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Artifactual and Natural Variation of *Oochoristica javaensis*: Statistical Evaluation of In Situ Fixation

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ABSTRACT: Lack of knowledge of the extent of natural morphological variation can undermine proper taxonomic decisions. Confounding this problem is artifactual variation that arises from improper fixation techniques. For the morphologically based taxonomy of the cestode genus *Oochoristica*, little information exists on the plasticity of important taxonomic characters. In addition, paratypes of several species of *Oochoristica* are highly contracted and contorted. These paratypes were recovered from preserved hosts; thus, the tapeworms were killed and fixed inside the host (in situ fixation). Experiments demonstrated that in situ fixation of *Oochoristica javaensis* results in highly contracted specimens, and statistical comparisons between relaxed and in situ-fixed tapeworms revealed significant differences for proglottid measurements. Natural variation for the paratypes recovered from preserved hosts is likely misrepresented by the artificial variation induced by in situ fixation. Lastly, data from natural infections suggested that proglottid characters of *O. javaensis* are plastic and may be subject to crowding effects.

KEY WORDS: *Oochoristica javaensis*, Cestoda, *Hemidactylus turcicus*, Mediterranean gecko, fixation techniques, crowding effects, morphological variation, taxonomy.

The taxonomy of the cestode genus *Oochoristica* Lühe, 1898, has been based solely on morphology without knowledge of the extent of natural intraspecific morphological variation. Parasite morphological variation may be the result of genetic determinants, host-induced effects, parasite intensity effects, or external habitat influences. Stunkard (1957) and Haley (1962) discussed the importance of environmental and host-induced variation for the systematics of helminth parasites, citing such factors as different host species, host age, host diet, or infection intensity as causes of variation. They also emphasized the need to assess experimentally the stability of taxonomic characters when identifying a species.

In addition to the lack of knowledge on intraspecific variation, natural variation of some species of *Oochoristica* may be masked by artificial morphological variation induced by fixation techniques. Several paratype specimens of *Oochoristica* examined from the U.S. National Parasite Collection (USNPC), Beltsville, Maryland, U.S.A. were highly contracted and contorted. Examination of the respective species descriptions revealed that these paratypes (listed below) were obtained from formalin-fixed hosts. That is, they were removed from host specimens deposited in museum collections without regard to the effects of host fixation on internal parasites. Bakke (1988) and others have qualitatively illustrated the distorting effects of improper fixation techniques on the morphology of soft-bodied helminths, but comparisons of fixation techniques have not been tested statistically to examine for quantitative differences in the measurements of important taxonomic characters.

The purpose of our report was to provide a quantitative assessment of the artifactual morphological variation induced by killing and fixing tapeworms within a host, i.e., in situ fixation. In order to address the effects that improper fixation methods may have on the morphologically based taxonomy of *Oochoristica*, statistical comparisons of in situ-fixed tapeworms to ones that were collected alive and killed in a relaxed state were conducted with specimens of *Oochoristica javaensis* Kennedy, Killick, and Beverley-Burton, 1982. In addition, we provide data regarding the effects of intensity on *O. javaensis* morphology.

Materials and Methods

Fixation experiments

To mimic lizard fixation techniques used for museum collections, 15 Mediterranean geckos, *Hemidactylus turcicus* (Linnaeus, 1758), were collected from the campus of Louisiana State University (LSU), Baton Rouge, Louisiana, U.S.A. (30°24.92′N; 91°10.81′W), where they were known to have a high prevalence of

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infection with *O. javaensis* (C. D. Criscione, unpublished data). Geckos were killed using an overdose of ether and immediately fixed via subcutaneous, oral cavity, and body cavity injections of unheated 10% formalin. Oral cavity injections insured that the tapeworms were killed immediately. After 6 days in 10% formalin, geckos were soaked in water for 24 hr to remove the formalin. Geckos were then transferred to 70% ethanol for storage until dissection 4 days later. In situ-fixed tapeworms recovered upon necropsy were stored in 70% ethanol, stained in Semichon's acetocarmine, dehydrated in ethanol, cleared in xylene, and mounted in Canada balsam. Comparisons were made with relaxed tapeworms that were killed with hot water (90°C) and fixed and stored in alcohol–formalin–acetic acid solution (AFA). Relaxed worms were obtained in a helminth survey of *H. turcicus* from LSU in the summer of 1998 (C. D. Criscione, unpublished data); staining and mounting techniques were the same as for the in situ-fixed specimens.

Quantitative analyses included measurements of mature proglottids from 5 relaxed and 5 in situ-fixed specimens of *O. javaensis*. Three mature proglottids, located just anterior to the first proglottid displaying evidence of egg production, were selected from each individual. Length and width were measured for each mature proglottid and for the ovary, vitellaria, and 1 testis within each proglottid. One testis was randomly selected from each proglottid, ovary length was measured for the ovary lobe opposite the genital atrium, and ovary width was measured across both lobes. Although multiple testes are present within a single proglottid, only 1 was chosen in order to facilitate the use of appropriate statistical tests. In order to test for in situ-fixation effects, a nested ANOVA design was used to control the pseudoreplication of measuring 3 proglottids from 1 tapeworm. Tapeworms nested within type of fixation constituted the experimental units, i.e., true replicates, with the error term being the proglottids, i.e., pseudoreplicates, nested within individual tapeworms. Principal components analysis (PCA) with Varimax rotation was used as a data reduction technique and to examine latent relationships among the variables. A variable was considered to load on a factor if its correlation to the factor was $>|0.5|$ (Hair et al., 1999). The resulting factors with their standardized factor scores were then tested for differences between relaxed and in situ-fixed tapeworms in the nested design. Statistical significance was determined at $P < 0.05$.

Analysis of natural morphological variation

The analysis of intensity effects on the morphology of *O. javaensis* included 5 tapeworms from each of 3 Mediterranean geckos that were naturally infected with 15, 28, and 64 tapeworms. Criteria and morphological characters used for measurements were the same as those used in the fixation experiments. Experimental design using factor scores from a PCA with Varimax rotation was also the same, in that a nested ANOVA was used to test for intensity effects. A priori contrasts of 15 versus 28 and 28 versus 64 were computed. In order to conduct this analysis, the 5 tapeworms from each intensity level were treated as true replicates, when in fact they were pseudoreplicates.

In addition, 10 relaxed tapeworms that were killed with hot water (90°C) were selected to provide measurements representative of *O. javaensis* recovered in a helminth survey of *H. turcicus* (C. D. Criscione, unpublished data). This representative data set included specimens from geckos with intensities ranging from 1 to 64, and had at least 1 tapeworm from each of 5 collection locations in southeastern Louisiana, U.S.A. [Bayou Segnette State Park in Westwego (29°53.18′N; 90°09.80′W); Fairview-Riverside State Park in Madisonville (30°24.55′N; 90°08.41′W); a residential neighborhood in Metairie (30°00.76′N; 90°08.90′W); Southeastern Louisiana University in Hammond (30°30.67′N; 90°27.98′W); and LSU]. PCA with Varimax rotation was applied to this data set to examine for latent relationships among the same variables used in the fixation and intensity analyses.

Specimens examined

Museum specimens examined from the USNPC and the Canadian Museum of Nature (CMNPA), Ottawa, Ontario, Canada included the following: *O. javaensis*, 2 paratypes (CMNPA nos. 1982-0693, 1982-0695); *Oochoristica anolis* Hardwood, 1932, 1 voucher (USNPC no. 75748) and the holotype (USNPC no. 30898); *Oochoristica bezyi* Bursey and Goldberg, 1992, 2 paratypes (USNPC no. 81874); *Oochoristica bresslaui* Fuhrmann, 1927, 1 voucher (USNPC no. 89087); *Oochoristica chinensis* Jensen, Schmidt, and Kuntz, 1983, 2 paratypes (USNPC no. 077168); *Oochoristica islandensis* Bursey and Goldberg, 1992, 1 paratype (USNPC no. 82225); *Oochoristica macallisteri* Bursey and Goldberg, 1996, 1 voucher (USNPC no. 89267) and 2 paratypes (USNPC no. 86196); *Oochoristica mccoyi* Bursey and Goldberg, 1996, 2 vouchers (USNPC nos. 85403, 85408) and 1 paratype (USNPC no. 86343); *Oochoristica novaezealandae* Schmidt and Allison, 1985, 5 paratypes (USNPC no. 78407); *Oochoristica osheroffi* Meggitt, 1934, 1 voucher (USNPC no. 80433); *Oochoristica parvula* (Stunkard, 1938), 3 vouchers (USNPC no. 84397); *Oochoristica piankai* Bursey, Goldberg, and Woolery, 1996, 1 voucher (USNPC no. 88189) and 1 paratype (USNPC no. 84589); *Oochoristica scelopori* Voge and Fox, 1950, 2 vouchers (USNPC nos. 84234, 87529). We deposited voucher specimens of *O. javaensis* from *H. turcicus* in the USNPC (nos. 90344–90348).

Results

For the fixation analysis, PCA revealed 3 latent variables from the 8 measured (Table 1). Examination of the variable factor loadings revealed that factor 1 consisted of all the vertical measurements, while factors 2 and 3 were characterized by horizontal measures. Factors 1, 2, and 3 were renamed vertical, horizontal-1, and horizontal-2, respectively. All 3 factors showed significant worm-to-worm variation within each type of fixation ($F_{\text{vertical}} = 9.20$, $F_{\text{horizontal-1}} =$

Table 1. *Oochoristica javaensis*: variable factor loadings, factor eigenvalues, and percent total variance accounted for by each factor from the Varimax rotated correlation matrix of the fixation data set.

	Varimax rotated loading matrix		
	Factor 1 (vertical)	Factor 2 (horizontal-1)	Factor 3 (horizontal-2)
Testis length	**0.917***	−0.112	0.209
Ovary length	**0.875**	−0.176	0.209
Vitellaria length	**0.863**	−0.237	0.262
Proglottid length	**0.805**	−0.497	0.023
Ovary width	−0.203	**0.912**	−0.082
Proglottid width	0.236	**0.869**	−0.096
Testis width	−0.092	0.469	**−0.835**
Vitellaria width	0.463	0.177	**0.793**
Eigenvalues	3.320	2.184	1.498
Percent of total variance explained by the factor	41.495	27.296	18.723

* Bold print shows loadings where variable loaded onto factor.

42.20, $F_{\text{horizontal-2}} = 5.31$, df = 8, 20, $P < 0.001$); thus, the fixation main effect was tested with the mean-square values of the subgroups, tapeworms nested within fixation method. The vertical factor showed a significant effect between in situ-fixed and relaxed tapeworms (Fig. 1) ($F_{1,8} = 11.927$, $P = 0.009$); however, neither horizontal factor was significant. Table 2 provides the raw measurements of the variables used in the analysis.

PCA revealed that the 8 variables used in the intensity data set constituted only 1 factor (Table 3), thus showing that all 8 characters from relaxed specimens varied together. Even though there was significant worm-to-worm variation for this factor ($F_{12,30} = 12.62$, $P < 0.001$), there was still a significant intensity effect ($F_{2,12} = 13.42$, $P < 0.001$) (Fig. 2). The a priori contrast between 15 and 28 was significant ($F_{1,12} = 10.58$, $P = 0.007$), but 28 versus 64 was not. Figure 3A–C displays mature proglottid variation for tapeworms recovered from intensities of 15, 28, and 64, and Table 4 gives the raw measurements of the variables. Measurements of 10 *O. javaensis* tapeworms from Louisiana are given in Table 5. Based on results from the fixation experiments, only tapeworms exhibiting little to no wrinkling (i.e., contraction) were used to provide the representative measurements of *O. javaensis* collected in our survey. The fact that the 8 variables in the representative data emerged as only 1 factor from the PCA (Table 3) again demonstrated that all 8 characters from relaxed specimens varied together.

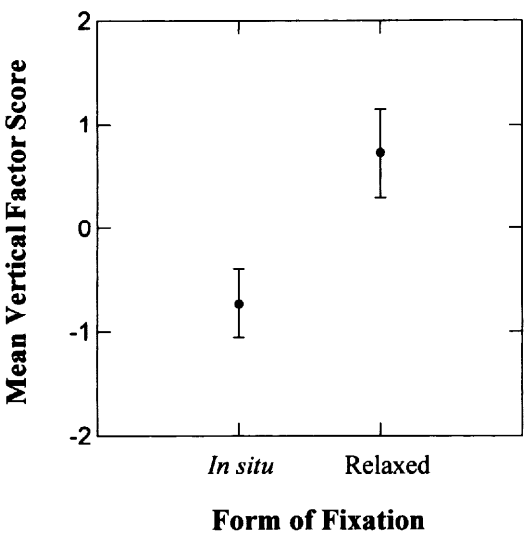

Figure 1. Plot of the mean factor scores and 95% confidence intervals for the vertical factor of relaxed and in situ-fixed specimens of *Oochoristica javaensis*.

Discussion

Variation resulting from different fixation techniques or conditions only confounds taxonomic problems in which the range of natural morphological variation is not known. Experimental data revealed 2 quantitative problems with using in situ-fixed tapeworms. The first was that a significant reduction in the vertical factor without a significant change in horizontal factors produced an accordion effect. High vertical factor scores were determined by large length measurements of the proglottid and its ovary, vitel-

Table 2. Measurements of mature proglottids from specimens of *Oochoristica javaensis* fixed in a relaxed state and specimens fixed in situ; all measurements are in μm.

	Sample size*	Relaxed $n = 5$†	In situ-fixed $n = 5$
Proglottid width	15	482–648 (589 ± 15.8)‡	403–845 (635 ± 34.1)
Proglottid length	15	356–640 (493 ± 25.8)	213–490 (312 ± 23.0)
Ovary width	15	246–351 (288 ± 8.06)	261–355 (310 ± 7.01)
Ovary length	15	152–238 (201 ± 6.63)	109–183 (144 ± 5.69)
Vitellaria width	15	125–195 (161 ± 5.11)	113–148 (130 ± 2.86)
Vitellaria length	15	90–137 (104 ± 3.83)	43–94 (70.5 ± 4.11)
Testis width	15	31–43 (38.7 ± 0.83)	35–47 (43.5 ± 0.95)
Testis length	15	27–47 (39.5 ± 1.23)	20–35 (27.1 ± 1.07)

* Sample size refers to the total number of each character that was measured.

† Number of tapeworms used in measurements.

‡ Range followed by mean ± 1 SE in parentheses.

laria, and testes (Table 2); thus, relaxed tapeworms had a higher mean factor score (Fig. 1). Contraction from in situ fixation resulted in mature proglottids wider than long (Fig. 3D–F), but completely relaxed tapeworms from hot-fixed specimens yielded mature proglottids longer than wide (Fig. 3A–C).

The second quantitative effect pertained to the correlative relationships among the mature proglottid characters and was revealed by the PCA itself. If only relaxed specimens are incorporated into the PCA, i.e., the intensity and representative data sets, all 8 characters vary together as 1 factor (Table 3); but when in situ-fixed tapeworms are incorporated into the PCA, i.e., the fixation data set, vertical measurements become independent of horizontal measurements (Table 1). Contraction of the in situ-fixed tapeworms altered the correlative nature of the 8 variables and divided 1 factor into 3 factors.

Contraction of helminth parasites resulting from improper fixation has been documented many times in the parasite literature (Bakke, 1988); however, our study may be the first to quantify the effects of different forms of fixation and to analyze the data statistically. The empirical evidence provided in the current study not only supported the conclusions of Bakke (1988)

Table 3. *Oochoristica javaensis*: variable factor loadings, factor eigenvalues, and percent total variance accounted for by each factor from the correlation matrix of the intensity data set and representative data set.

	Loading matrices for the	
	Intensity data set Factor 1	Representative data set Factor 1
Ovary width	**0.924***	**0.909**
Vitellaria width	**0.921**	**0.854**
Ovary length	**0.916**	**0.814**
Proglottid width	**0.893**	**0.851**
Vitellaria length	**0.866**	**0.784**
Testis width	**0.841**	**0.825**
Testis length	**0.753**	**0.749**
Proglottid length	**0.751**	0.443
Eigenvalues	5.929	4.996
Percentage of total variance explained by the factor	74.116	62.445

* Bold print shows loadings where variable loaded onto factor.

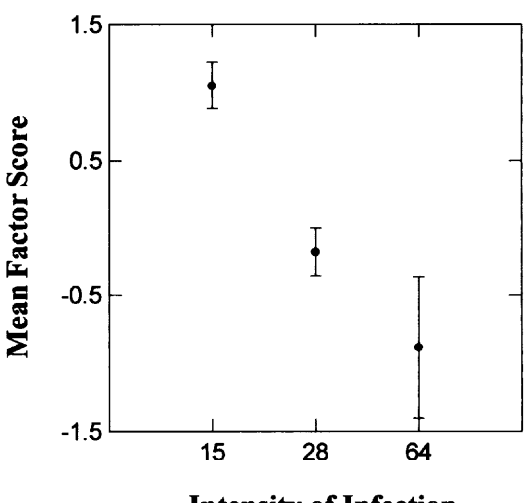

Figure 2. Plot of the mean factor scores and 95% confidence intervals for tapeworms collected at intensities of 15, 28, and 64.

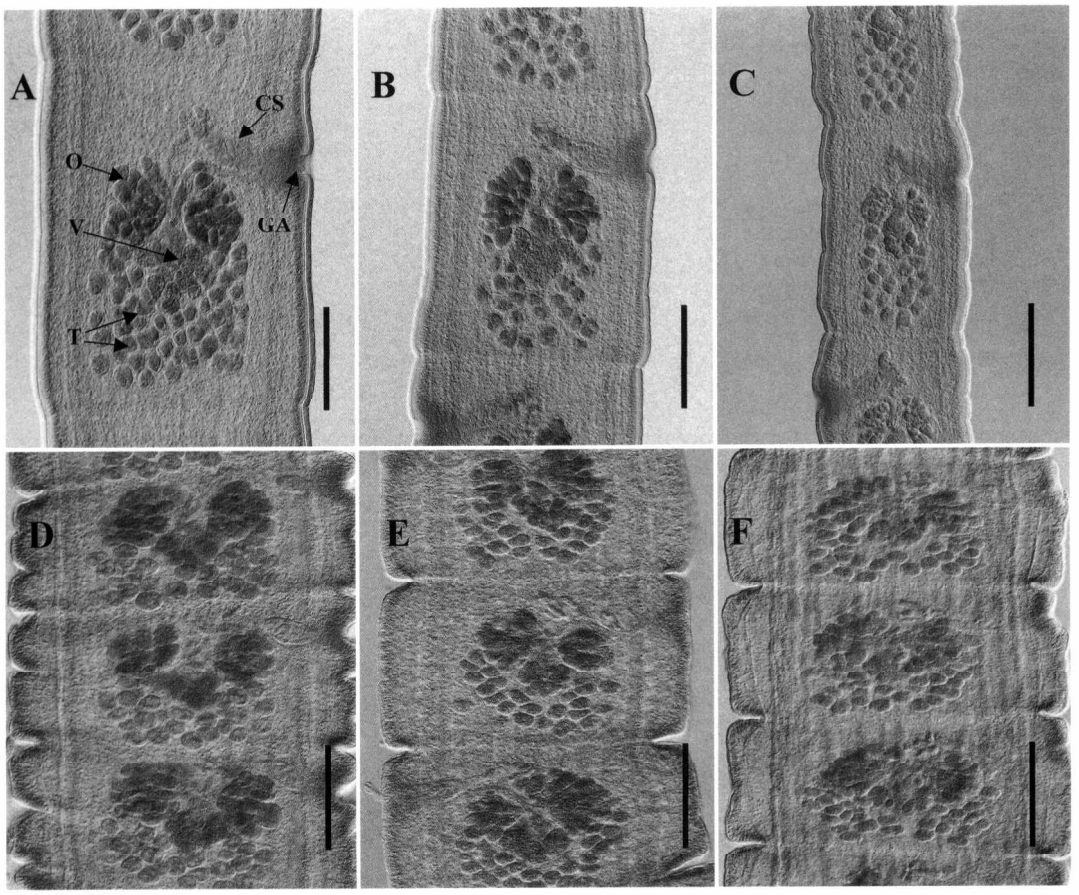

Figure 3. Mature proglottid variation of *Oochoristica javaensis* from *Hemidactylus turcicus*. Photomicrographs were taken with differential interference contrast. A–C. Natural variation of specimens from intensities of 15, 28, and 64, respectively; all were fixed in a relaxed state. D–F. Artificial variation showing contraction that resulted from in situ fixation. Bars = 200 μm. CS = cirrus sac, GA = genital atrium, O = ovary, T = testis, and V = vitellaria.

Table 4. Mature proglottid measurements of *Oochoristica javaensis* from *Hemidactylus turcicus* with intensities of 15, 28, and 64; all measurements are in μm.

Level of intensity	Sample size*	15 $n = 5$†	28 $n = 5$	64 $n = 5$
Proglottid width	15	506–616 (560 ± 7.06)‡	387–450 (409 ± 5)	269–545 (387 ± 31.7)
Proglottid length	15	545–751 (624 ± 14.5)	395–553 (470 ± 14.7)	419–545 (463 ± 8.7)
Ovary width	15	238–293 (268 ± 3.89)	195–254 (225 ± 4.85)	121–281 (198 ± 14.2)
Ovary length	15	156–226 (189 ± 4.48)	125–179 (160 ± 3.83)	78–183 (126 ± 7.9)
Vitellaria width	15	129–203 (161 ± 5.78)	86–133 (117 ± 3.37)	59–117 (90.4 ± 5.19)
Vitellaria length	15	74–137 (105 ± 4.95)	70–113 (90.3 ± 3.3)	43–101 (73.5 ± 4.91)
Testis width	15	39–47 (42.7 ± 0.61)	35–43 (39 ± 0.78)	27–43 (35 ± 1.29)
Testis length	15	39–51 (43 ± 0.87)	31–47 (39 ± 1.03)	27–47 (36.6 ± 1.4)

* Sample size refers to the total number of each character that was measured.

† Number of tapeworms used in measurements.

‡ Range followed by mean ± 1 SE in parentheses.

Table 5. Measurements of *Oochoristica javaensis* from naturally infected *Hemidactylus turcicus* in southeastern Louisiana; measurements in μm unless noted otherwise.

Variable		Sample size*	n = 10†
Total	L‡ (mm)	10	22.2–105 (53.4 ± 7.4)§,‖
Proglottid number		10	86–164 (131 ± 7.8)‖
Neck	W	10	158–237 (205 ± 8.1)‖
	L (mm)	10	1.12–1.58 (1.38 ± 0.05)‖
Scolex	W	10	148–246 (195 ± 9.1)‖
	L	10	98–183 (140 ± 8.9)
Sucker	W	10	51–90 (74.1 ± 3.9)‖
	L	10	62–117 (89 ± 5.3)‖
Immature proglottid	W	30	261–506 (408 ± 14.5)
	L	30	237–371 (297 ± 7.6)
Genital pore position#		30	0.24–0.33 (0.28 ± 0.004)‖
Mature proglottid	W	30	277–648 (490 ± 19.5)‖
	L	30	395–751 (509 ± 17.8)‖
Cirrus sac	W	30	43–55 (47.7 ± 0.58)
	L	30	86–144 (116 ± 2.8)‖
Ovary	W	30	133–390 (268 ± 11.8)‖
	L	30	78–316 (184 ± 0.6)‖
Vitellaria	W	30	62–269 (144 ± 8.9)‖
	L	30	43–221 (106 ± 6.6)‖
Testis	W	30	27–51 (40.2 ± 0.98)‖
	L	30	31–47 (40.9 ± 0.9)
Testes number		30	17–46 (26.6 ± 1.4)‖
Gravid proglottid	W	30	158–650 (492 ± 29.4)‖
	L (mm)	30	0.85–1.99 (1.26 ± 0.05)‖
Oncosphere	W	30	20–34 (25.3 ± 0.69)‖
	L	30	18–28 (23.2 ± 0.52)‖
Hook	L	30	8–12 (11.5 ± 0.18)‖

* Sample size refers to the total number of each character that was measured.

† Number of tapeworms measured.

‡ L = length, W = width.

§ Range followed by mean ± 1 SE in parentheses.

‖ Indicates that the range of the character extends values reported in the original description (Kennedy et al., 1982).

Genital pore position was calculated as a ratio of the position along the length of the mature proglottid from the anterior end (length to the center of the genital pore ÷ length of proglottid).

but also quantitatively demonstrated the misleading representation of morphological characters used in the taxonomy of *Oochoristica* resulting from in situ fixation. We believe that our more rigorous analysis is important because, despite the elegant studies of Bakke (1988) and previous workers, the practice of describing improperly fixed specimens is still widespread among parasite taxonomists.

In addition to the quantitative changes resulting from in situ fixation, 2 qualitative effects further demonstrated the inappropriateness of using in situ-fixed specimens in species descriptions. Distortion of the scolex (Fig. 4A, B) and proglottids (Fig. 4C, D) prevented accurate measurements of these characters. This is not to say that every scolex and mature proglottid fixed in situ will be rendered useless for species identification, but the number of appropriate characters available for analysis will be greatly reduced. As seen in Figure 4A–D, one would have difficulty in finding a true scolex width, and contortion in the strobila would limit the number of proglottids suitable for examination.

Species descriptions based on contracted or disfigured specimens will misrepresent the true natural variation by decreasing the means of vertical characters and artificially inflating the dispersion of measurements if used in conjunction with relaxed specimens. Such may be the case with several paratype (*O. bezyi, O. islandensis, O. macallisteri, O. mccoyi, O. piankai*) and voucher (*O. mccoyi, O. parvula, O. piankai, O. scelopori*) specimens examined in our study. These specimens may represent true species, but their reported natural variation is more than like-

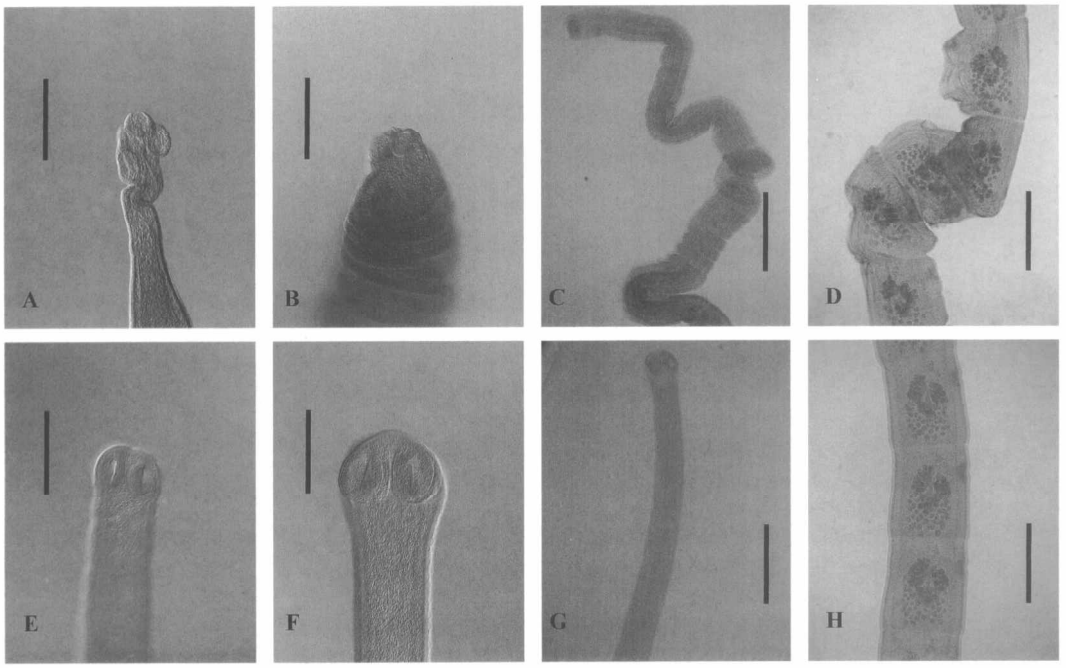

Figure 4. Comparisons of relaxed and in situ-fixed specimens of *Oochoristica javaensis*. A–D. Distorting effects of in situ fixation on scolices and proglottids. E–H. Relaxed specimens. Bars = 200 μm for A, B, E, and F (differential interference contrast). Bars = 500 μm for C, D, G, and H (brightfield).

ly masked within the artificial variation induced by in situ fixation. Characters that do not reflect their natural variation should not be used to describe species. *Oochoristica* spp. recovered from fixed museum hosts may provide historical abundance data, but identification of these specimens should be made with extreme caution, and ideally, in conjunction with specimens fixed appropriately.

Intensity effects were examined because a crowding effect has been documented as a cause of variation in the size of tapeworms (Read, 1951), and because of the occurrence of different intensities in naturally infected *H. turcicus*. PCA for the intensity data set produced 1 factor in which all 8 variables loaded high (Table 3); therefore, individual proglottids with large measurement values received high factor scores. Although not quantified, there was no apparent crowding effect observed for natural infections with intensities between 1 and 15. Tapeworms from an intensity of 15 had significantly greater factor scores than specimens from 28 and 64 (Fig. 2), thus indicating that crowding reduces the size of the respective morphological characters (Table 4) in *O. javaensis*. Brooks and Mayes (1976) reported similar crowding effects for *O. bivitellobata* recovered from the prairie racerunner, *Cnemidophorus sexlineatus* Lowe, 1966. Their results and ours, however, should be considered only preliminary for 2 reasons. First, data were obtained from natural infections; thus other factors that induce variation were not controlled. Second, the intensity levels were not replicated. Ideally, one would wish to sample tapeworms from multiple hosts harboring all possible intensity levels. Both reports, however, suggest that morphological characters for species of *Oochoristica* can be variable and may be subject to intensity levels.

Measurements of the 10 *O. javaensis* specimens given in Table 5 extend the ranges of several characters provided in the original description of *O. javaensis* (Kennedy et al., 1982). These measurements were based on specimens with little or no contraction and are provided to give a representation of *O. javaensis* collected from *H. turcicus* in southeastern Louisiana. Means of several characters (Table 5) do not match those provided by Kennedy et al. (1982); however, based on the lack of host specificity displayed in laboratory experiments (Criscione

and Font, 2001), the indication of plasticity in morphological characters (Table 4), and the examination of *O. javaensis* paratypes, it was determined that the specimens from *H. turcicus* in southeastern Louisiana were *O. javaensis*.

In summary, statistical analyses demonstrated that measurements of in situ-fixed tapeworms, i.e., specimens recovered from preserved hosts, distorted the true natural variation of *O. javaensis*. The intraspecific variation of several species of *Oochoristica* may be misrepresented because they were described from highly contracted, in situ-fixed specimens. Additionally, morphological characters used in the taxonomy of *Oochoristica* have not been examined for their stability when exposed to different environmental or host-induced conditions. Our analyses indicated that proglottid morphology was highly variable and that this plasticity may have resulted from crowding.

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