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Comparative morphometry of *Fasciola gigantica* (Cobbold, 1855) and *Fasciola hepatica* (Linnaeus, 1758) coexisting in Philippine Carabao (*Bubalus bubalis*)

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ABSTRACT
Coexisting individuals of *Fasciola hepatica* and *Fasciola gigantica* in livers of slaughtered carabaos grazed in Tacurong City, Sultan Kudarat, Mindanao, Philippines were compared based on their body length and widest body width, body width at ovary level, cone width along the proximal end of acetabulum, the distance from the anterior body to the proximal end of the acetabulum; and the distance from the posterior end of the acetabulum to the posterior body end. Flukes were relaxed in phosphate buffered saline solution containing $MgCl_2$ crystals, and were fixed in alcohol formalin acetic acid. In the four infected livers, *F. gigantica* was the dominant species (66.7-83.3%) relative to *F. hepatica* (16.7-33.3%). While *F. gigantica* was longer (16-39 mm), with 64.0% of the flukes in the range 30-39 mm, they had narrower bodies (4-10 mm) compared to *F. hepatica* (7-11 mm). Body width measurements at the ovary level revealed a wider range in *F. gigantica* (2-9 mm) compared to *F. hepatica* (4-8 mm). Cone width ranged 2-4 mm and overlapped between the species. The distance from the anterior body end to the proximal end of the acetabulum of almost 96.0% of the flukes was 2-3 mm. The measurement from the distal end of the acetabulum to the posterior end of the body in 65% individuals of *F. gigantica* ranged 26-37 mm, and the other worms overlapped with those of *F. hepatica*. Although there were overlaps in body measurements between the two species, only the differences in the mean body length; mean widest body width; and mean of the distance from the posterior end of the acetabulum to the posterior end of body were significant ($p \leq 0.05$). While the significant difference in the morphometrical values noted in the current study alongside marked difference in the appearance/shape are clearly useful in the identification of *Fasciola* spp., the commonly reported coexistence of both forms together with the existence of intermediate forms in the liver of any susceptible mammalian host species may point to seem to these merely as polymorphic populations, and not necessarily as separate species. It would be interesting to examine various aspects of the reproductive organs of flukes in view of the recognized differences in testicular and ovarian patterns (Bergeon and Laurent, 1970) between the species.

Keywords: *Fasciola* spp.; Philippine; water buffaloes; Carabao; morphometrics.

INTRODUCTION

Liver flukes inhabit large biliary ducts of herbivorous mammals and cause the disease fascioliasis, with *Fasciola hepatica* and *Fasciola gigantica* recognized as the most common etiologic agents (Haseeb *et al*., 2002; Ashrafi *et al*., 2006b; Kimura *et al*., 1984). Worldwide *F. hepatica* has the widest distribution, while *F. gigantica* is more widespread in the tropical and subtropical regions (Mas-coma 2005; Ashrafi *et al*., 2004; Moghaddam *et al*., 2004; Esteban *et al*., 2003).

Fasciolids are identified primarily on differences in body shape and size of adults, with the smaller *F. hepatica* exhibiting wide and defined shoulders compared to the slender *F. gigantica* having less defined shoulders and shorter cephalic cones (Merck Veterinary Manual, 2008; Kimura *et al*., 1984). Overlaps in the body morphometrics of adult *F. hepatica* and *F. gigantica* infecting water buffaloes and cattle are common (Ashrafi *et al*., 2006b; Periago *et al*., 2006). Moreover, sequences of mtDNA, rDNA and 28S rDNA genes (Marcilla *et al*., 2002; Itagaki *et al*., 2001; Blair and Mcmanus, 1989) and protein banding patterns (De Vera *et al*., 2008) of both forms likewise have similarities and sequence overlaps.

In the Philippines, bovine and bubaline fascioliasis is common (Molina *et al*., 2005; Intong *et al*., 2003). Based on morphological characters and measurements of body length and width, Kimura *et al*. (1984) inferred infection of water buffaloes with three species, namely *F. hepatica*, *F. gigantica* and *Fasciola indica*. This contradicts earlier findings of molecular evidence of natural hybridization between *F. hepatica* and *F. gigantica* by Agatsuma *et al*. (2000), who then proposed that the small and large forms are essentially of the same species. The present work sought to compare other morphometrical parameters, in addition to the standard body length and width between the two forms of fasciolids that coexisted in livers of slaughtered Philippine water buffaloes.

MATERIALS AND METHODS

Isolation, relaxation and fixation of worms

Livers were obtained from six slaughtered water buffaloes that originated from Tacurong City, Sultan Kudarat, Mindanao, and were kept in an ice-filled cooler en route to the Parasitology Research Laboratory. With a forceps, worms were removed from the hepatic ducts and ductules (Fig. 1), sliced approximately 3 cm thick. Intact and broken flukes were placed in beakers containing phosphate buffered saline (PBS) solution. The worm burden per liver per host was noted. Worms were initially segregated based on body shape and size and microscopically-viewed morphological features as *F. hepatica* and *F. gigantica* (Ashrafi *et al*., 2006a; Periago *et al*., 2006; Moghaddam *et al*., 2004; Kimura *et a*l., 1984). Severely broken worms whose anterior body parts were no longer distinguishable were excluded in the initial segregation of species.

The worms were relaxed in PBS solution to which about three pinches of $MgCl_2$ crystals were added. When necessary, more $MgCl_2$ crystals were added. Completely relaxed flukes were preserved in alcohol formalin acetic acid (AFA) (Ahmedullah *et al*., 2007). Flukes that contracted post-addition of AFA were returned in PBS solution containing $MgCl_2$ crystals. Only intact and completely relaxed worms were used in morphometrical analysis.

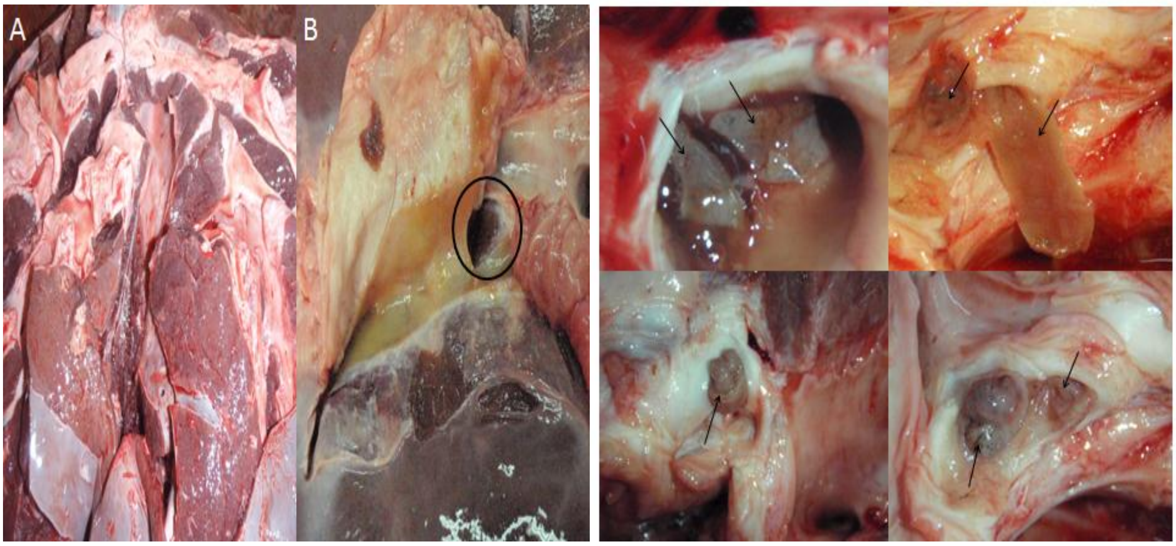

Figure 1. Liver samples. A. Normal. B. Infected. Note the yellowish tissue and larger biliary duct (encircled) and flukes in the bile ducts (arrows).

Morphometrical data gathering and analysis

With the aid of a stereoscope and a fine ruler, six morphometrical characters of intact worms were measured: a. body length; b. widest body width; c. body width at ovary level at distal end of acetabulum; d. cone width at proximal end of acetabulum; e. distance from anterior end of body to proximal end of acetabulum; and f. distance from distal end of acetabulum to posterior body end (Periago *et al*., 2006). The Difference in mean values obtained from *F. hepatica* and *F. gigantica* were analyzed using the student *t*-test ($p \leqq 0.05$).

RESULTS

Four (66.6%) of the slaughtered water buffaloes had fascioliasis. Recovered from the livers of three livers were between 26-37 worms (Table 1). Owing to damage to some flukes during removal, only 105 out of 131 worms were used in the initial segregation, showing the dominance of *F. gigantica* (Table 1; Fig. 2). Morphometrical data obtained from 77 fully-relaxed whole worms of *F. gigantica* and 15 of *F. hepatica* revealed longer *F. gigantica* (16-39 mm), with almost 64.0% measuring 30-39 mm, and showed overlap in body length between the species (Fig. 3).

Table 1. Comparison of the number of flukes recovered from four infected water buffaloes according to species.

Liver sample	Total	*F. gigantica* Number (%)	*F. hepatica* Number (%)
1	36	30 (83.3)	6 (16.7)
2	26	21 (80.8)	5 (19.2)
3	6	4 (66.7)	2 (33.3)
4	37	30 (81.1)	7 (18.9)
Total	105	84 (80.0)	21 (20.0)

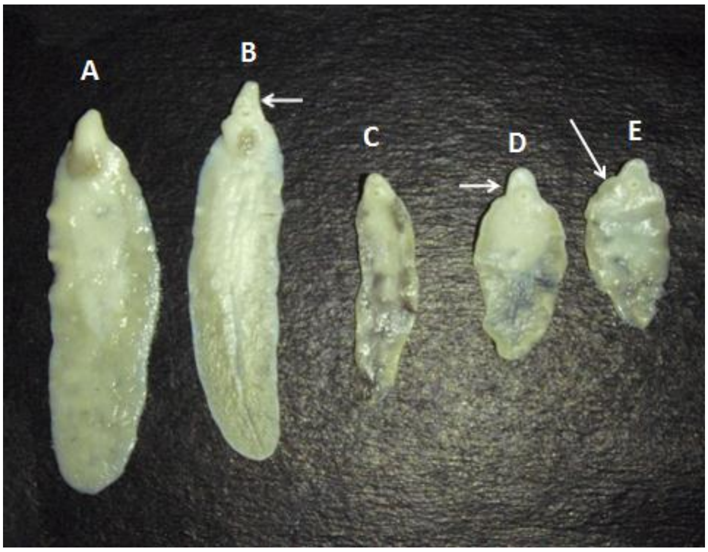

Figure 2. A, B & C. *Fasciola gigantica* (21.5-35.0 mm long). Note elongate and narrower body, and shape and height of cone (arrow). Worm in C is immature. D & E. *Fasciola hepatica* (15.5-17.8 mm long), with broad body, prominent shoulder (long arrow) and short and blunt anterior end (short arrow).

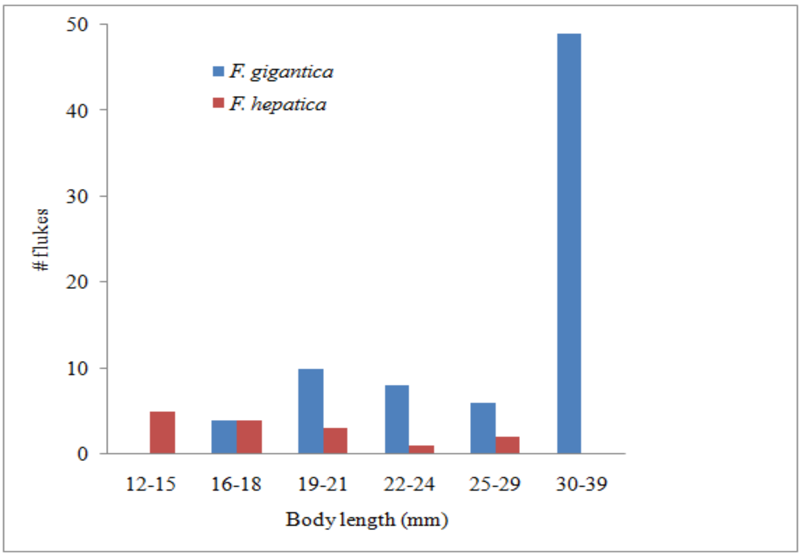

Figure 3. Comparison of the body length of *F. gigantica* and *F. hepatica*.
Note the number of *F. gigantica* in the 30-39mm range.

F. gigantica had narrower bodies (4-10 mm) compared to *F. hepatica* (7-11 mm) (Fig. 4). Body width measurements at the ovary level revealed a wider range in *F. gigantica* (2-9 mm) relative to *F. hepatica* (4-8 mm) (Table 2). Cone width ranged between 2-4 mm, with overlap at 2-3 mm (Table 2). The distance from the anterior body end to the proximal end of the acetabulum ranged 1-3 mm, and 2-3 mm in almost 96.0% of the worms (Table 2). In about 65.0% (n=50) of the *F. gigantica* worms, the length from the distal end of the acetabulum to the posterior body ranged 26-37 mm (Fig. 5).

Analysis of morphometrical values between the species revealed significant ($p \leq 0.05$) difference in body length, widest body length, and length from the distal end of the acetabulum to the posterior body end (Table 2).

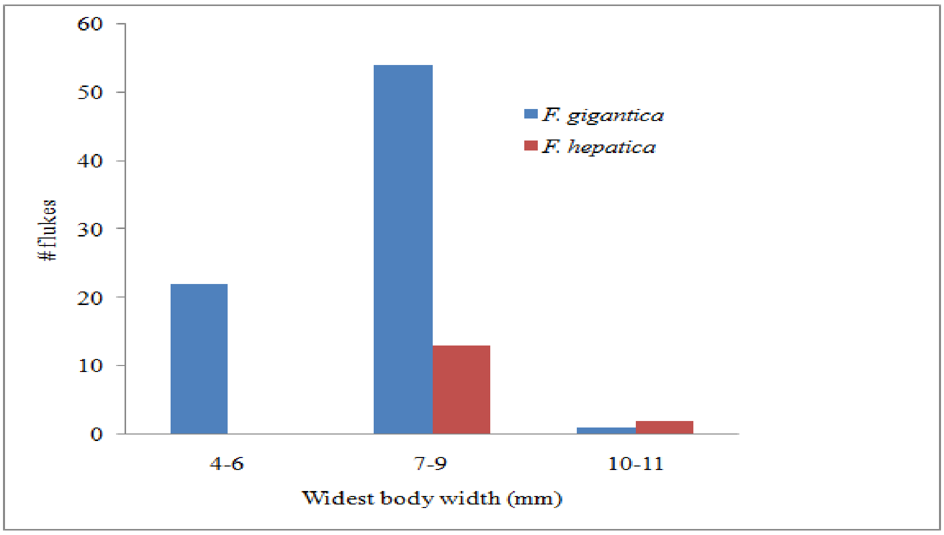

Figure 4. Comparison of the widest body width of *F. gigantica* and *F. hepatica*.

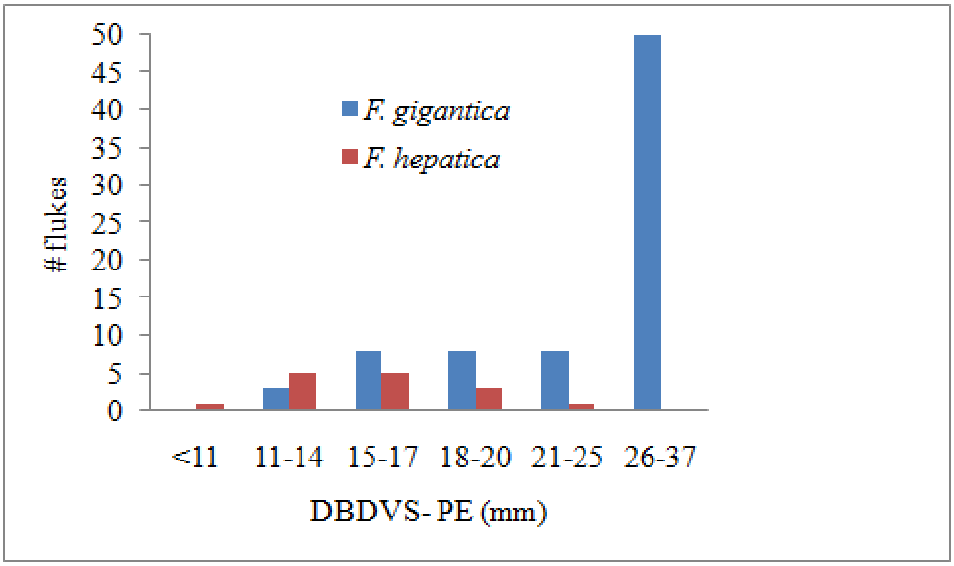

Figure 5. Comparison of the measurements from the distal end of the acetabulum to posterior end of the body (DBDVS-PE) between the species.

Table 2. Summary of ranges, mean ± SD of morphometrical values of *F. hepatica* and *F. gigantica*.

Measured Body Part	*F. hepatica* n=15 Range (mm) Mean ±SD	*F. gigantica* n=77 Range (mm) Mean ±SD
Body Length	12-29 18.7 ±4.91[a]	16-39 29.3 ±6.18[b]
Maximum body width	7-11 8.2 ±1.26[a]	4-10 7.4 ±1.43[b]
Body width at ovary level at distal end of acetabulum	4-8 5.6 ± 1.40	2-9 5.7 ±1.61
Cone width in proximal end of acetabulum	2-3 2.7 ±0.49	2-4 2.7 ±0.74
Distance from anterior body to proximal end of acetabulum	2-3 2.2 ±0.41	1-3 2.1 ±0.47
Length from the distal end of acetabulum to posterior end of body	9-25 15.3 ±3.91[a]	11-37 26.1 ±6.37[b]

[a-b] $p \leqq 0.05$ significant

DISCUSSION

Present observation of the coexistence of *F. hepatica* and *F. gigantica* as well as, the preponderance of *F. gigantica* in water buffaloes is consistent with earlier reports in the country (Molina *et al*., 2005; Intong *et al*., 2003). The morphological attributes and morphometrical data obtained in the current survey do not support earlier claim by Kimura *et al*. (1984), of the existence of *F. indica*, a polymorphic/morph of flukes earlier described as the same as *F. gigantica* based on mitochondrial DNA analysis by Agatsuma *et al*. (2000).

Individuals of *F. gigantica* in the current study were generally shorter compared to those obtained from Philippine buffaloes (25-37 mm; χ = 31.2 mm) earlier reported by Kimura *et al*. (1984), and Iranian buffaloes (28.6-48.7 mm; χ = 38.0 ± 0.42 mm) as well as, with those isolated from Iranian (22.7-59.2 mm; χ = 37.7 ± 0.27 mm) and African (30.7-52.0 mm; χ = 39.5 ± 0.84 mm) cattle (Ashrafi *et al*., 2006b). While the widest body width of flukes in the current study (4-10 mm; χ = 7.4 ± 1.4 mm) approximates that of the *F. gigantica* infecting Iranian cattle (3.5-9.8 mm; χ = 6.4 ± 0.04 mm), it is narrower compared to those infecting Philippine buffaloes (7.1-10.2; χ = 8.5) and African cattle (6.5-11.4 mm; χ = 8.9 ± 0.16) (Kimura *et al.*, 1984; Ashrafi *et al*., 2006b). In the case of *F. hepatica*, data on body length (12-29 mm; χ = 18.7 ±4.91) are consistent with the findings of Kimura *et al*. (1984) at 15.1-20.5 mm (χ = 18.3), and with those infecting the Bolivian cattle (11.7-28.7 mm; χ = 19.6 ± 0.34), and among slightly gravid flukes infecting the Iranian cattle (8.1-29.1 mm; χ = 17.4 ± 1.5) (Ashrafi *et al*., 2006b). The remaining measurements such as, the body width at ovary level at the distal end of acetabulum, cone width along the proximal end of acetabulum, distance from anterior body to the proximal end of acetabulum in both species concur with the findings of Ashrafi *et al.* (2006b) and Kimura *et al*. (1984).

The difference in the body length and width of the *F. gigantica* between the present and earlier works may be geographically-influenced. Also, the fixation of individual flukes in the earlier surveys between glass slides or between a glass slide and cover slip as against the use of a relaxant in the current study may have also unnaturally overstretched or distended the worms. While there were clear overlaps in all the measurements obtained between the *F. hepatica* and *F. gigantica*, they manifested somehow marked differences in their mean body length, widest body width, and distance from the distal end of the acetabulum to the posterior end of the body. As the small and recognized as two species. But do they really represent indubitably separate species or do they represent instead well established polymorphic populations? The coexistence of both the small and large forms in susceptible hosts is commonly observed worldwide (Ashrafi *et al*., 2004, 2006b; Intong *et al*., 2003; Rhee *et al.*, 1987), and presumably such condition provides greater possibilities of cross-fertilization between them, and consequently producing intermediate forms that manifest morphological/morphometrical overlaps. Mayr (1969) attributes polymorphic populations as an outcome of the simultaneous occurrence of several discontinuous phenotypes or genes in a population and further asserts that morphs in polymorphic populations have no taxonomic status and deserve no formal recognition. To this day, considering that morphological, morphometrical and molecular data have consistently documented overlaps between the small and large forms, alongside the existence of intermediate forms, the species status of *F. hepatica* and *F. gigantica* remains dubious (De Vera *et al*., 2008; Ali *et al*., 2008; Lin *et al*., 2007; Periago *et al.,* 2006; Ashrafi *et al*., 2006b; Adlard *et al*., 1993). It would be interesting to examine various aspects of the reproductive organs of flukes in view of the recognized differences in testicular and ovarian patterns between the species (Bergeon and Laurent, 1970).

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