Prevalence and Clinico-Pathological Studies on Trematodiasis in Marine Turtles in Indonesia

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Abstract

The purpose of this study was to determine the prevalence and clinico-pathological changes associated with cardiovascular fluke infections in marine turtles in Indonesia. Prevalence and clinico-pathological changes associated with flukes infesting other organs were also determined. The heart and associated blood vessels from all samples were examined for the presence of flukes. The heart flukes from a sub-sample of 224 sea turtles were collected and counted to determine the level of infection. Pathological examination was done on 224 sea turtles selected for determination of the level of infection. Blood samples were collected from 134 C mydas, for RBC, PCV and Hb analyses. The prevalence of infection in both species was 100%. The intensity of infection varied between 9 - 197 flukes per turtle. Anaemia, hydropericardium, hydroperitoneum, thickening and hardening of arterial walls, as well as the presence of flukes in the heart, were characteristic gross lesions observed. Microscopically, egg granulomas, presence of flukes, endarteritis and/or perivascular cuffing, and central necrosis were prominent. Other flukes were also consistently found (100%) in all parts of the gastrointestinal tract, and in 40% and 48% of urinary and gall bladders examined, respectively. Negative correlations were found between the total number of cardiovascular flukes and RBC (Rs = -0.55, P = 0.001), PCV (Rs = -0.56, P = 0.001), and Hb concentration (Rs = -0.64, P = 0.001), indicating that their presence was associated with some degree of anaemia. On the other hand, the total number of flukes present in places other than the heart and adjacent blood vessel were correlated neither with blood parameters nor bodyweight/curved carapace length ratio. Findings in this study indicated that cardiovascular fluke infection is a major problem in marine turtles in Indonesia, with anaemia and lodgement of adult flukes and/or their eggs causing life threatening illness.

Key word: *Indonesia, trematodiasis, cardiovascular fluke, prevalence, Pathology*

I. Introduction

Before 1980 most reports of diseases in marine turtles concerned those in farms or oceanariums, and generally associated with husbandry factors such as improper construction of tanks, inadequate nutrition and sanitation, poor water quality and overcrowding, and biting behavior.[1,2,34,5] More recently however, concern about the survivability of marine turtles in the wild, and the impact of human activities on this has grown, as has interest in diseases or potential diseases that may affect them. Diseases such as cutaneous fibropapillomatosis[6,7,8,9], spirorchidiosis[10,11,12,13], ectoparasitism[14,15,16], and coccidiosis[16] have all been identified as possible contributors to the decreasing of population of wild sea turtles.

As well as cutaneous fibropapillomatosis, which is of major interest[9], cardiovascular fluke (CVF) infection (spirorchidiosis) is another potentially life threatening disease of marine turtles in the wild[10,12,13,17,18]. These digenetic trematodes belong to the family Spirorchiidae[19,20,21,22,23]. This disease is characterized by a focus of inflammatory cells associated with flukes or their released eggs surrounding blood vessels in any tissue[10,12,13,17,23].

The prevalence and epidemiology of CVF have been described in green and loggerhead turtles[22,23] and however, as such study was mainly based on field necropsy of dead or moribund turtles, the results may be misleading if used to estimate the actual prevalence of infection and its impact on the wild population of sea turtles[24].

The purpose of the present study was to determine the prevalence and clinico-pathological changes associated with spirorchids infections in slaughtered marine turtles in Indonesia. These animals are more likely to indicate the levels of disease in wild populations than are the moribund animals examined in previous studies. In addition, prevalence and clinico-pathological changes associated with flukes infesting other sites such as those in the intestine, urinary bladder, and gall bladder were also determined. This study was part of a larger study entitled "Diseases and Husbandty Aspects of Wild-Caught Marine Turtles in Indonesia".

II. Materials and Methods

Collection of material

Observations were made and samples were collected at a turtle abattoir near Tanjung Benoa Bay, Bali, during January 1994 to December 1994, five years before all species of sea turtles are protected in Indonesia. The curved carapace length (CCL) of the turtles varied between 39 cm - 116.5 cm (mean ± SD = 76.8 cm ± 22.6) and originating from various capture sites in the Indonesian seas (**Fig 1**). Turtles were transported to Bali by boat and were slaughtered one to 20 days following their arrival. Details of turtles examined for this purpose, in relation to capture sites, maturity status, and sex are presented in **Table 1**.

Table 1: Total turtles examined clinically for trematodiasis and after slaughter in abattoirs in Bali, in relation to their capture site, maturity status, and sex.

	Chelonia mydas				*Eretmochelys imbricata*			
	Mature		Immature		Mature		Immature	
	Male	Female	Male	Female	Male	Female	Male	Female
Java Sea	19	132	77	205	-	-	20	71
Teluk Cempi	0	12	11	39	-	-	9	30

Wakatobi	10	37	8	9	1	1	1	7
Aru	41	140	7	6	-	-	-	-
Berau	56	119	4	7	-	-	-	-
Bird's Head of Papua	39	128	-	-	-	-	-	-
Sukamade	44	102	-	2	-	-	-	-
Pangumbahan	51	104	-	-	-	-	-	-
Total	260	774	107	268	1	1	30	108

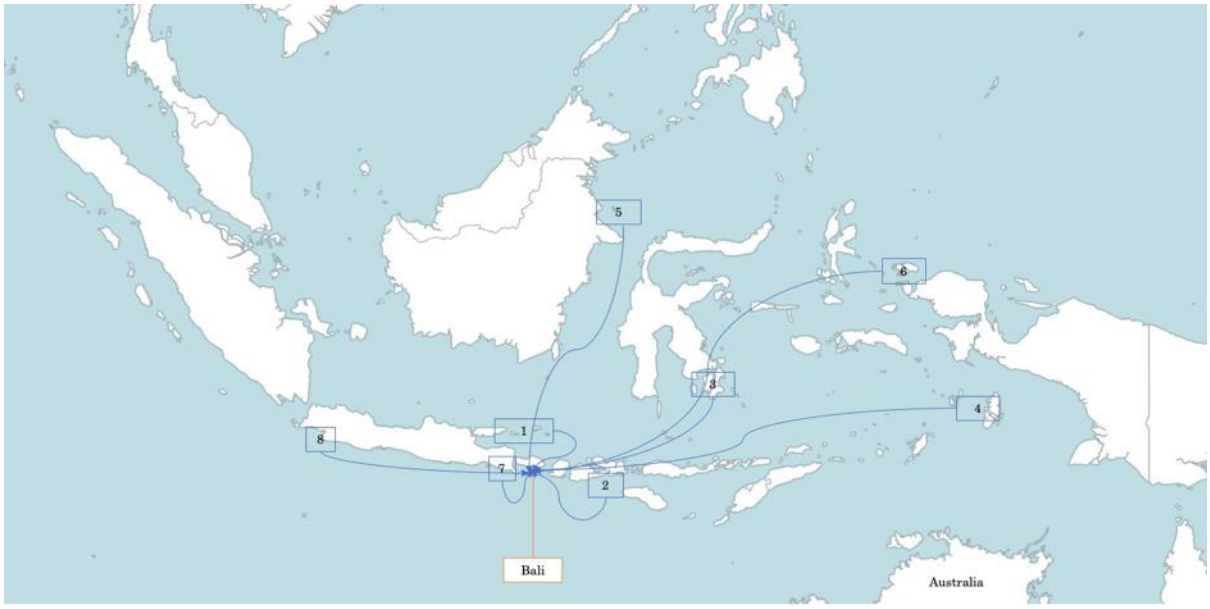


Fig 1: Capture sites of marine turtles examined during the study. 1 (Java Sea), 2 Teluk Cempi, Sumbawa), 3 (Wakatobi, South-East Sulawesi), 4 (Aru), 5 (Berau, East Kalimantan), 6 (Bird's Head of Papua), 7 (Sukamade, East Java), and 8 (Pangumbahan, West Java).

Determining the prevalence of infection

During slaughtering, the heart and associated blood vessels from each of 1409 *C mydas* and 140 *E imbricata* were examined for the presence of flukes. To determine the level of infection of CVF, the heart flukes from a sub-sample of 195 *C mydas* and 29 *E imbricata* were collected and counted. The prevalence and levels of infection with flukes in the gut, urinary bladder, and gall bladder, however, were determined in only 134 *C mydas*.

Collection, fixation, staining procedures, and identification of flukes were done as follows. After the turtles were slaughtered and the plastron was removed, the organs, were separated into: (a) the alimentary tract, (b) urinary bladder, (c) gall bladder, and (d) heart and adjacent blood vessels (approximately 10 cm length). The alimentary tract was then separated into stomach, small intestine, and the large intestine. All of these organs were put into separate buckets (volume approximately 2 litres) filled with a saline solution (formula: 1 part sea water and 3 parts tap water). It should be noted that an attempt was also made to collect flukes from

the liver, spleen, kidneys (with attached ovary/testes), and lungs (including trachea), by slicing them into several pieces (almost all the way through), followed by the procedure applied also for the heart and adjacent blood vessels, gut, gall and urinary bladder. However, as this practice inconvenienced the butcher and the abattoir owner, it was discontinued.

The presence of flukes in the gastrointestinal tract, urinary bladder, the gall bladder, and the heart were evaluated after these organs were cut open, then turned inside-out, and all their contents washed carefully into saline (the surfaces being rubbed gently to dislodge the parasites). In addition, these organs were agitated in the sea water filled buckets every few minutes for about 30 minutes, after which they were removed, and the buckets and their contents, were then swirled repeatedly, allowing intervals of a few minutes for particulate matter to settle. Sea water was then decanted gently. This washing/sedimentation process was repeated until the supernatant was clear, and when appropriate, was transferred to a smaller container. After the water was reduced as much as possible, hot formalin (10% neutral buffered formalin) was poured on top of the flukes quickly (to prevent the flukes from being curled), and then, they were removed into petri dishes, and immediately observed, identified and counted under the dissecting microscope.

Parasites were washed in distilled water, stained by placing them in glass cavity blocks filled with 3-5 ml Gower's Carmine or Delafield's solution[25]. After approximately 24 hours, the stain was discarded, and the worms were rewashed in distilled water. Depending on the intensity of the stain taken by the worms, they were de-stained by 0.5% HCL solution, until the stain was removed from the parenchyma, but the gonads were pale pink. After being partially de-stained in this manner, the worms were put into Scott's substitute tap-water for about five minutes, followed by dehydration through an alcohol series (70, 90, 95, and 100%). Dehydration in each concentration of alcohol was for about 30 minutes. Clearing was done by xylol, and Canada balsam or permount were then used to mount the worms before labelling. Identification was done using light microscopy based on manual provided by Blair (unpublished) [26].

Pathological examinations

A complete pathological examination was done on 224 sea turtles selected for determination of the level of infection of CVF. At slaughter of the turtles, organs were first examined in situ, then a more detailed examination was done after viscera were removed. The slaughter was done by a standard butchering procedure applied in the turtle abattoir.

For histopathology, irrespective of whether or not gross changes were noted, representative tissues from 224 marine turtles were taken. Included were all major organs viz. brain, eyes, salt-secreting gland, lung, liver (sometimes with gall bladder), kidney, urinary bladder, spleen, heart, gonads, and gastrointestinal tract with pancreas. These were fixed in 10% neutral buffered formalin, and after paraffin embedding, sections cut at 6µm, were stained with haematoxylin and eosin.

Haematological examination

Blood samples were collected from 134 *C mydas* in which the flukes in organs such as the heart, gut, urinary and gall bladders were collected and counted. Blood collection was done for adult (CCL more than 85 cm)[27] and immature turtles[28]. All samples were sent on ice, directly to the Disease Investigation Center office in Bali for determination of RBC, PCV, and Hb.

Determining correlations between total heart flukes or total flukes found in other organs, with bodyweight/CCL ratio and some blood parameters

Such correlations were ascertained in the 134 green turtles from which blood was collected as above. These turtles were of varying size (CCL = 37.2 cm to 110.2 cm). All had been captured in approximately the same habitat (ie waters around the Java Sea in the eastern part of Java), during May 1994. Following their capture, all turtles experienced an "out of water" period of approximately nine days during transportation to Bali, and while being kept in the temporary holding areas for two to three days before slaughter. Bodyweight was measured and blood samples taken twice, ie immediately after the arrival of the turtles (at the unloading site), and immediately before slaughter.

Statistical analysis

Flukes were grouped into two groups according to location where they were found; those found within the heart, and those collected in the gut, urinary and the gall bladders. Total flukes in each of these two broad locations were then correlated with the bodyweight/CCL ratio and blood variables such as RBC, PCV, and Hb by means of a bivariate Spearman Rank Correlation test[29].

III. Results

Prevalence of the cardiovascular fluke's infection

All samples examined harbored cardiovascular flukes (CVF). The prevalence of infection in both *C mydas* and *E imbricata* was 100%. Based on the examination of 195 *C mydas* and 29 *E imbricata*, flukes of six genera of the family Spirorchiidae were associated with cardiovascular lesions. In order of decreasing prevalence, they were Learedius (89.7%), Hapalotrema (36%), Neospirorchis (12.5%), Amphiorchis (1.3%), Spirorchis (0.9%), and Monticellius (0.9%). All of these flukes were identified in *C mydas*, but only four genera namely Learedius, Hapalotrema, Neospirorchis, and Amphiorchis were found in *E imbricata* (**Table 2**).

Learedius sp was the most prevalent CVF in *C mydas*, as evidenced by its prevalence in 193 of 195 (99%) green turtles examined. *Learedius sp* was found either as a single invader (116 of 193 - 60.1%), or in combination with other heart flukes (**Table 3**). In 77 *C mydas* infected with two or more genera of heart flukes, a combination involving Learedius and Hapalotrema was the most common (57 of 77 - 74%).

Table 2. Prevalence of cardiovascular infection, in relation to the genera of the fluke, in a total of 224 turtles, composed of 195 *C mydas* and 29 *E imbricata*. Numbers in brackets are the total numbers of each species of turtle infected.

Fluke	Species and number of infected turtles	Total turtles infected	Prevalence (%)
Learedius	*C mydas* (193) *E imbricata* (8)	201	89.7
Hapalotrema	*C mydas* (60) *E imbricata* (22)	82	36
Neospirorchis	*C mydas* (27) *E imbricata* (1)	28	12.5
Amphiorchis	*C mydas* (2) *E imbricata* (1)	3	1.3
Spirorchis	*C mydas* (2)	2	0.9
Monticellius	*C mydas* (2)	2	0.9

Hapalotrema spp. was another CVF that was of probable importance in *C mydas*. Its prevalence was 30.3% (59 of 193) turtles. It was present as the only genus of CVF found in two *C mydas* but was in combination with other heart flukes in the remaining 57. Other spirorchids namely Neospirorchis, Amphiorchis, Spirorchis, and Monticellius were less. Prevalent in green turtles. None was found as a single invader, but in combination with *Learedius sp* (**Table 3**).

Table 3. Cardiovascular flukes found in a total of 195 green turtles.

Flukes	No. infested with particular fluke(s)	Prevalence (%)
Learedius (single infestation)	116	59.5
Learedius and Hapalotrema	45	23.2
Learedius and Neospiroschis	17	8.7
Learedius and Amphiorchis	1	0.5
Learedius and Monticellius	1	0.5
Learedius, Hapalotrema, and Neospirorchis	10	5.1
Learedius, Hapalotrema, and Spirorchis	1	0.5
Learedius, Hapalotrema, and Monticellius	1	0.5
Learedius, Hapalotrema, Amphiorchis, and	1	0.5
Hapalotrema (single infestation)	2	1
Total infested green turtles	195	100

Unlike in green turtles where Learedius was the most commonly found, in hawksbill turtle, Hapalotrema was more important. Overall prevalence of Hapalotrema infestation in hawksbills was 69% (22 of 29 examined); in two hawksbills this fluke was in combination with other flukes (**Table 4**).

Table 4. Cardiovascular flukes found in a total of 29 hawksbill turtles examined.

Flukes	No. infested with particular fluke(s)	Prevalence (%)
Hapalotrema	20	69
Learedius	5	17.1
Neospirorchis	1	3.5
Hapalotrema and *Learedius*	2	6.9
Learedius and *Amphiorchis*	1	3.5
Total infested hawksbills	29	100

Learedius and Neospirorchis were two genera that were also found independently in hawksbills. Their respective prevalence was 17.2% (5 of 29) and 3.5% (1 of 29). As in green turtles, Amphiorchis in hawksbills was in combination with another fluke *(*Learedius*)*, probably indicating a minor role of this fluke in the hawksbill turtle.

Level of infection

The intensity of infection varied greatly between 9 and 197 (35 ± 30 – Mean ± SD), and relatively few turtles (28 of 224 - 12.5%) had burdens of more than 65 CVF. This feature was apparent for both turtle species. In green turtles, the average CVF burden was 37 ± 37 (range = 9 - 197), with only a small proportion of turtles (27 of 195 - 12.1 %) having more than 65 CVF. In the hawksbill, the average CVF burden (21 ± 16, range = 9 - 79) was less and the

majority (23 of 29 - 79.3%) had fewer than 25 CVF.

Clinical signs

Marine turtles from Indonesia examined during this study were wild-captured, and apart from changes such as debilitation, lacerations and fibropapillomatosis[30] they showed no overt clinical signs of illness. It was difficult to ascertain to what extent debilitation was due to fluke infection. Most turtles were emaciated, as reflected by a concave appearance of the plastron and a "sunken" appearance of both eyes. They were generally immobile, and unresponsive when approached. Some turtles were reported to have exhibited a buoyancy abnormality and were observed floating in the water. A bloody discharge from the mouth, with a frequent forced expiratory effort was noticed in one green turtle. A continuous shaking of the head to the right side was noted in another green turtle. Additionally, most of the turtles had an indication of external trauma such as wounds on of the front or hind flippers. One had a large irregularly shaped deep wound on the ventral side of the neck.

Pathological changes associated with cardiovascular fluke infection

In general, pathological changes observed in CVF-affected green turtles and hawksbills were comparable to those described in previous reports for loggerheads[12], green turtles[10,11,13,17], and hawksbills[11]. Emphasis here is therefore placed on exceptional or unusual findings not previously described.

Gross findings

Gross changes observed in green and hawksbill turtles were similar. The lesions observed and their prevalence are tabulated in **Table 5**. Anaemia, indicated by paleness and lack of normal coloration of all organs were observed in 34 green turtles and five hawksbills. A total of 29 *C mydas* and four *E imbricata* that were heavily infected by spirorchids (with fluke burdens of more than 60 per animal) all had an excess of one to two liters of clear pericardial (hydropericardium) and peritoneal fluid (hydroperitoneum) as compared to turtles infected lightly.

Flukes were found in any part of the heart chamber (**Fig 2a**), but were mostly on the endocardium of both auricles, especially on the atrioventricular valves. Thickening and hardening of arterial walls, which was sometimes accompanied by thrombosis (either with or without the visible presence of flukes) was frequent (43 of 233 – 18.5%) in the aortic arches (**Fig 2b and c**) in green turtles with a total of at least 60 flukes. A similar lesion was also found

in other blood vessels, such as those beneath the serosa of the gastrointestinal tract (38 of 233 - 16.3%), or within the meninges (35 of 63 – 55.6%) (**Fig 2d**).

Table 5 : The prevalence of gross lesions observed in a total of 231 sea turtles in Indonesia.

Gross lesions	***C mydas* (N=200)**	***E imbricata* (N=31)**
General:		
Anaemia	17	16.1
Hydropericardium and Hydroperitoneum	14.5	12.9
Heart and major vessels:		
Sub-endocardial petechiae	22	32.3
Arterial aneurisms with or without thrombosis	18.5	12.9
Para–aneurismal cysts	3.5	0
Calipee: Mucoid degeneration	94	87.1
Gastrointestinal tract:		
Multiple nodules in the serosa and muscularis layer of the stomach and/or intestine	61.5	38.7
Raised linear and darkly pigmented lesions in the intestine	9.5	3.2
Brain: Aneurisms and mineralization of the meningeal vessels	56.4	25.8

Fig 2: Gross pathology associated with cardiovascular fluke infection in the heart and blood vessels. (**a**) Blood flukes (Digenea: Spirorchiidae) within the heart chamber of a green turtle. Note that the flukes (arrows) are very small (about 0.1 to 0.2 cm length and 0.04 to 0.06 cm wide) and are largely transparent except for the dark, laterally-located vitelline glands and gut. These flukes were identified as Hapalotrema which is one of the largest heart flukes in the sea turtle. (**b**) Thickening and aneurysmal dilation of the wall of the aortic arch of a green turtle as a result of entrapped flukes or their eggs. Bar = 20 mm. This lesion, which was frequently accompanied by thrombosis and mineralization, seemed to be associated with the severity of infection as manifested by the total flukes found in the heart chamber. Note heart (short arrow) and portion of trachea (long arrow). (**c**) Section following formalin fixation of the aortic arch of a green turtle infected with cardiovascular flukes. Note irregular thickness of vessel wall and focal haemorrhagic lesions (arrow heads). (**d**) Thickening of the wall of the meningeal vessels of a green turtle as result of entrapped flukes or their eggs. Bar = 10mm.

Histopathological changes

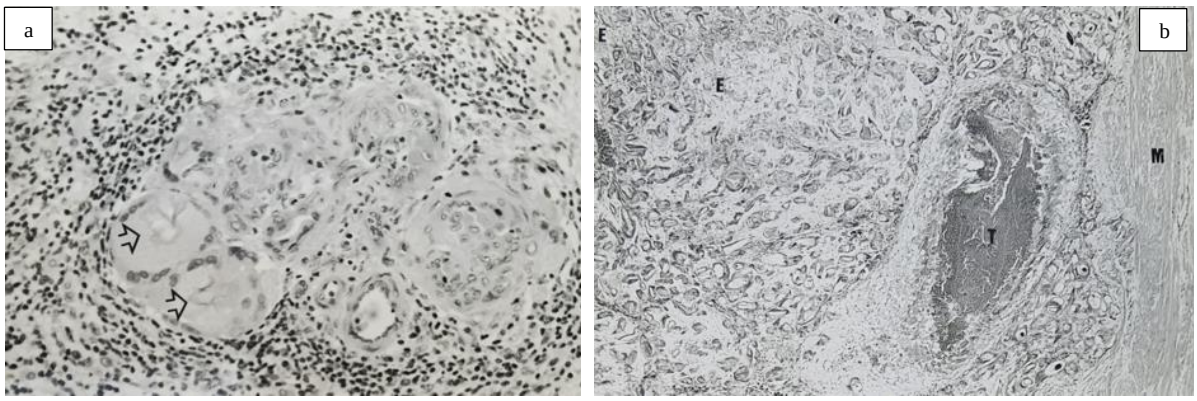
Microscopically, the prominent features of spirorchidiosis were egg granulomas, presence of fluke's endarteritis and/or perivascular cuffing, and central necrosis. In each infected turtle, single or multiple egg granulomas in different stages of development were observed in most tissues examined including brain and meninges, choroid layer of the eyes, salt-secreting glands, skin, lung, trachea, oesophagus, stomach, small intestine (including pancreas), pseudo-caecum, large intestine, liver and gall bladder, adrenal, kidney, urinary bladder, gonads, and heart and major blood vessels. A characteristic feature of granulomas was the presence of intact eggs or remnants of them surrounded by giant cells, macrophages, lymphocytes, and plasma cells after contained by a fibrous capsule (**Fig 3a**). The latter granulomas were found especially in lung, kidney, liver, and spleen. Whether granulomas were focal or multifocal depended on the organ involved, and the severity of the infection. In most organs namely heart, skin, kidney, urinary bladder, gonads, trachea, and oesophagus, focal granulomas associated with a solitary intact or disintegrating fluke egg were common. In spleen, lungs, gut, choroid layer of the eye, brain, and major blood vessels, however, multifocal granulomas, which were associated with numerous eggs (**Fig 3b**) were more frequent and were observed not only in the intima of blood vessels, but also scattered within their vasa vasorum. In the meningeal vessels and within the parenchyma of the brain, presence of numerous eggs and/or adult flukes (**Fig 3c**) sometimes elicited hemorrhages.

Adult flukes (intact or disintegrating) were also frequently encountered in sections (**Fig 3d**). In order of decreasing prevalence, they were found within blood vessels of the brain (64 of 79 examined - 81%), gut (86 of 212 - 40.6%), salt-secreting gland (18 of 54 - 33.3%), heart (49 of 201 - 24.4%), spleen (49 of 219 - 22.4%), kidney (33 of 185 - 17.8%), lungs (33 of 217 - 15.2%), adrenal (29 of 209 - 13.9%), and liver (25 of 193 - 13%). In the brain, flukes were especially present in the meningeal vessels (64 of 64 - 100%), and much less often (9 of 64 - 14.1%) were they found to penetrate into the arterioles of the brain parenchyma. In the

gastrointestinal tract, flukes were of comparable prevalence in all regions such as the stomach (64 of 86 - 74%), small - (56 of 86 - 65%), and large intestines (58 of 86 - 67.5%). In all of these sections, blood vessels of the submucosa and beneath the serosa seemed to be the most common sites. In the heart, flukes were usually buried between myocardial fibres. Blood vessels in the medulla (16 of 18 - 88.9%) of salt-secreting glands were more likely to be the sites of flukes than vessels in the cortex (4 of 18 - 22%). In kidney and adrenal however, flukes were mostly observed in the cortical blood vessels (25 of 33 - 75%, 25 of 29 - 85%, respectively) rather than in the medulla (15 of 33 - 45.8% and 6 of 29 - 20%, respectively). In the spleen, arteries in the trabeculae were the sites of the flukes, and in the lungs, arteries adjacent to large bronchi were the most frequent site where flukes were found. On two occasions, however, disintegrating flukes were within capillaries of the interalveolar septa. Of 25 specimens with flukes in the liver, 23 had flukes within the veins of portal area. In one case, flukes encapsulated by fibrous tissue, macrophages, and pseudo-pallisaded giant cells, were on the capsule of the liver, adjacent to fluke migration tracts and necrotic debris that were also surrounded by fibrous tissue, macrophages, and giant cells.

Granulomas in which neither eggs nor flukes could be demonstrated were often observed surrounding a zone of central necrosis, which in turn contained flukes or persistent remnants of them, such as rod-shaped disintegrating vitellaria or sperm of the flukes.

Because of shrinkage and distortion of flukes and/or their eggs during histological processing, their generic identification based on morphology was seldom possible. Presence of a golden-brown egg with an elongated tail, or of a spined cuticle, however, justified their identification as *Laredius sp* and/or *Hapalotrema* sp, respectively.



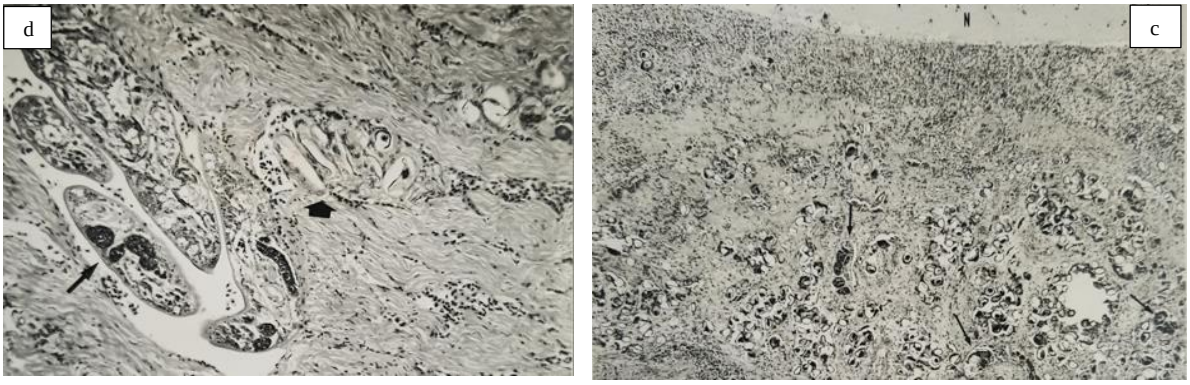


Fig 3: Histopathology spirorchidiosis in the heart, blood vessel, and meningen.
(**a**) A typical granuloma associated with blood fluke infection. Note the disintegrating remnants of fluke egg(s) (arrows) within multinucleated giant cells and surrounding mononuclear cells. Haematoxylin and eosin x275. (**b)** Microscopic appearance of lesions shown in **Figure 2a**. Note muscularis of the vessel (M), numerous eggs (E), and central necrosis (T) which was probably the tract of a fluke. Haematoxylin and eosin x48. (**c)** Severe meningeal lesions associated with spirorchid infection. Note normal brain parenchyma (N) and the lodgement of remnants of adult flukes (arrows). Haematoxylin and eosin x45. (**d)** Adult flukes (long arrow) and formation of their eggs (short arrow) in the heart muscle of a green turtle severely infected by blood flukes (Digenea: Spirorchiidae). Haematoxylin and eosin x 114.

Another interesting feature that was probably associated with this infection was the presence of nodules within the serosa and/or muscularis of the gut in a total of 123 C *mydas* and 12 E *imbricata* examined (**Fig 4a**). These nodules were white or darkly pigmented and fibrotic, measuring about 0.4 to 0.6 cm in diameter. They were most frequently observed in the small intestine, followed by the stomach and then large intestine. Microscopically, nodules consisted of a thick fibrous wall surrounding necrotic debris (**Fig 4b**). Sometimes fragments of eosinophilic cuticle-like material, and sperm–like particles resembling those in the tegument and testes, respectively, of the flukes, were observed within lesions. Raised, darkly pigmented linear lesions, measuring about 2 - 8 cm long and 0.3 x 0.5 cm wide were occasionally observed within or beneath the mucosa of the large intestine of a total of nine C *mydas* and one *E imbricata* (**Fig 4c**). Microscopically, these lesions were seen to consist of masses of fluke eggs (**Fig 4d**).

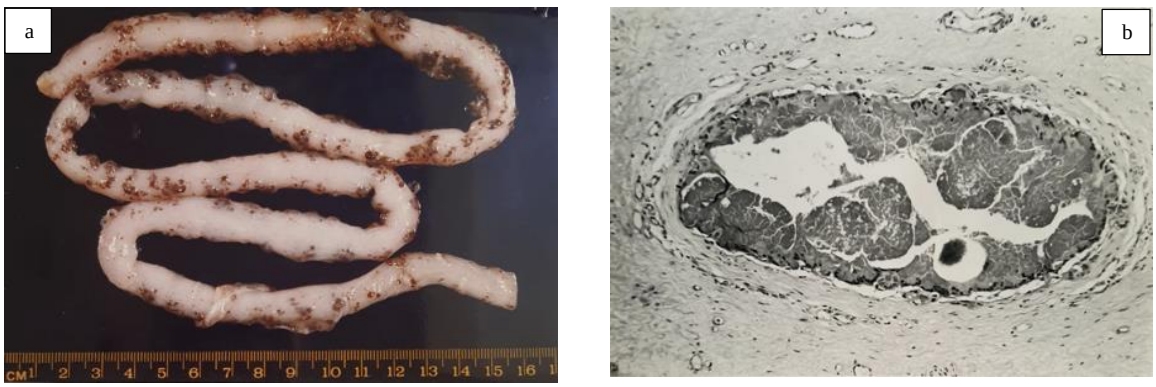


Fig 4: Gross and microscopic pathology blood fluke in the gastrointestinal.
(**a**) Multiple dark-pigmented intra-serosal nodules in the intestine of a green turtle severely infected by blood flukes. (**b)** Microscopic appearance of a nodule as shown in **Figure 4a**. A granulomatous reaction and a ring of fibrosis surrounds necrotic debris, which probably indicates the tract of the fluke. Haematoxylin and eosin x 114. (**c)** A raised darkly pigmented linear swelling in the mucosa of pseudocaecum of a green turtle. Bar = 12 mm. (**d)** Microscopic appearance of lesion shown in **Figure 4c**. Note masses of eggs entrapped in the submucosa of the pseudo-caecum. Haematoxylin and eosin x45.

Infection of flukes other than spirorchids

Prevalence and the level of infection

As is in the heart and associated blood vessels, flukes were also consistently found (134 of 134 - 100%) in all parts of the gastrointestinal tract such as stomach, small intestine, and the large intestine. In the urinary and gall bladders, however, their prevalence was 39.6% (53 of 134) and 47.8% (64 of 134), respectively.

Various kinds of flukes (**Table 6**) and a wide range in the level of infection (**Table 7**) in each organ was found. The highest number was in the large intestine (200 ± 397), followed by the stomach (120 ± 196), small intestine (61 ± 92), gall (16 ± 18), and urinary bladders (8 ± 15).

Table 6: Prevalence of flukes in the gastrointestinal tract, gall, and urinary bladders of a total of 134 *C mydas* in Indonesia.

Organs	Family	Genera	Prevalence
Stomach	Pronocephalidae	not determined	134/134
	Microscaphiidae	not determined	42/134
Small intestine	Pronocephalidae	not determined	44/134
	Microscaphidiidae	not determined	134/134
	Angiodictyidae	not determined	9b/134
	Rhytidodiidae	not determined	17/134
Large intestine	Microscaphiidae	not determined	122/134
	Angiodictyiidae	not determined	134/134
	Paramphistomiidae	not determined	88/134
	Rhytidodiidae	not determined	19/134
Urinary bladder	Pronocephalidae	*Pyelosum*	50/134

Gall bladder	Gorgoderidae	*Plesiochorus*	5/134
	Rhytidodidae	*Rhytidodoides*	64/134
	Telorchiidae	*Orchidasma*	20/134
	Unidentified	Unidentified	3/134

Table 7: Mean number of flukes found in a total of 134 green turtles in Indonesia, in relation to the organs where they were observed (except the heart and associated blood vessels).

Organs	Mean	SD	Minimum	Maximum	N
Stomach	120	196	10	1253	134
Small intestine	61	92	10	567	134
Large intestine	200	397	20	2726	134
Urinary bladder	8	15	1	81	53*
Gall bladder	16	18	1	84	64*

* Total *C mydas* with flukes

Pathological changes possibly associated with flukes other than spirorchids

Apart from petechial haemorrhages, there were no obvious pathological changes associated with flukes other than spirorchids. Such haemorrhages were mostly observed in the gastrointestinal tract (**Fig 5a**), in which its prevalence was 30.4% (41 of 134). Petechial haemorrhages were more frequently observed in the stomach (24 of 134 - 17.9%) than in the large intestine (14 of 134 - 10.4%) or small intestine (3 of 134 - 2.2%). Petechial haemorrhages were also occasionally found in the urinary bladder (**Fig 5b**) or the gall bladder; prevalence in those organs were 9.4% (5 of 53) and 4.7% (3 of 64), respectively. Presence of petechial haemorrhages in the gut, gall, and urinary bladders did not appear to be related to total numbers of flukes in that organ (**Table 8**).

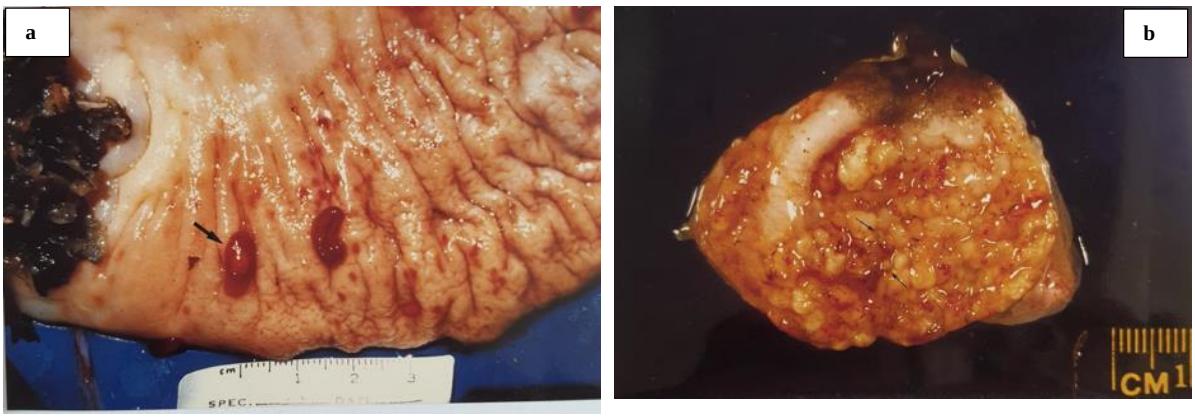


Fig 5: Pathological changes associated with flukes other than spirorchids.
(**a**) Petechial haemorrhages in the mucosa of the stomach of a green turtle, presumed to be due to infection with pronocephalids which are engorged with blood (arrow). (**b**) Petechial haemorrhages in the mucosa of the urinary bladder of a green turtle, possibly associated with pronocephalids infection (arrows). Note the multi-nodular appearance of the mucosa, which is normal in sea turtles.

Table 8: Prevalence of petechial haemorrhage in the gut, gall, and urinary bladders, presumed to be associated with flukes in marine turtles in Indonesia.

Organs (and number of flukes present)	Prevalence (%)		Range of total number of flukes per turtle	
			In those with petechiae	In those without petechiae
Stomach (134)	17.9	(24 of 134)	18 to 1197	10 to 1253
Small intestine (134)	2.2	(3 of 134)	10 to 567	11 to 440
Large intestine (134)	10.4	(14 of 134)	20 to 1355	20 to 2726
Gall bladder (64)	4.7	(3 of 64)	15 to 81	1 to 84
Urinary bladder (53)	9.4	(5 of 53)	14 to 52	1 to 81

Correlation between fluke burdens with bodyweight/CCL ratio, and some blood variables

Significant negative correlations with high correlation coefficients (Table 9) were found between the total number of CVF and: red blood cell count (rs=-0.55, P = 0.001), packed cell volume (rs = -0.56, P = 0.001), and haemoglobin concentration (rs = -0.64, P = 0.001), indicating that the presence of heart flukes was associated with some degree of anaemia.

Table 9: Relationships between total cardiovascular flukes (CVF) or total other flukes (non-CVF), with bodyweight/CCL (B/C) ratio, red blood cell (RBC) count, packed cell volume (PCV), and haemoglobin concentration [Hb] in green turtles in Indonesia.

Parameters	Spearman correlation coefficient (r_s)	Significance (P value)
Total CVF and B/C ratio	-0.21	0.050
Total CVF and RBC	-0.55	0.001
Total CVF and PCV	-0.56	0.001
Total CVF and [Hb]	-0.64	0.001
Total non-CVF and B/C ratio	0.12	0.078
Total non-CVF and RBC	0.17	0.193
Total non-CVF and PCV	0.07	0.124
Total non-CVF and [Hb]	0.14	0.143

Application of the Bivariate Spearman Correlation test revealed that the total number of flukes present in places other than the heart and adjacent blood vessel were correlated neither with blood parameters (RBC, PCV, or Hb) nor bodyweight/CCL ratio (**Table 9)**. This emphasizes the important of blood flukes in the general health status of marine turtles.

IV. Discussion

The first report on cardiovascular flukes (Digenea: Spirorchiidae) infection in green and hawksbill turtles in Indonesia is provided. Five genera namely *Learedius, Hapalotrema, Neospirorchis, Amphiorchis,* and *Monticellius* were identified in C *mydas* and *E imbricata*

captured from 11 widely dispersed capture sites, representing a wide geographical range. The large number of samples examined, provided abundant and presumably unbiased evidence that cardiovascular fluke infection is widespread in wild sea turtles in Indonesia. The fact that the prevalence of trematodiasis in both species (C *mydas* and *E imbricata)* was 100%, in which their presence was significantly correlated with certain blood variables, may be of particular importance in view of the endangered status of these species and increasing human encroachment on their habitat in Indonesian waters. The association between total spirorchids found within the heart chamber and adjacent blood vessels with the presence of vascular lesions in the aortic and brachiocephalic arches, and the discovery of a mixed infection composed of two to four genera of flukes both in C *mydas* and *E imbricata* in the present study appear to be new findings. Presence of flukes other than spirorchids did not seem to be significantly correlated with the general health status of the green turtles, as reflected by some blood variables and bodyweight/CCL ratio.

Spirorchidiasis in the present study was diagnosed with confidence, as it was based not only on histopathology, but also on direct observation of flukes in the heart and adjacent major blood vessels. The prevalence of the CVF infection in wild C *mydas* and *E imbricata* in Indonesia (100%) was higher than that reported in wild sea turtles in other regions such as Puerto Rico (25%)[31], Florida (33%)[12], Bermuda (55%)[13], Australia (72%)[11], Italy (17,5 – 33,3%)[24], and Hawaii (90%)[23]. Other workers in Hawaii[32], who used an ELISA to detect the presence of blood flukes in green turtles, found a prevalence of 80%. One or a combination of the following reasons might explain the higher prevalence in Indonesia. First, transmission of the disease was probably more efficient under tropical conditions in Indonesia. For example, C *mydas* in some habitats such as near Bunaken in the southeast Celebes feed predominantly on sea grasses such as *Thalassic hemprichii* and *Enhalus acoroides*[33], which are known to support large populations of marine gastropods. The significant differences in ELISA-Ab titre among various groups of green turtles from different geographical regions has been reported in Hawaii[32]. A second explanation may be differences in the methods of diagnosis. Whereas in Australia and Hawaii, the workers searched visually for flukes in the heart and associated blood vessels[11,12], the heart and associated blood vessels did not wash to dislodge the flukes as was the practice in the present study. Some flukes, especially those belonging to the genus *Learedius* are very small and are not detected on gross examination, particularly if any blood clots remain in the heart. Thus, diagnosis based solely on visual observation with the unaided eye is inaccurate. Other workers, who grossly examined squashed preparations of brain, liver, kidney, or spleen, scrapings of intestinal wall, or fixed mesenteric vessels in an attempt to find

adult spirorchids and/or their eggs[12,13], did not confirm the presence of flukes in the heart or associated blood vessels. As the predilection site of spirorchids is within the heart chamber, searching for these flukes in places other than the heart must result in a lower prevalence being recorded.

The use of ELISA provides a new approach to the detection of the blood flukes in marine turtles[24], particularly as this technique can be applied to live turtles. However, as the life cycle of these flukes has not yet been defined, caution in interpretation of results is needed. The cut-off value of any negative control using captive-reared turtles must be carefully defined, as they may also be infected[11,17], though perhaps at a low level. Another consideration is, that the prevalence survey of spirorchid infection conducted in Hawaiian green turtles used only three genera of blood flukes viz. *Carettacola, Hapalotrema,* and *Learedius* as sources of antigen[32], so that other genera present might not be detected. It has been known that there are at least eight genera of spirorchids are harboured by marine turtles[11].

A third possible explanation for the different in fluke prevalence is the condition of turtles examined. Results on prevalence of CVF reported so far were mostly based on the examination of turtles found dead which may thus have autolyzed before being necropsied[12,13]. Examination for flukes in the present study was always conducted immediately after turtles were killed.

In the present study, the level of infestation of heart flukes in *C mydas* was higher than those found in *E imbricata.* Host size, and presumably age seemed unlikely to be the cause of difference in this study; as most hawksbills examined were immature, they could be expected to harbour more flukes rather than the green turtles that were mostly mature. Difference in habitat of these two species may explain the contrasting fluke prevalence. Whereas C *mydas* is a herbivore that feeds mainly on sea grasses and algae[34], *E imbricata* is considered an omnivore that feeds particularly on coral reefs and rocky outcrops[35,36]. However, whether the intermediate host(s) of the heart flukes is more abundant in the C *mydas* feeding ground rather than in *E imbricata* feeding areas needs to be explained.

The intensity of infection of heart flukes in infected turtles found in this study was comparable to those reported from C *mydas* in the Great Barrier Reef, Australia[11], but higher than those reported from Bermuda[13] or Hawaii[21]. As in regard to prevalence, factors such as local environment, methods of diagnosis, and condition of turtles examined probably explain the varying severity of infection.

An interesting finding in the present study was that in C *mydas,* the CVF burden decreased with increasing size, and presumably age of turtles. Smaller turtles, ie those with a CCL up to 60 cm were found to harbour more flukes than those with a CCL between 61 cm

and 122 cm. The reason for this phenomenon is not clear, but the development of an immune response might be involved. The first contact of C *mydas* with snails is probably during the post-hatchling period, when the young turtles occupy open ocean pelagic habitats. This was indicated by the finding that the stomach of a post-hatchling C *mydas*, removed from the stomach of a shark caught near Durban, South Africa, was filled with the pelagic snail *Janthina janthina*[37]. Whether this snail is an intermediate host for CVF, is unknown. More intensive contact between green turtles and the suspected snails probably occurs when they arrive at benthic foraging areas, where they begin feeding on seagrasses and algae. It has been suggested that marine gastropods were known to be well supported by these two floras[11]. It seems likely that this enhanced contact between the turtle and the parasite might stimulate an immune response against the flukes. Turtles in general, are reported to have a capability to form antibody and immunological memory when they are challenged with diverse soluble and particulate immunogens[38].

Two genera namely *Learedius* and *Hapalotrema* were the most prevalent spirorchids in green and hawksbill turtles. This finding was comparable to those reported in the Cayman Islands[17], Bermuda[13], North Queensland, Australia[11], Hawaii[23], and Puerto Rico[31,39].

Dual infection with these flukes in the one host was reported in north Queensland, Australia[11] and in Hawaii[23] who also observed concurrent infections with three genera *(Learedius, Hapalotrema,* and *Carettacola)* in C *mydas*. In the present study however simultaneous infection of turtles with four genera of spirorchids was found. Given the complexity of life history of sea turtles and the varied marine environment in which they live, these findings were not surprising. Marine turtles are known to migrate extensively, thus enhancing the possibility of infection with several or more flukes. However, as there is no indication about what species of snail might be the intermediate host, research in regard to this aspect is paramount.

Pathological changes associated with blood fluke infection in this study are comparable to those previously reported[11,12,13,17]. Importantly, however, in the present study, the severity of lesions in the brain and aortic arch ie aneurisms and thickening and hardening of arterial walls – sometimes accompanied by thrombosis, seemed to be related to the number blood flukes recovered from the turtle. This might explain why such severe lesions were seldom observed by other workers who noted fewer parasites. Except for several heavily infected (average of 36 flukes per turtle) wild C *mydas* in Australia[11] in which the fluke burdens were high (between 23 - 167), these lesions so far have not been reported in sea turtles from other countries, nor in farmed or oceanarium-reared green turtles harbouring up to six and 23 flukes,

respectively[40].

A total of 35 turtles (55.6% of those in which brain was examined) was observed to have spirorchidiosis with severe brain involvement. Haemorrhages associated with the lodgement of flukes and/or their eggs in the meningeal vessels and within the brain parenchyma, was, again, correlated with the number of flukes found within the heart and adjacent blood vessels (at least 60 flukes per turtle). Spirorchidiosis with brain involvement has been reported in wild and oceanarium-rear green turtles from Australia[10,11], but the brain lesions observed did not seem to be as severe as in the present study.

Cysts (Nodular granulomas) adjacent to but distinctively separate from arterial aneurisms were another interesting finding in this study. Microscopically, these Nodular granulomas cysts contained flukes and/or eggs morphologically similar to those within the heart, so it seems likely they might indicate ectopic spirorchid infection. The means by which the blood flukes and/or their eggs escape from the blood vessels is, however, undetermined.

Another lesion observed in this study that apparently has not been reported previously was the presence of multiple darkly pigmented nodules in the muscularis and/or serosa of the stomach, small, and large intestine. Although the presence of eosinophilic cuticle-like material, resembling that found in the tegument of the flukes suggested these lesions were associated with flukes, it was not determined whether it was solely associated with spirorchid or other flukes.

The consistent finding of a large number and variety of trematodes other than spirorchids probably suggests that such flukes are unlikely to affect the general health and survival of the green turtles in Indonesia adversely. This was suggested by the finding of no significant correlations between the total numbers of these flukes with bodyweight/CCL ratio and some blood variables such as RBC, PCV, and Hb. A further detailed study on the species of flukes associated with petechial haemorrhages in the gut, gall, and urinary bladder need to be done.

Spirorchid infection, especially when severe and involving in excess of, for example, 60 flukes per turtle, is clearly a major problem in marine turtles in Indonesia. Anaemia as indicated by negative correlations between spirorchid burden and bodyweight/CCL ratio, and erythrocytes parameters such as RBC, PCV, and Hb, is an obvious consequence of this disease in marine turtles. Lodgement of adult flukes and/or their eggs in almost all tissues examined, particularly the brain, heart, lungs, spleen, and liver, is clearly threatening the life of the turtles.

V. Conclusion

The prevalence of spirorchidiasis in both *C mydas* and *E imbricata* were 100%, involving flukes of six genera of the family Spirorchiidae in *C mydas*, and four genera in *E imbricata*. A single, or multiple species could be found in a turtle, with the intensity of infection varied between 9 -197 flukes per turtle. Depending on the intensity of infection, the pathological effects associated with cardiovascular flukes included anaemia, hydropericardium, hydroperitoneum, thickening and hardening of arterial walls. Microscopically, egg granulomas, presence of flukes, endarteritis and/or perivascular cuffing, and central necrosis were prominent. Other flukes were also consistently found (100%) in all parts of the gastrointestinal tract, 40% in urinary bladder, and 48% in gall bladder. The total number of flukes present in the places other than the cardiovascular system were correlated neither with blood parameters nor bodyweight/curved carapace length ratio. Findings in this study indicated that cardiovascular fluke infection is a major problem in marine turtles in Indonesia, with anaemia and lodgement of adult flukes and/or their eggs causing life threatening illness.

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