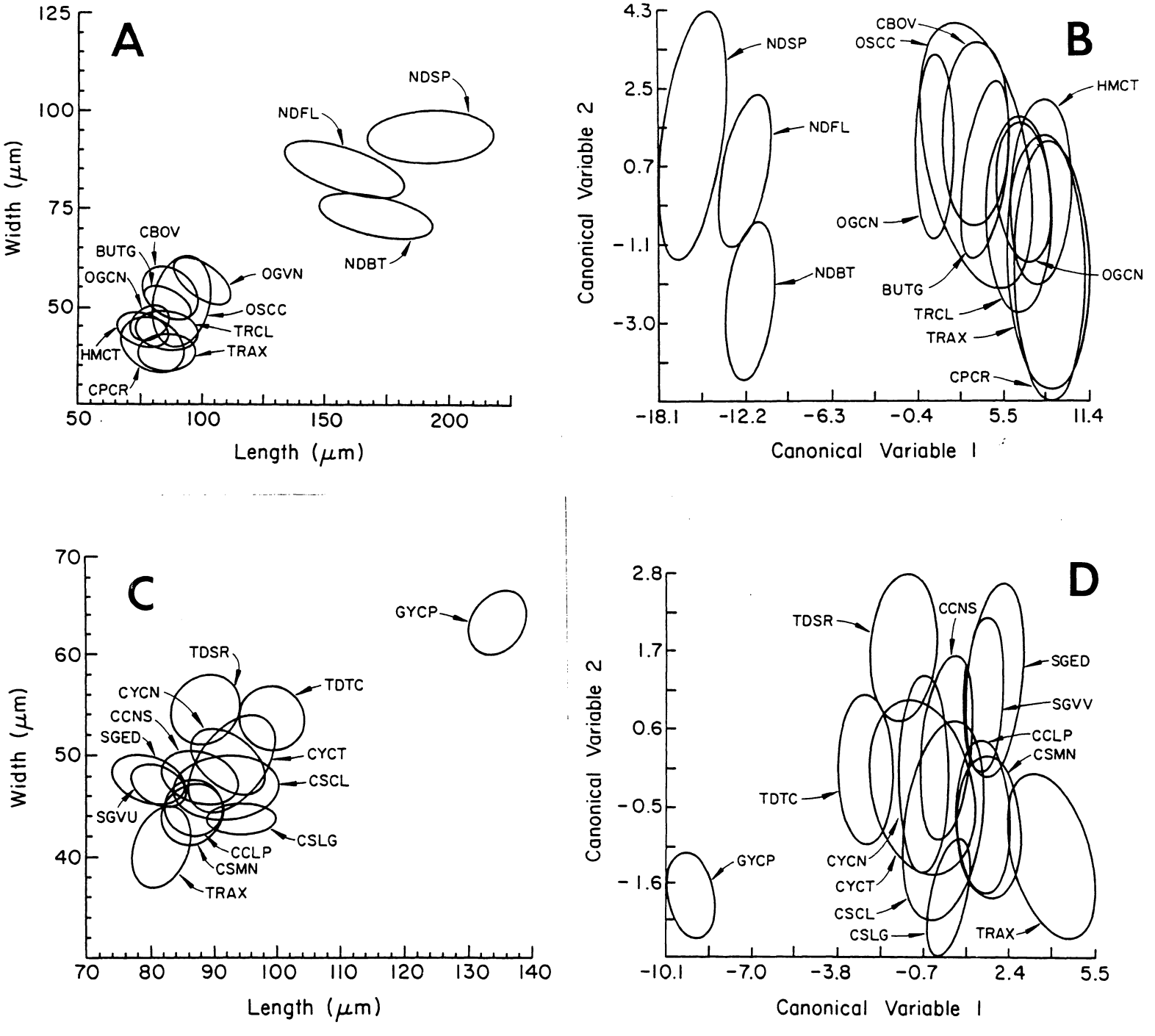

D
150
100
50
width = maximum value of $y_2 - y_1$
x_1, y_2
width
x_1, y_1
50
100
150
μm

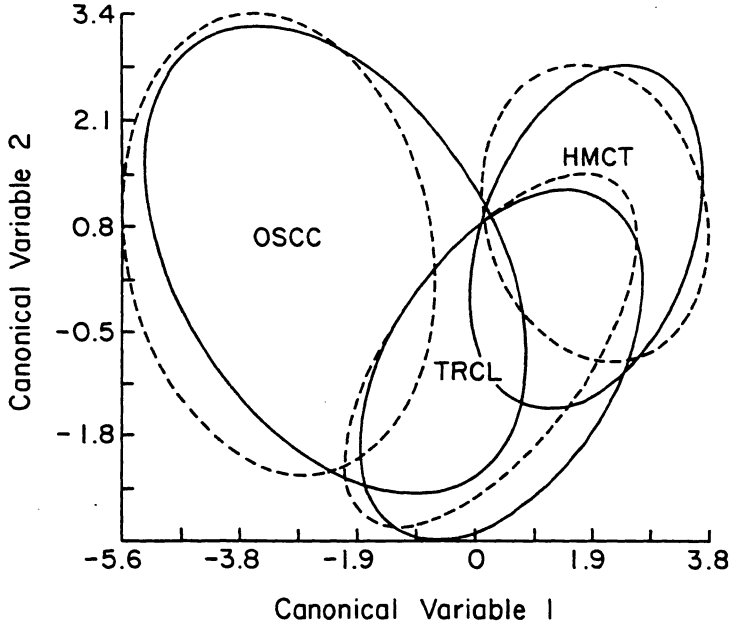

E
150
100
50
Area = Sum of area of trapezoids
Perimeter = Sum of distances between adjacent points
50
100
150
μm


F
150
100
50
Areas and arc lengths of poles calculated in the same manner
5%
5%
50
100
150
μm


Haemonchus contortus
Trichostrongylus colubriformis
Both species
Frequency
15
10
5
70
75
80
85
90
95
Length (μm)


A
UNST
ANCN
HMCT
Width (μm)
Length (μm)
70
60
50
40
30
40
50
60
70
80
90
100
B
UNST
ANCN
HMCT
Canonical Variable 2
Canonical Variable 1
5.0
3.2
1.4
-0.4
-2.2
-4.0
-5.0
-2.8
-0.6
1.6
3.8
6.0


A
125
100
75
50
Width (μm)
50
100
150
200
Length (μm)
NDSP
NDFL
NDBT
CBOV
BUTG
OGCN
OGVN
OSCC
TRCL
TRAX
HMCT
CPCR
B
4.3
2.5
0.7
-1.1
-3.0
Canonical Variable 2
-18.1
-12.2
-6.3
-0.4
5.5
11.4
Canonical Variable 1
NDSP
NDFL
NDBT
CBOV
OSCC
HMCT
OGCN
BUTG
TRCL
TRAX
CPCR
C
70
60
50
40
Width (μm)
70
80
90
100
110
120
130
140
Length (μm)
GYCP
TDSR
TDTC
CYCN
CCNS
SGED
CYCT
CSCL
SGVU
CSLG
CCLP
CSMN
TRAX
D
2.8
1.7
0.6
-0.5
-1.6
Canonical Variable 2
-10.1
-7.0
-3.8
-0.7
2.4
5.5
Canonical Variable 1
TDSR
CCNS
SGED
SGVV
CCLP
CSMN
TDTC
GYCP
CYCN
CYCT
CSCL
CSLG
TRAX


3.4
2.1
0.8
-0.5
-1.8
Canonical Variable 2
OSCC
HMCT
TRCL
-5.6
-3.8
-1.9
0
1.9
3.8
Canonical Variable 1