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การศึกษาการเจริญเติบโตของตัวอ่อนพยาธิและความดกไข่ของพยาธิใบไม้ในลำไส้ *Echinostoma revolutum* ในไก่บ้าน

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บทคัดย่อ: การศึกษาวิจัยการตรวจพบการเจริญเติบโตของไข่พยาธิ และความไข่ดกของพยาธิใบไม้ในลำไส้ *Echinostoma revolutum* ในไก่บ้าน (*Gallus gallus domesticus*) โดยทำการคัดแยกตัวอ่อนพยาธิใบไม้ *E. revolutum* ระยะเมตาเซอร์คาเรียจากหอยขม (*Filopaludina martensi martensi*) และทำการป้อนระยะติดต่อตัวอ่อนพยาธิใบไม้ระยะเมตาเซอร์คาเรียจำนวน 50 ตัว ต่อลูกไก่บ้านอายุ 3 วัน 1 ตัว (n=60 ตัว) จากนั้นทำการตรวจหาไข่พยาธิจากอุจจาระไก่ เพื่อหาจำนวนไข่และตรวจนับไข่พยาธิในมดลูกของพยาธิ ตรวจนับปริมาณไข่ต่อกรัมมูลไก่ (EPG) และศึกษาสัณฐานวิทยาและการเจริญของไข่พยาธิ จากผลการศึกษาวิจัยพบว่าพยาธิใบไม้ชนิดนี้สามารถดำรงชีวิตอยู่ในตัวไก่บ้านได้ 36 วัน และหลังจากการป้อนตัวอ่อนระยะติดต่อพยาธิใบไม้ชนิดนี้ พบว่าค่าความชุกของการติดเชื้อเท่ากับ 60.0% (36/60) และจากการป้อนตัวอ่อนพยาธิใบไม้ระยะติดต่อทั้งหมด 1,800 ตัวในไก่บ้าน สามารถตรวจพบตัวพยาธิใบไม้ 465 ตัว (27.1%) ส่วนใหญ่จะพบในลำไส้ ส่วน jejunum และ ileum และมีส่วนน้อยที่พบใน caeca และนอกจากนั้นยังพบว่าพยาธิใบไม้ระยะโตเต็มวัยสามารถสร้างไข่ได้ประมาณวันที่ 10 หลังจากทำให้ติดโรค และสามารถตรวจพบไข่พยาธิได้ในมูลไก่บ้านจากการใช้วิธีตรวจด้วยเทคนิคการตกตะกอน formalin-ether ได้นับตั้งแต่วันที่ 10 หลังการป้อนตัวอ่อนพยาธิใบไม้เข้าไป สำหรับการนับจำนวนไข่พยาธิ หรือ EPG นั้นพบว่าปริมาณไข่จะเพิ่มขึ้นตั้งแต่วันที่ 10-16 จากนั้นจะเริ่มคงที่ไปจนถึงวันที่ 36 หลังการป้อนตัวอ่อนพยาธิใบไม้ ไข่ของพยาธิจะเริ่มมีการเจริญเติบโตตั้งแต่วันแรกจนถึงวันที่ 10 ภายหลังจากที่วางไข่ปนออกมากับอุจจาระ และสามารถเจริญเป็นตัวอ่อนระยะที่ 1 ไมราซีเดียม ที่สมบูรณ์ได้หลังจากวันที่ 10 ไปแล้วก่อนที่มันจะฟักออกมาจากไข่ในภายหลัง

คำสำคัญ: *Echinostoma revolutum* ไก่บ้าน ความดกไข่ การเจริญของไข่

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Echinostoma revolutum in Domestic Chickens: Developmental Larval Stages and Fecundity of an Intestinal Trematode

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Abstract: This study was conducted to observe the recovery and fecundity of intestinal trematode, *Echinostoma revolutum* in domestic chick (*Gallus gallus domesticus*) and notes on their egg development. Each 60 three days old domestic chicks were fed 50 cysts of *E. revolutum* isolated from *Filopaludina martensi martensi*. Worm recovery, uterine egg counts, numbers of eggs per gram of feces (EPG), egg morphology and development of *E. revolutum* during of experimental infection in chicks were analyzed. The worms survived in chicks for 36 days post infection (PI). The incidence of infection was 60.0% (36/60). Of the 1,800 cysts fed to chicks, 465 (27.1%) worms mainly recovered from the jejunum and ileum, occasionally in caeca. The worms became ovigerous by day 10 and produced eggs, which were detected in feces as early as 10 days PI. The number of EPG, determined by a modified formalin-ether concentration technique, as well as EPG per worm increased slowly during day 10-16 PI and then remaining stable and showed a little fluctuation until day 36 PI. Additionally, egg development was characterized from day 0 to 10 post embryonation. Eggs developed fully formed miracidia from chicks after 10 days and emerged later.

Keywords: *Echinostoma revolutum*, Domestic chicks, Fecundity, Egg development

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Introduction

Echinostoma revolutum (Froelich, 1802) Looss, 1899 (Digenea: Echinostomatidae) is an intestinal parasite of a broad range of vertebrate hosts, such as birds, mammals, including human and occasionally reptiles and fishes (Kanev, 1994; Fried and Graczyk, 2004). *E. revolutum* is cosmopolitan and infect a

large number of different hosts, both in nature and the laboratory. This worm has a wide range of experimental definitive hosts, though the compatibility may differ considerably between species (Fried, 1984; Franco *et al.*, 1986; Humphries *et al*., 1997). The establishment of *E. revolutum* in experimental animals has been studied in mice (Hosier and Fried, 1986), golden hamsters (Chai *et al*., 2011; Chantima *et al*., 2013) and domestic chicks (Fried *et al*., 1997). Several studies have been reported the fecundity of echinostomes, which had made on the basis of eggs per gram of feces (Odaibo *et al*., 1988, 1989), uterine egg counts (Christensen *et al*., 1990) and total amount of eggs in the feces of the host (Toledo *et al*., 2003; Munoz-Antoli *et al*., 2007). Detailed information concerning uterine egg counts and egg output to determine fecundity appears limited to infections with *E. revolutum* in experimental animals. However, the fecundity of *E. revolutum* in experimental animals has been studied in detail, but no report for studying in details of the uterine egg counts and egg output of this worm infection in domestic chicks.

Echinostome eggs are unembryonated when laid and require a period of incubation in the environment to form mature miracidia capable of hatching (Huffman and Fried, 1990). A comparative photographic study of the eggs of echinostomes, including *E. paraensei*, *E. trivolvis* and *E. caproni* was done by Krejci and Fried (1994) and Fujino *et al*. (2000). There are not morphologically different from other echinostome eggs, whereas the ultrastructure of *E. revolutum* eggs was not reported previously. Incubation of *E. revolutum* eggs from laboratory infected hosts used to study in avian hosts (Kanev, 1994). In addition, the studied on storage and incubation of this worm eggs recovered from wild Canada geese was done by Davis (2005). Most studies concerned about the hatchability of miracidium in various conditions, without observations on the development of *E. revolutum* egg to form mature miracidia. The developments of *E. revolutum* eggs are not well understood, and there is no report on egg development of this worm recovered from the domestic chicks. Therefore, the present study was aimed to determine the worm recovery and fecundity of *E. revolutum* in an experimental chick. Moreover, the authors have described the ultrastructure and development of eggs to form mature miracidia.

Materials and Methods

Experimental infections and worm recovery

Three days old domestic chicks (*Gullus gallus domesticus*), weighing between 48-60 g were used as the experimental hosts.

Metacercariae of *E. revolutum* were collected from naturally infected snails, *Filopaludina martensi martensi* from Chiang Mai province, Thailand. They were collected by the crushing method and isolated using a sharp pin, gently covered with a coverslip, and observed under a light microscope. The presence of a head collar with 37 spines was highly indicative of *E. revolutum.* Fifty encysted metacercariae of *E. revolutum* were orally forced fed to each chick. Each 60 chicks were sacrificed daily at day 1-60 post-infection (PI) by excess diethyl ether for examination of the parasite. All experimental hosts were managed according to the guideline approved by the Animal Ethics Committee of the Faculty of Science, Chiang Mai University. All Chick digestive tracts were roughly divided into the esophagus, crop, stomach (proventriculus and gizzard), small intestine (duodenum, jejunum, ileum), caecum and rectum, longitudinally with a pair of scissors and placed in 0.85% NaCl. Worms were collected and examined under a stereo microscope. The number of recoveries was recorded to determine the infectivity and worm recovery.

Fecundity of the worm

Data on fecundity were determined by uterine egg counts (UEC) and a number of eggs per gram feces (EPG). Fecal samples were examined for determining the EPG. The chick feces were collected separately, incubated at 60°C for 24 hours, weighed and fixed in 10% formalin before an examination. Chick feces were checked daily by a modification of the formalin-ether concentration technique (MFECT) (Elkins *et al*., 1991). Eggs per gram of feces were determined according to Odaibo *et al*. (1988, 1989). The number of eggs in the uterus of 10 worms from each day during early appeared in uterus (10 days PI) to the end of experimental was determined by dissection (Christensen *et al*., 1990). The correlation was used to quantify the association between the worm recovery and UEC, and/or EPG. In addition, the correlation of UEC per worm (UEC/worm) and EPG per worm (EPG/worm) was determined.

Egg morphology

The eggs were photographed, measured and illustrated under a compound microscope for morphological study. Morphological traits of eggs (n=30) were studied and measured by an Olympus eye-piece micrometer. Some eggs were studied for the surface ultrastructure with scanning electron microscope (SEM). Briefly, the eggs were rinsed several times in 0.1 M phosphate buffer, pH 7.4, and fixed in 2.5% glutaraldehyde at 4°C for 24 hours, and post-

fixed with 1% osmium tetroxide for 3 hours. Then they were dehydrated in a graded alcohol series, transferred into acetone, and finally dried in a critical-point dryer. The specimens were mounted on stubs and then coated with gold. The specimens were observed and photographed using a JEOL JSM-5400LV SEM.

Observations of egg development

Mature unembryonated eggs were purified from chick feces by filtration through a series of graded sieves, then observed under a stereo microscope. The eggs were washed in 0.85% NaCl several times and then incubated in multi-well plate cultures containing 2 ml of distilled water. The eggs were incubated at room temperature (25-28°C) with ambient room lighting for at least 2 weeks to observe the final hatch to obtain eggs with fully developed miracidia. Egg development was observed under a compound microscope from live eggs prepared as wet mounts. Egg development was monitored daily. Developmental stages were photographed using a compound microscope (OLYMPUS DP20, Olympus).

Results

Worm recovery

After orally introduced to chicks, the metacercariae excysted and developed into adults in the small intestine. From day 1 to day 36 PI, a total of 465 worms were recovered from 36 chicks that had been infected with a total of 1,800 metacercariae. The incidence of infection was 60.0% (36/60) and the average worm recovery per chick was 27.1%, which varied from 2.0 to 74.0%. Metacercariae of *E. revolutum* developed into mature adults after 8 days PI and ovigerous adults developed after 9 days PI. The worms were mostly recovered from the jejunum (75.2%), ileum (23.4%), and some from the caecum (1.4%) while not found the worms in other parts of the digestive tract. The worms were survived until day 36 PI.

Fecundity of worm

The worms vigerous and began to produce eggs on day 10 PI. Based on the eggs in feces (Figure 1), they firstly appeared at 10 days PI and egg was released continuously from the first day until day 36 PI. Egg per gram of feces per worm (EPG/worm) increased slowly and showed a little fluctuation during day 10-22 PI and then slowly decreased until day 36 PI. After that, the worms were expelled from the chicks by day 37 PI. Figure 1 also shows that the uterine egg counts per worm (UEC/worm) during early appeared in uterus (10 days PI) to the end of experimental. The UEC/worm rapidly increased during the first two weeks of

infection and the mean number of UEC/worm observed daily was 172.8±64.6. When the UEC/worm and EPG/worm were plotted, no correlation was observed between those 2 parameters (*r=0.06*) whereas the worm recovery and the number of EPG/worm were highly correlated (*r=0.98*), also the correlation coefficient of worm recovery and UEC/worm were relatively high (*r=0.58*) (Figure 2).

Egg morphology

The morphology of *E. revolutum* eggs were photographed under a light microscope (LM) and SEM (Figure 3). LM observations revealed the structures of the operculum and abopercular region. The eggs were ovoid, pyriform or elliptical, operculate and yellow-brown. They are unembryonated, with a smooth shell with a definite knob like thickening at the abopercular end of the shell. The eggs of *E. revolutum* were 113-133 μm long and 60- 0 μm wide. As determined by SEM, the surface ultrastructure of the eggs showed that the egg shell surface is smooth and operculate. The operculum was smooth and surrounded by an opercular junction (Figure 3C). The abopercular knob wrinkled invaginates the eggshell (Figure 3D).

Egg development

Egg development showed in Figure 4. The mature eggs were unembryonated when laid (day 0). In 1-3 days old eggs, there are clusters of vitellocytes, which are small cells peripheral to the early embryo, like foam bubbles. The vitellocystes became progressively smaller and then not apparent. In 4 day-old eggs, the vitellocytes surrounded the embryo. In 5 day-old eggs, there was a spherical core of cells forming the developing embryo with clusters of vitellocytes. In 6 day-old eggs, the growing embryo is surrounded by a layer of vitellocytes. In 7 day-old eggs, there was larger enlarged embryo, contained in defined clusters of vitellocystes and balloon-like vesicles. From day 8 to 9, the embryo began to resemble a miracidium, contained balloon-like vesicles. The embryo developed a body with cilia and the embryo had attained nearly the same size as the miracidium with apical papillae and the eye spots obvious in 9 day-old eggs. At day 10, the miracidia was oriented along the long axis of the egg and the balloon-like vacuoles filled with a clear refrainment fluid. The vacuoles incompletely encircled the miracidium and they fill a large space between the shell and the miracidium. Most eggs had two vesicles, but some three or only one fully developed miracidia observed by day 10-11 and hatched later.

Miracidia formed within a week and developed slowly. The first miracidia hatched at day 10 and eggs usually hatched in greater

numbers by day 11. The miracidia showed slight motility within the eggs, with the larva moving longitudinally from the posterior end to the anterior end of the egg. The miracidial tegument is ciliated and began to beat and showed bending movements. Newly hatched miracidia swim rapidly, changing direction from time to time.

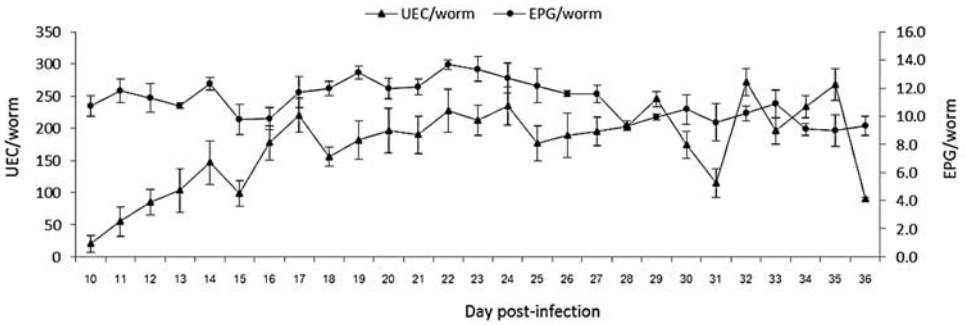

Figure 1. The number of uterine egg counts per worm (UEC/worm) and egg per gram of feces per worm (EPG/worm) detected during experimental from day 10-36 post-infection. Vertical bars represent the standard deviation.

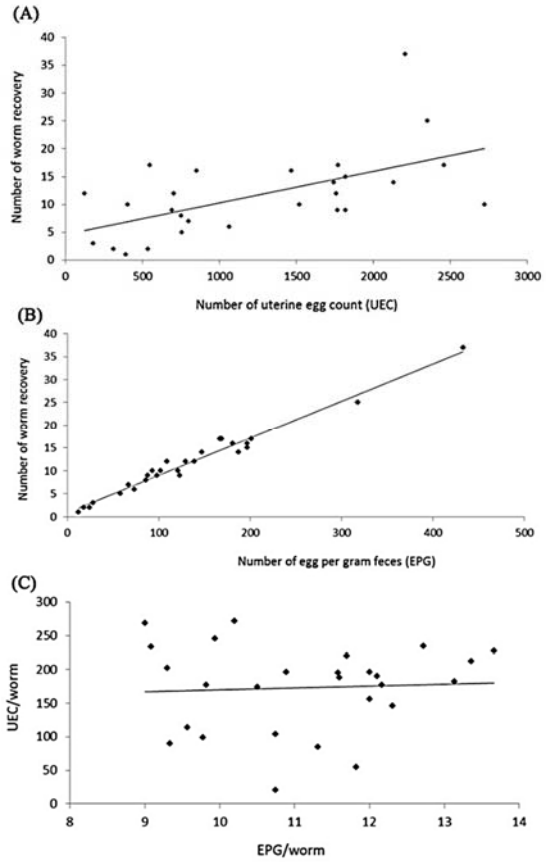

Figure 2. Illustrated the correlation among the parameters used in this experiment: (A) Correlation of the worm recovery and uterine egg counts (UEC); (B) Correlation of worm recovery and egg per gram of feces (EPG); (C) Correlation of UEC per worm (UEC/worm) and EPG per worm (EPG/worm).

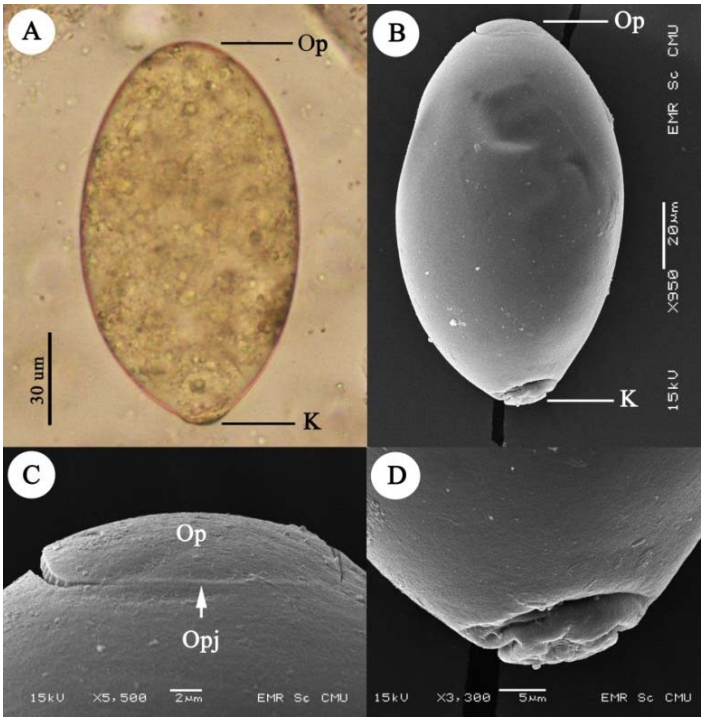

Figure 3. The eggs of *E. revolutum*. (A) Newly laid egg collected from chick feces showing the operculum (Op) and abopercular knob (K); (B) Surface ultrastructure of the egg showing the smooth egg shell surface with the operculum and abopercular knob; (C) Enlarged view of the operculum showing the operculum and operular junction (OpJ); (D) Abopercular knob.

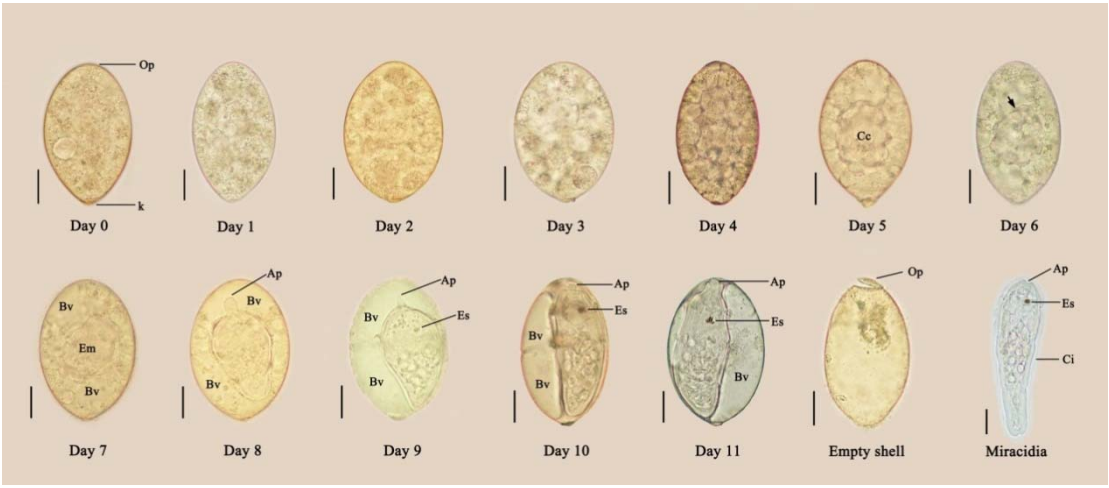

Figure 4. Light micrographs of egg development to form mature miracidia of *E. revolutum*. Note the operculum (Op), abopercular knob (K), the central core cell (CC), the embryo (Em), balloon-like vesicle (Bv), apical papilla (Ap), eye spots (Es), cilia (Ci) and layer of vitellocytes (arrowhead); scale bar = 30 µm.

Discussion

The results of this study has been pointed out that the worm recovery and fecundity of *E. revolutum* in the experimental chicks. The average of worm recovery was high (27.1%) and maintained thus in domestic

chick until day 36 PI. The worm recovery rate in this study was relatively higher than previous reports in the domestic chick by Fried *et al*. (1997). Based on the distribution of worm indicate that this worm will invade the jejunum and ileum, and some in the caecum. Nevertheless, no study about the longevity of *E. revolutum* in domestic chick was found. Kanev (1994) reported the longevity of *E. revolutum* in pigeons lived between 4 and 8 weeks under laboratory conditions, while in this study they were survived in domestic chick intestine for 36 days (5 weeks).

The eggs of *E. revolutum* usually appeared in feces on day 10 PI of young pigeons and day 11 or 12 PI in hamster (Kanev, 1994), while the worms in this study were ovigerous and began to produce eggs on day 10 PI. These findings suggested the birds (chicks) were compatible host than mammalian hosts. Several studies used quantitative methods for measuring fecundity of echinostomes (Toledo, 2009). The quantitative measurements for fecundity of *E. revolutum* are not well understood and had no report. The fecundity of *E. revolutum* in this study was measured by the UEC and EPG. The patterns of EPG/worm is similar to *E. malayanum* in mice (Srisawangwong *et al*., 2004), *E. friedi* in rat (Toledo *et al*., 2006) and *E. caproni* in mice (Munoz-Antoli *et al*., 2007) and hamster and rat (Toledo *et al*., 2004), that gradually increases and high egg output observed on day 22. The UEC of *E. revolutum* was not constant during this experiment. The UEC rapidly increased during the first two weeks of infection, which probably in relation to the progressive maturation of the adult worms. The UEC/worm in *E. revolutum* infection in domestic chick in the present study is similar to that observed for the closely related *E. caproni* in NMRI mice (Odaibo *et al*., 1988, 1989). Recovered worm dependent constraint on worm fecundity was observed in these studies. The correlation coefficient indicates that the worm recovery and EPG/worm were highly correlated. This result also showed the positive correlation between worm recovery and UEC/worm. In other words, an increase of recovered worm may reveal higher the egg production in uterus and egg output in feces. In contrast, the correlation between EPG/worm and UEC/worm are not correlated. Therefore, the numbers of egg in uterus may not reflect the egg release of worm. The authors prefer both parameters, UEC/worm and EPG/worm can be used to determine the fecundity of *E. revolutum* in experimental chick. Nevertheless, the egg production on the basis of UEC/worm and EPG/worm constitute only partial measurement of fecundity, because the egg output of echinostomes depends on

a number of factors. Toledo (2009) suggests that these factors include echinostome species, population density, age of infection and host species. Uterine egg counts and the number of egg releases as measurements of the fecundity in *E. revolutum* infections in other host species needs to be re-evaluated.

Furthermore, there was considering the morphology of eggs worm. LM investigations revealed the opercular and abopercular region in eggs of *E. revolutum*, which agrees with Chai *et al*. (2011). Moreover, eggs of *E. revolutum* are similar morphologically to other echinostome, including *E. paraensei, E. caproni* and *E. trivolvis* (Fujino *et al*., 2000). The SEM of *E. revolutum* eggs has not been documented previously. The present results reveal that the surface ultrastructure is similar to other echinostomes (Krejci and Fried, 1994; Fujino *et al*., 2000). Eggs are characterized by a smooth shell, opercular area with an opercular junction and an abopercular knob. The opercular junction was not conspicuous as with the eggs of other trematodes, heterophyid and opisthorchiid (Lee *et al*., 2012). The abopercular knob has winkles, deep invagination and infolding of the shell, while that of other echinostomes (*E. paraensei*, *E. caproni* and *E. trivolvis*) has superficial winkles and shallow infolding (Krejci and Fried, 1994; Fujino *et al*., 2000). This distinguishes this worm from other echinostomes. Additionally, the present SEM finding of this worm egg was newly added for study and significant finding of abopercular knob were obtained.

The egg development of *E. revolutum* has only one other reported (Davis, 2005). Direct comparison between their studies and this study is not able to be made because of differences in the methods of egg preparation and incubation. Davis (2005) determines the effect of cold storage duration on the incubation success of *E. revolutum* eggs. The eggs were stored at 4-8 ℃ for up to 72 weeks before being examined, whereas this study were used fresh eggs isolated from host feces which were undeveloped (unembryonated) when laid. The eggs contained clusters of vitellocytes at an early phase of development which was also described in *E. caproni* eggs by Schmidt (1998), while the vitellocystes in other trematode, *Fasciola hepatica* termed as yolk cells (Hussein *et al*., 2010). During the late phase of egg development, balloon-like vesicles are formed and filled with a clear refrainment fluid. Schmidt (1998) suggested that these vesicles are probably cell debris left from the vitellocytes which are dispersed by movements of the miracidia. The ballon-like vesicles seem to be identical to those in *E. caproni* (Idris and Fried, 1996; Schmidt, 1998). These vesicles in *E. caproni* described as

glycan vesicles become altered during the late phase of development of the miracidium. They fuse into larger entities ending up as one or two vesicles per egg (Schmidt, 1998).

In the present study, miracidia become fully developed and hatch at as early as 10 days, but maximal miracidia hatching occurred in day 11. In comparison, initial hatching of *E. trivolvis* occurred after 11 days and hatching of *E. caproni* from hamster-source eggs in day 11 and from mouse-source eggs after 13 days (Behrens and Nollen, 1993; Nollen, 1994). However, the authors did not study the viability of egg produced from experimental host. More detail about viability of eggs need to be included in the determination of the fecundity and parasite reproductive success.

Conclusion

Conclusively, the authors evaluated the worm recovery and fecundity of *E. revolutum* in the experimental chick. The results presented in this study provide the most extensive up to date of experimental host for this worm. The authors provided a background for further studies on the fecundity of this worm infection and use of the *E. revolutum*/chick system as a model for the study of host-parasite relationships. Additionally, the present report is the first report reveals the development of eggs to form miracidium of this worm. Such information provides an essential background for the further development of this worm as an applied for treatments and management of this parasite.

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