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Comparative morphology of eggs of the Haplorchiinae (Trematoda: Heterophyidae) and some other medically important heterophyid and opisthorchiid flukes

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Abstract. The egg morphology of the following medically important small flukes from Southeast Asia and Far East were studied: *Opisthorchis viverrini, Clonorchis sinensis* (Opisthorchiidae), *Haplorchis taichui, H. pumilio, H. yokogawai, Stellantchasmus falcatus* and *Metagonimus* sp. (Heterophyidae). This study revealed a great intraspecific variability and interspecific similarity in size and shape of eggs. The eggs shape does not seem to be suitable for species identification. On the other hand, biometrical analysis of egg size enabled us to divide eggs from the species studied into four distinct groups according to the Faust-Meleney index (FMI) characterizing egg size rather than the length and width of eggs. The surface structures of eggs, delineated by using a scanning electron microscope (SEM), appeared to be a suitable morphological feature for distinguishing some groups of small flukes. Eggs from the Haplorchiinae were typified by the characteristic filamentous mesh structure. The problems of identification of eggs in human stool samples and suitability of using morphological criteria such as shape and size of eggs are discussed herein.

In some geographical areas, where raw fish is a favourite meal, people are often parasitized by small flukes, particularly from the families Opisthorchiidae and Heterophyidae. The crucial problem of correct species diagnosis is the identification of individual species on the basis of eggs found in the sample of human stools. During the study of opisthorchosis carried out in Laos in 1989*, a common occurrence of heterophyid flukes of the subfamily Haplorchiinae in human population was shown to exist in that country (Ditrich et al. 1990a; Giboda et al. 1991). This assumption was confirmed by a serological investigation (Ditrich et al. 1992). Since sufficient material for a comparative study of egg morphology was obtained, the present paper attempts to distinguish eggs of the Haplorchiinae subfamily from those of some other heterophyid and opisthorchiid trematodes infecting humans in Far East and Southeast Asia.

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MATERIALS AND METHODS

The eggs from the following trematode species were studied: *Haplorchis taichui* (Nishigori, 1924), *H. pumilio* (Looss, 1896), *H. yokogawai* (Katsuta, 1932) and *Stellantchasmus falcatus* Onji et Nishio, 1916 (Heterophyidae: Haplorchiinae); *Metagonimus* sp. (Heterophyidae: Heterophyinae); *Opisthorchis viverrini* (Poirier, 1886) and *Clonorchis sinensis* (Cobbold, 1875) (Opisthorchiidae).

The identification of all species except *Metagonimus* sp. was based on adult trematodes. The eggs from haplorchiin flukes were obtained from gravid adults found in cats captured from the surrounding areas at the Nam Ngum Dam reservoir in central Laos (Ditrich et al. 1990a). Only mature eggs from the anterior third of the uterus were used.

In addition, the following eggs were also studied: *H. taichui* adults obtained from a stool sample of a praziquantel treated Laotian; *Metagonimus* sp. from a Korean student's stool sample; *O. viverrini* obtained from adult worms found in the liver of a cat captured in Vientiane, Laos, and from flukes recovered from the stool sample of a praziquantel treated Laotian; *C. sinensis* obtained from an experimentally infected cat in Khabarovsk region of the Russia.

The measured eggs were fixed with 4% formaldehyde. In all cases, 30 eggs were measured (length, width and operculum diameter), and standard deviations were counted.

Index of Faust and Meleney (1924) (FMI = $1 \times w^2$ where "l" is the length of the egg and "w" is the width of the egg) and ratio of length and width (LWR = 1:w) were used for evaluating egg size. Measurements were assessed statistically using the analysis of variation (ANOVA).

For scanning electron microscopy (SEM), the eggs were fixed with 4% buffered formaldehyde prepared from paraformaldehyde, postfixed with OsO_4, dehydrated with alcohol series, transferred into acetone, dried using the method of critical point (CO_2), mounted on stubs, coated with gold and examined with the scanning electron microscope Tesla BS 300. For transmission electron microscopy, adults of *H. taichui* were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and postfixed with 2% OsO_4. After dehydration in acetone, flukes were embedded in Durcupan. Ultrathin sections from the anterior part of the body containing mature eggs were stained with uranyl acetate and lead citrate and examined with Philips EM 420 microscope.

RESULTS

Egg shape

Light microscopical examination revealed that the morphology of eggs from all species studied was very similar (Pl. I). In addition, the egg shape varied tremendously even within individual species samples (compare Pl. I, Figs. 8 and 9). The morphology of the operculum seems to be also rather variable. The prominent margin of the operculum appearing as "shoulder", commonly used for distinguishing the opisthorchiid and heterophyid eggs, can be observed in eggs of both families (compare Pl. I, Figs. 1–2 and Figs. 4–5).

Egg size

Biometrical evaluation of eggs showed that egg size may be a suitably distinct characteristic used for species identification only in some cases (Table 1). Analysis of the variance revealed that out of the four variables shown in Table 1, the FMI seems to be the most advantageous for distinguishing single species eggs. While length, width and LWR varied to a great extent even within one species (compare *O. viverrini* and *H. taichui* from cat and man), FMI enabled us to divide the eggs under study into four distinct groups differing from each other at a probability level

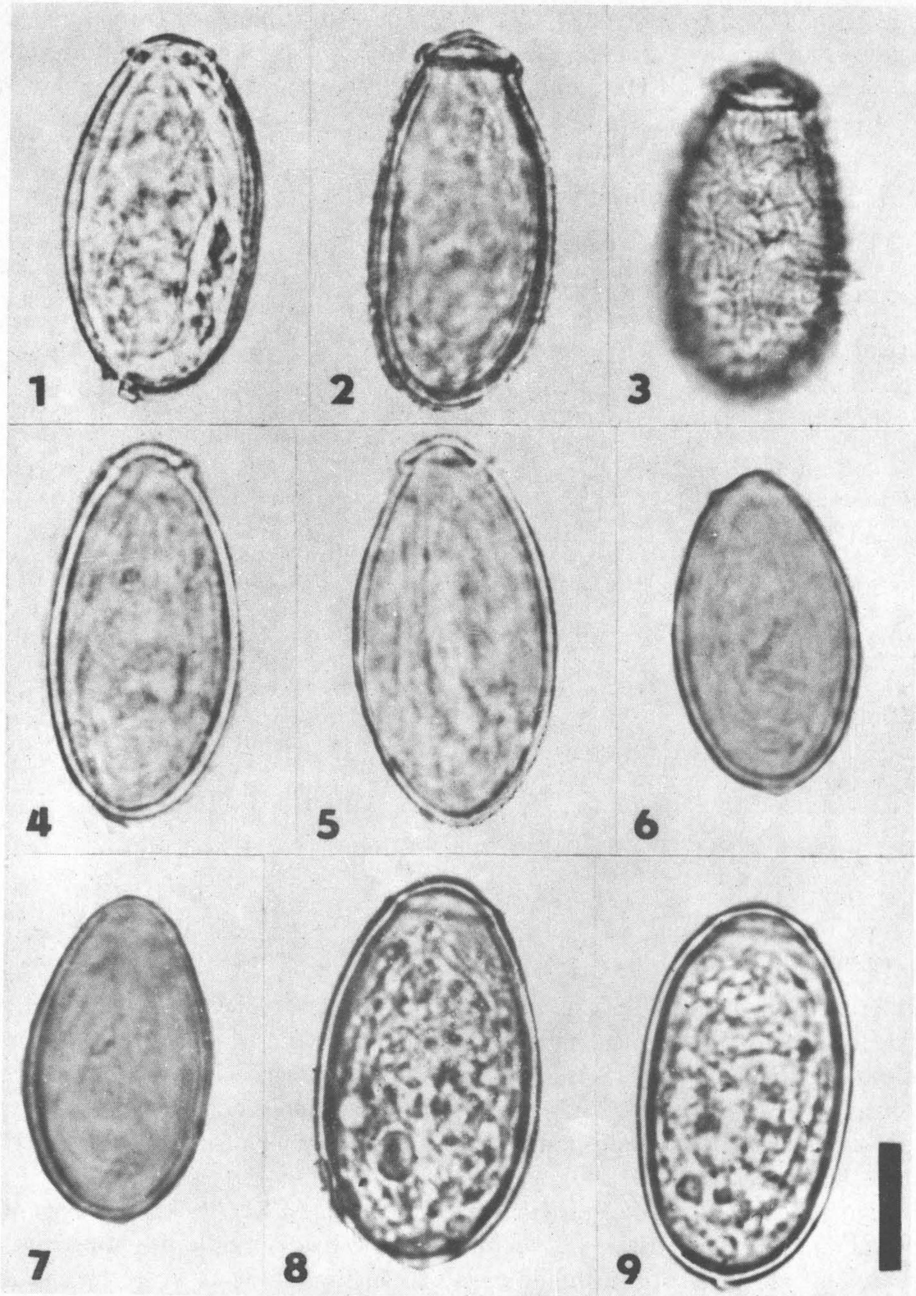

Plate I. Light microscopy of eggs. **Fig. 1.** *Opisthorchis viverrini* – cat, Vientiane province, Laos. **Figs. 2, 3.** *Clonorchis sinensis* – cat, Khabarovsk region, Russia (note typical muskmelon structure in Fig. 3). **Fig. 4.** *Haplorchis taichui* – cat, Vientiane province, Laos. **Fig. 5.** *Haplorchis pumilio* – cat, Vientiane province, Laos. **Fig. 6.** *Haplorchis yokogawai* – cat, Vientiane province, Laos. **Fig. 7.** *Stellantchasmus falcatus* – cat, Vientiane province, Laos. **Figs. 8 and 9.** *Metagonimus* sp. – man (stool sample), Korea. Bar = 10 µm.

of 0.05 (Fig. 1). The eggs from *S. falcatus* can be distinguished from eggs of all species by their smaller size as expressed by FMI. *H. pumilio* eggs exhibit the highest value of FMI out of all Haplorchiinae studied; they are, however, smaller than those from *Metagonimus* sp.

Table 1. Measurements of eggs under study

Fluke species	Length (mm)	Width (mm)	FMI*	LWR**
Haplorchis taichui (cat, Laos)	27.8 ± 0.6	13.7 ± 0.8	5252 ± 586	2.0 ± 0.1
Haplorchis taichui (man, Laos)	27.0 ± 1.7	14.1 ± 1.1	5348 ± 746	1.9 ± 0.2
Haplorchis yokogawai (cat, Laos)	28.6 ± 0.7	15.4 ± 0.5	6777 ± 456	1.9 ± 0.2
Haplorchis pumilio (cat, Laos)	30.8 ± 0.8	17.3 ± 0.7	9210 ± 808	1.8 ± 0.2
Stellantchasmus falcatus (cat, Laos)	22.1 ± 0.9	12.3 ± 0.4	3345 ± 279	1.9 ± 0.1
Metagonimus sp. (man, Korea)	32.6 ± 0.8	18.6 ± 1.3	11331 ± 1899	1.8 ± 0.2
Opisthorchis viverrini (cat, Laos)	28.3 ± 1.1	14.4 ± 1.3	5985 ± 1193	2.0 ± 0.2
Opisthorchis viverrini (man, Laos)	27.1 ± 2.6	15.1 ± 1.7	6256 ± 1612	1.8 ± 0.2
Clonorchis sinensis (cat, Russia)	28.5 ± 1.5	14.8 ± 1.3	6279 ± 1251	1.9 ± 0.2

* FMI = length × width^2 (Faust and Meleney 1924)
** LWR = length : width

Surface morphology

Differences in the surface structure of the egg seem to be a relatively suitable marker because of the typical muskmelon-like structure of the surface of opisthorchiid eggs. This structure is well visible using light microscope with the immerse oil objective (Pl. I, Fig. 3). In this case, the microscope must be focused to the surface of the egg shell since this structure disappears when focused at the median level of the egg.

The surface structures of egg shells are visible using SEM (Pl. II). The eggs of all Haplorchiinae studied possess a structure which is as distinctly different from the muskmelon pattern of opisthorchiid eggs (Pl. II, Figs. 1–3) as from the egg shell structure of another heterophyid fluke, *Metagonimus* sp. (subfamily Heterophyinae) (Pl. II, Fig. 4), which is formed from fine granular tubercles. This filamentous structure is typical of *H. taichui, H. yokogawai* and *S. falcatus*. It is only visible on the surface of some eggs (Pl. II, Figs. 6, 11) because it is mostly overlaid with an amorphous substance (Pl. II, Figs. 5, 8–10). The structure consists of a mesh of tiny,

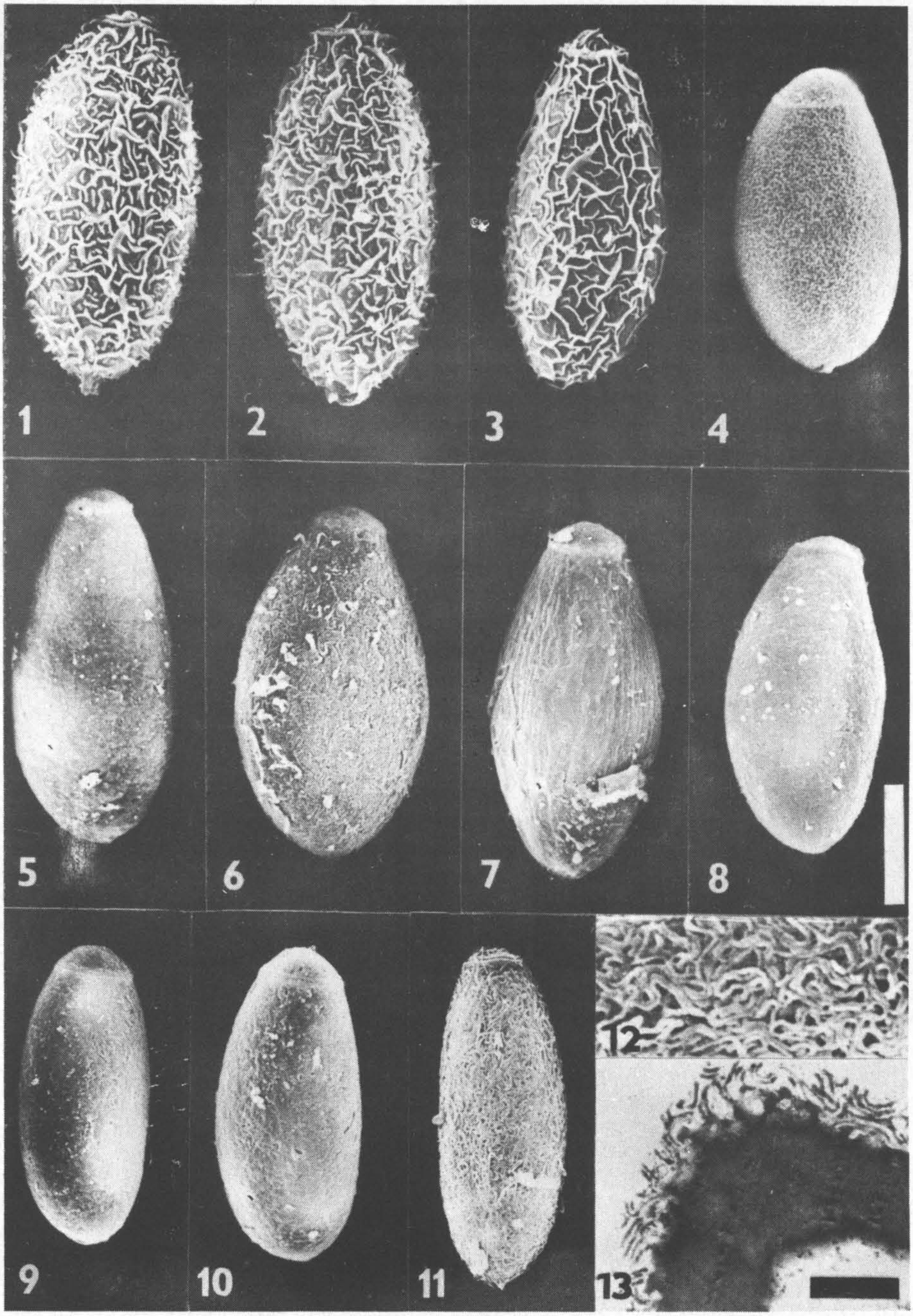

Plate II. Electron microscopy of eggs. **Fig. 1.** *Opisthorchis viverrini* – cat, Vientiane province, Laos. **Fig. 2.** *O. viverrini* – worms excreted from man after praziquantel treatment, Laos. **Fig. 3.** *Clonorchis sinensis* – cat, Khabarovsk region, Russia. **Fig. 4.** *Metagonimus* sp. – man (stool sample), Korea. **Figs. 5 and 6.** *Stellantchasmus falcatus* – cat, Vientiane province, Laos. **Fig. 7.** *Haplorchis pumilio* – cat, Vientiane province, Laos. **Fig. 8.** *Haplorchis yokogawai* – cat, Vientiane province, Laos. **Figs. 9 and 10.** *Haplorchis taichui* – cat, Vientiane province, Laos. **Fig. 11.** *H. taichui* – worms excreted from man after praziquantel treatment, Laos. **Fig. 12.** *H. taichui* – detail of surface structure of the same egg. **Fig. 13.** *H. taichui* – cross section of egg shell (TEM); egg in uterus of fluke, cat, Vientiane province, Laos. Bar = 10 μm (Figs. 1–11), 1.5 μm (Figs. 12–13).

irregularly coiled filaments about 0.1 μm thick (Pl. II, Fig. 12). The cross section through a *H. taichui* egg shows the ultrastructure of the egg shell including the filamentous structure forming its surface (Pl. II, Fig. 13). The surface structure of *H. pumilio* is somewhat different (Pl. II, Fig. 7): the filaments are thicker (about 0.2 μm), closely attached to the egg shell and resemble irregular ridges. They are positioned, predominately, in lenghtwise direction and do not form a typical mesh.

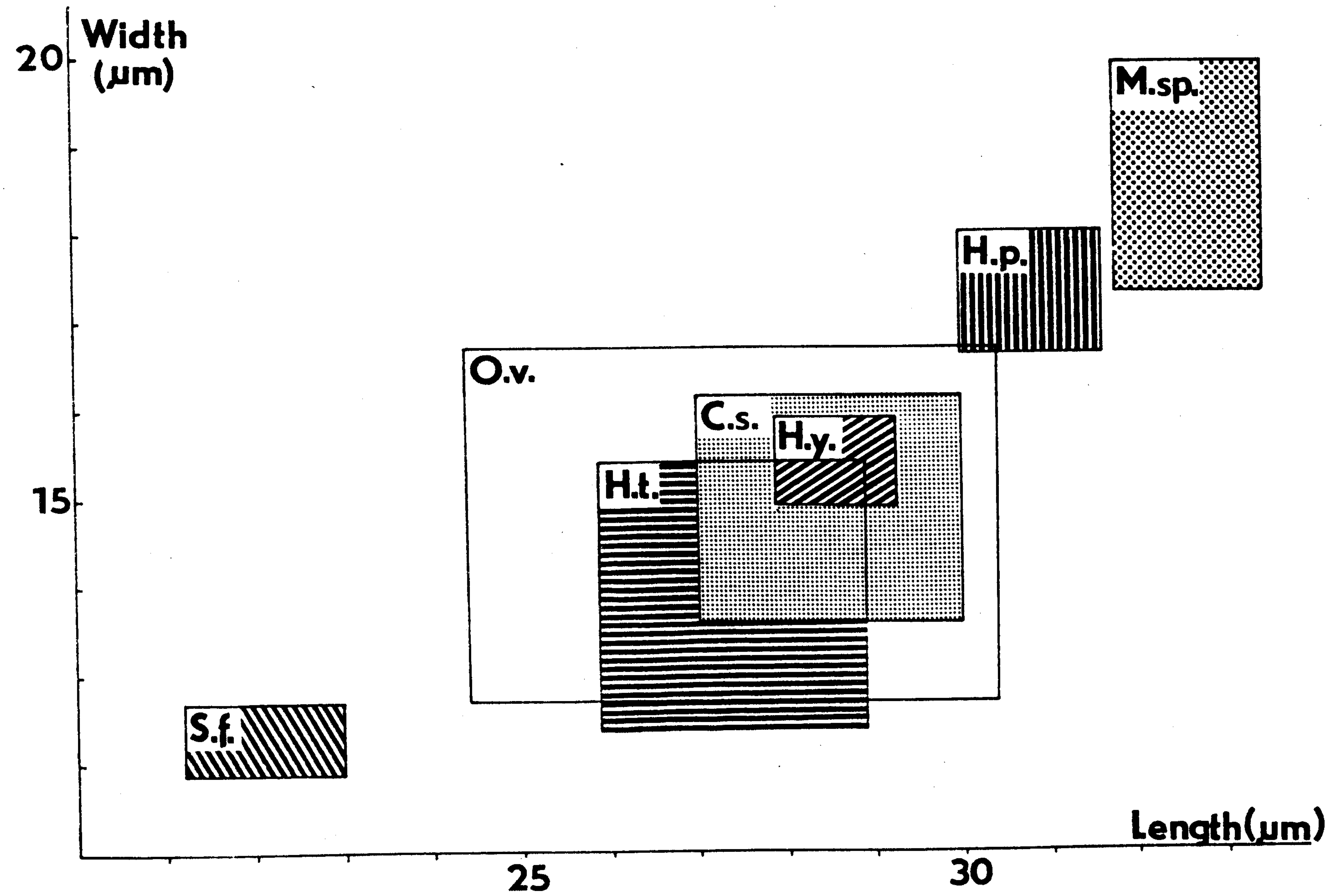

Fig. 1. Comparison of egg measurements expressed as standard deviations from arithmetical mean. C. s. – *Clonorchis sinensis*; H. p. – *Haplorchis pumilio*; H. t. – *H. taichui*; H. y. – *H. yokogawai*; M. sp. – *Metagonimus* sp.; O. v. – *Opisthorchis viverrini*; S. f. – *Stellantchasmus falcatus*.

DISCUSSION

Infections of humans with small intestinal and liver flukes represent in some areas serious medical problem. Diagnostics of these parasites are mostly only based on finding typical eggs in stool. However, species variability and morphological similarity do not enable appropriate species identification. Thus, some authors introduced the term "*Opisthorchis* complex" to avoid obstacles in species determination of the eggs from small flukes. Comprehensive studies involving helminthological examination during autopsies (Manning et al. 1971, Kliks and Tantachamrun 1974) and searching for adult flukes expelled from patients via their stool after praziquantel treatment (Radomyos et al. 1990, Giboda et al. 1991) or after bithionol treatment (Seo et al. 1984) revealed the occurrence of intestinal flukes of various species in humans. Most of these fluke species exhibit a low specificity in definitive hosts. This feature is characteristic mainly for members of the subfamily Haplorchiinae that parasitize fish-eating birds and various mammals including man (Pearson 1964, Pearson and Ow-Yang 1982). Their prevalence in humans might be much higher than in was previously thought to be (Ditrich et al. 1991, Giboda et al. 1991). Trematodes of this subfamily have been reported as causing disturbance in man and the clinical symptoms due to their presence were often considered as negligible. However, their eggs may occasionally enter the blood stream and be carried to various organs of the body, including brain and heart (Radomyos et al. 1990). Visceral complication of intestinal infection resulting in fatal heart failure, cerebral hemorrhage and neurological symptoms have also been reported (Africa and Garcia 1935, Africa et al. 1940, Chandler and Read 1961, Faust and Russell 1964).

Considering the above mentioned medical importance of the flukes of the subfamily Haplorchiinae, a necessity for reliable identification is evident. It is the initial basis underlying the effort to accurately identify their eggs in human stool. Moreover, effective dosage of praziquantel, the drug used against heterophyid intestinal flukes, is over two times lower than the dosage used in cases of human infection by opisthorchiid liver flukes (Cho et al. 1984).

Differentiation of eggs according to their size is rather problematic. Our results showed statistically significant differences in egg size of some species. On the other hand, intraspecific variability in the egg size of specimens from various hosts was not significant. However, it should be emphasized that our results concern only the material under study and cannot be overestimated. The size and shape of eggs may be also influenced by their origin. However, all the eggs under study, with the exception of *Metagonimus* sp., originated from the uterus of adults. In addition, according to our previous observations, the influence of host and source of material (eggs from stool or from fluke uterus) does not seem to be significant. A comparison of our results with data published in literature showed that they corresponded with data of most authors studying egg morphology of small flukes. However, there exist some notable differences in literature (Table 2). These differences not only might be influenced by subjective factors but also by geographical origin of species studied. In our opinion, some of the discrepancies in egg size referred to in the literature can hardly be explained by intraspecific variability; e.g. all measurements presented by Fujino et al. (1989) are about 1.5× smaller than those presented by other authors including the present data (Table 2). In such case, a measurement fault or miscalculation of scale could explain these differences.

Correct species identification of *Metagonimus* eggs from Korea was not possible owing to the lack of adult worms. According to the egg size, our material resembles the eggs from *M. takahashii* Suzuki, 1930. Moreover, the surface structure of the egg resembled the surface structure of this species as presented by Fujino et al. (1989) – granular tubercles appeared to be more conspicuous than those of *M. yokogawai* Katsurada, 1912. However, the species *M. takahashii* has been referred to only occur in Japan (Saito 1972).

According to our results, the eggs of Haplorchiinae can be recognized through

Table 2. Comparison of egg size of heterophyid and opisthorchiid eggs

Trematode	Host	Geo-graphical origin	Length range (mean) mm	Width range (mean) mm	Author (year)
Haplorchis taichui	hamster	Taiwan	(18)	(8)	Fujino et al. (1989)
	hamster	India	29.0–31.0	(14)	Pande and Shukla (1973)
	man	Thailand	23–32 (28)	10–16 (14)	Manning et al. (1971)
	cat	Laos	25.8–28.9 (27.8)	12.4–15.5 (13.7)	present authors
	man	Laos	24.0–29.9 (27.0)	12.0–15.6 (14.1)	present authors
Haplorchis pumilio	hamster	Taiwan	22–23	10	Fujino et al. (1989)
	hamster	India	30.0–34.0	15–19	Pande and Shukla (1973)
	cat	Laos	30.0–32.0 (30.8)	15.0–18.1 (17.3)	present authors
Haplorchis yokogawai	hamster	India	31.0–32.0	12.0–15.0	Pande and Shukla (1973)
	man	Thailand	28–35 (31)	13–18 (16)	Manning et al. (1971)
	pigeon	India	26–32	12–14	Nath (1973)
	dog	India	28–34	12–14	Nath (1973)
	cat	Laos	26.9–30.0 (28.7)	13.4–16.0 (15.4)	present authors
Stellantchasmus falcatus	man	Korea	25.3–29.2 (27.2)	11.1–13.3 (12.2)	Lee et al. (1984)
	man	Korea	(27)	(12)	Seo et al. (1984)
	cat	Hawaii	19–25 (22)	12–16 (13)	Noda (1959)
	man	Thailand	22.1–23.0 (22.5)	11.3–11.7 (11.4)	Radomyos et al. (1990)
	cat	Laos	20.7–23.2 (22.1)	11.4–13.0 (12.3)	present authors
Metagonimus yokogawai	man	Korea	26.9–31.6 (28.5)	14.2–18.2 (16.8)	Lee et al. (1984)
	hamster	Taiwan	(20)	12–13	Fujino et al. (1989)
	hamster	Japan	23.5–31.5 (27.0)	14.5–18.1 (16.5)	Saito (1972)
	man	Japan	30–36	18–23	Suzuki (1983)
Metagonimus takahashii	hamster	Japan	29.0–34.0 (31.8)	18.1–21.3 (19.4)	Saito (1972)
Metagonimus sp.	man	Korea	32 0–33.6 (32.6)	17.6–20.8 (18.6)	present authors
Clonorchis sinensis	man	Korea	25.3–33.2 (28.3)	14.2–17.4 (15.9)	Lee et al. (1984)
	man	Japan	27–32	15–18	Suzuki (1983)
	cat	USSR	26.3–31.1 (28.5)	12.0–16.8 (14.8)	present authors
Opisthorchis viverrinii	man, cat	Thailand	22–32 (28)	11–22 (16)	Wykoff et al. (1965)
	man	Thailand	19–29 (26.7)	12–17 (15)	Sadun (1955)
	cat	Malaysia	26–28	13–15	Bisseru et Chong (1969)
	cat	Laos	25.8–31.0 (28.3)	12.4–17.6 (14.5)	present authors
	man	Laos	22.8–34.7 (27.1)	12.0–19.2 (15.1)	present authors

their typical filamentous surface structure. This surface is different from the egg surface of members of other heterophyid subfamilies studied: *Heterophyes heterophyes nocens* – Chai et al. (1984) and *Metagonimus* spp. (Heterophyinae) – Suzuki (1983), Fujino et al. (1989) and Ditrich et al. (1990b), *Crypto-*

cotyle lingua (Cryptocotylinae) – Krupa (1974) and *Pygidiopsis ardeae* (Pygidiopsinae) – Køie (1990).

The filamentous structure present on the egg surface of some Haplorchiinae was described as "irregularly coiled rope-like ridges" in *H. taichui* and as "longitudinal irregular ridges of various lengths whose surfaces were tuberculate" in *H. pumilio* by Fujino et al. (1989). Other authors (Radomyos et al. 1990), who studied eggs using a scanning electron microscope overlooked this structure because of the amorphous substance mentioned above and noted only the lack of the muskmelon pattern.

The similar surface structure was described by Krupa (1974) on the egg shell of *Cryptocotyle lingua*. This structure, appearing as surface elevations and forming an undulating pattern, was designated by the author as villosities. In comparison with these villosities, the filaments covering eggs of Haplorchiinae flukes are more dense. Krupa (1974) also recognized in flukes sectioned for transmission electrone microscopy fuzzy, filamentous material discernible among villosities resembling an amorphous substance covering the egg shell of trematodes of the subfamily Haplorchiinae studied.

The total shape of eggs and such morphological details as the "shoulder" created by prominent margin of the operculum or "knob" in abopercular end may be useful in some cases. However, these features should not be overestimated since the variability of egg shape mostly varies to a far greater extent. By contrast, surface structures are very constant and may serve for distinguishing subfamilies or even genera of trematodes infecting human beings.

On the basis of the present data and our previous results (Ditrich et al. 1990b), we would like to add some methodological remarks concerning the identification of trematode eggs in stool samples. Eggs should be studied in native samples or in stool fixed by solutions containing formaldehyde. Data obtained from stained slides are not reliable because of deformations during staining procedures. More than 10 eggs should be examined to get an accurate picture of the shape and, the measurements of at least 30 eggs are necessary for statistical evaluation of egg size. The arithmetic mean and standard deviation is more appropriate for size comparison than the range minimum–maximum. Value of FMI seems also to be very useful for this purpose.

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