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内　　容

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The office of publisher would like to apologize to make errors in the text of two publications which belong to "Hideo Kumazawa, Ryoji Suzuki, Toshihiko Okura" and "Yoshihisa Hashiguchi" published in vol. 9, No. 1 respectively. Please correct the mistakes following the errata.

編集事務の誤りで9巻1号掲載の著者に御迷惑をおかけしました。別表正誤表に従って御訂正下さい。

I. 高知県における大複殖門条虫症の第4例

熊沢秀雄，鈴木了司，大倉俊彦（和文論文）

訂正箇所	誤	正
1頁 左段本文下から 3行目	特続	持続
〃 上から 11行目	1571	1971
右段本文上から 1行目	判断	診断
〃 〃 3行目	同月29日午前7時頃…	同月29日午前3時頃から複鳴持続し，午前7時頃に
〃 下から 10行目	6％	0％
2頁 左段本文下から 9行目	縦走節	縦走筋，横走筋髄層の
〃 〃 7行目	横断図	横断面
〃 〃 5行目	腔	膣
3頁 右段本文下から 11行目	1981	1891
〃 〃 1行目	*D. guandis*	*D. grandis*
4頁 右段本文上から 3行目	海産類	海産魚
5頁 左段本文下から 4行目	下痢腹鳴	下痢，腹鳴
右段 〃 上から 1行目	2例	2列
文献		
1) 2行目	(Cestoda; Pserdophyllidea)	(Cestoda; Pseudophyllidea)
5) 2行目	*D. balaneopterae*	*D. balaenopterae*
9) 1行目	大複殖門条虫成熟虫寄生例	大複殖門条虫未成熟虫寄生例
12) 2行目	k892	1892
14) 1行目	*Diphyllobothrim*	*Diphyllobothrium*
16) 1行目	Additional II cases	Additional 11 cases

II. Examinations on the protective immunity to the common liver fluke, fasciola in rats, using immunosuppressants or diffusion chamber techniques

YOSHIHISA HASHIGUCHI

		error	correct
p. 10	Seventh line from the bottom	batach	batch
p. 11	Fifth line 〃	50 minutes	50 minutes.
p. 12	Twentieth line from the Top	$\bar{C}—\bar{I}\times 100$	$\bar{C}—\bar{I}/\bar{C}\times 100$
p. 14	Eighth line 〃	blbumin,	albumin,
p. 14	Figure 1	C: betaglobulin,	C: beta-globulin,
p. 15	Column in the Table 3	without chambers	without chamber
p. 16	Fifteenth line from the Top	copared	compared
p. 16	Column in the Table 5	No. serum sampler	No. serum samples
p. 17	Figure 2	B: alphaglobulin	B: alpha-globulin
p. 19	Fourth line from the bottom	theat	that
p. 21	REFERENCE 25)	*Fasciola hepatic*	*Fasciola hepatica*
p. 22	和文抄録本文上から 1行目	*Fasciola Repatica*	*Fasciola hepatica*
〃	〃 〃 11行目	Ouchterlong	Ouchterlony
〃	〃 下から 8行目	蛋白画像	蛋白分画像
〃	〃 〃 6行目	Dexamethazdne	Dexamethasone
〃	〃 〃 5行目	防御免の	防御免疫の
〃	〃 〃 4行目	Ouchterlong	Ouchterlony
〃	〃 〃 2行目	r-globulin	γ-globulin

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高知県における大複殖門条虫症の第4例

熊沢秀雄[1]，鈴木了司[1]，大倉俊彦[2]

和和56年2月9日　受付

緒　　言

大複殖門条虫　*Diplogonoporus grandis* (*Blanchard*, 1894) Lühe, 1899 の人体寄生例は静岡，鳥取，長崎をはじめとする19府県から，すでに85例が知られている。しかしこの中には断片的な記録しか残っていないものも多く，感染経路を含む疫学上の諸問題や，この条虫をめぐる分類学上の問題は未解決のままであり，個々の症例を精確に記録してゆく必要性は今も変わらない。

筆者らはすでに高知県から，本条虫の未熟虫体寄生の2例を報告したが，本条虫の感染源が小型の海産魚と推定されること（Kamo *et al.*, 1571）などから，高知県における本条虫症例は少なくないことが予想された（熊沢ら，1981）。最近，ひとりの患者が前後少なくとも3回に亘って成熟片節を自然排出した1症例を得たので，ここに報告する。

症　　例

○岡○。45歳。男。高知県安芸市赤野在住。農業のかたわらで養鰻および沿岸漁業に従事。

既往歴，家族歴には特記すべきことはない。

現病歴。生来格別病気をしたことはなかったが，飲酒後下痢をするのが常であった。1980年1月26日から連夜飲酒，同月27日，下痢便とともに虫体（目測で約1m）を自然排出したが捨てたという。この頃から腹鳴が特続するようになり，時折腹痛もあったが放置。同月29日早朝，虫体（標本番号1）を下痢便とともに排出し，虫体を携えて受診した。虫体から大複殖門条虫症と判断し，駆虫を勧めたが患者多忙のためその後来院しなかった。同年2月下旬より飲酒の機会多く，同月29日午前7時頃に軟便とともに虫体を排出（標本番号2）。さらに同年3月18日午前8時30分頃，下痢便とともに標本番号3a, 3b, 3cの虫体断片をこの順序で排出した。但し標本3bは患者が持参しなかったので測定することはできなかったが，患者によれば約20cmの長さであったという。いずれの場合も虫体排出後，腹鳴は治まったが，軟便傾向は持続した。駆虫を勧めたがその後患者からの連絡がなく現在に至っている。

理学的所見。特記すべきことはない。

尿検査所見。比重1.015。pH 6.0。蛋白，糖，ケトン体，潜血反応，ピリミジン，いずれも（−）。ウロビリノーゲン，正常。

糞便検査は行なわなかった。

血液学的検査。赤血球数 452×10^4/cmm. Hb 15.0 g/dl. 血色素指数1.04。白血球数 9,100/cmm（好中球，1核3%，2核16%，3核21%，他20%，リンパ球25%，単球7%，好酸球8%，好塩基球6%）。

血液化学検査。LDH 210 単位。GOT 7 単位。GPT 8 単位。Al-p 6.6 単位。CHE 0.65 ⊿。ZTT 7.0 単位。黄疸指数7単位。総蛋白量 6.6 g/dl。

患者の嗜好。自宅の前の海で捕れたアジ，サバの刺身やイワシの酢物を常食し，好物のカツオを除き市場の海産魚介類は食べない。川魚は食べない。豚肉は好食するが牛肉は特に好まない。養鰻のためイトミミズを誤まって口にすることがあった。

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虫体観察所見

回収した虫体は次の4断片で，これらはすべて，クロロホルム加冷水道水で弛緩させて計測したのち，5%ホルマリンで固定した。

標本番号1　長さ 494 cm. 最大幅 19.5 mm（前端から 300 cm)。

標本番号2　長さ 352 cm. 最大幅 20.5 mm（前端から 280 cm)。

標本番号3a　長さ 310 cm. 最大幅 21.5 mm (前端から 260 cm)。

標本番号3c　長さ 111 cm. 最大幅 13.0 mm (後端)。

このうち3aと3cは連続して排出されたもので，3cの後端の幅は3aの前端の幅（16.0 mm）よりやや小さい。いずれの断片も2列の生殖器列を有するほか，2, 3a, 3cの断片では1～3個の過剰生殖器を含む片節（Figure 1）が見られる。いずれの断片にも頭節はない。どの断片でも片節は縦径よりも横径が大で，ストロビラの縁はやや鋸歯状を呈し，体表面には，生殖器と神経幹にそれぞれ対応する部位にやや深い縦溝があるほか，両生殖器間に5～7条，生殖器と神経幹の間に片側2～4条，神経幹より外側に片側5～7条の極めて弱い縦溝が認められる。両神経幹の間の縦溝は背腹で必ずしも一致せず，縦溝の数は腹側がやや多い。

Table 1 は虫体断片1の最大幅付近の切片標本についての計測値である。片節の幅径は縦径の18.6倍，生殖口間の距離は片節幅の25%となる。虫体の断面は外側から角皮，角皮下細胞，皮質層，縦走節，髄層の各層から成る（Figure 2)。

1対の神経幹と2本以上の排泄管が髄層内にあり，前者の横断図は背腹にやや扁平な楕円形である。貯精嚢は陰茎嚢の背後方に，これと鋭角をなして接続する。輸精管と腔は共に生殖窦に開口し，後者は生殖口として片節縦径の前端から約1/3の部位に開口する。子宮口はそれより約 200 μ 後方にある（Figure 3)。

精巣は陰茎嚢の周辺を除く髄層内のやや背側寄りに一層に配列し，横断切片では背腹に扁平な楕円形で，両陰茎嚢の間に28～31個，陰茎嚢より外側に片側6～31個が数えられる。精巣の配列は片節間で中断することなく連続している。

卵黄腺は角皮下細胞近くの皮質層内にあり，圧平標本では径 32～81 μ（平均 52 μ）の不規則な円形をなし，その分布は陰茎嚢や子宮と重なることはなく，また陰茎嚢の前や子宮の後で左右が連続することはない。他の部分では卵黄腺は均一に分布する。子宮ループは片側で2～5本が認めら

Table 1　Measurements (μ) of the *Diplogonoporus* specimen*.

segment	
length	955 ± 95
width	17800 ±271
thickness	600 ± 35
distance between	
two genital organs	4500 ± 95
genital organ and nerve trunk	2240 ±141
g.o. and excretory canal	1390 ±190
thickness of	
tegument	1.9± 0.6
tegument cell layer	44.3± 7.0
cortical parenchyma	89.2± 20.5
longitudinal muscle	74.7± 7.3
transverse muscle	35.0± 8.4
medullary parenchyma	108.0± 21.8
nerve trunk, diameter	
dorsoventral	69.4± 6.5
horizontal	86.4± 9.1
cirrus sac	
length	286.1± 18.1
width	240.4± 6.9
wall thickness	6.5± 3.4
seminal vesicle	
length	117.3± 5.1
width	87.3± 8.8
wall thickness	19.2± 9.5
testis, diameter	
dorsoventral	45.5± 7.5
horizontal	62.1± 10.7

* expressed as mean±standard deviation.

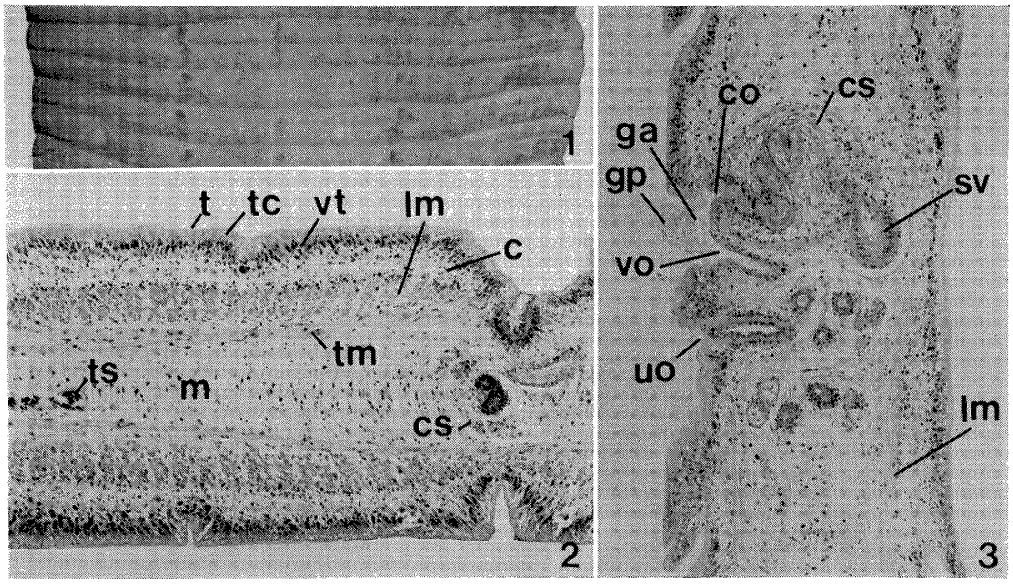

Figure 1 A part of the strobila no. 2 near its maximum width showing four rows of genital organs. Stained with alum carmine. ×2.3.

Figure 2 Transverse section of the strobila no. 1. Stained with Mayer hematoxylin and eosin. ×67.

Figure 3 Sagittal section of the strobila no. 1. Stained with Mayer hematoxylin and eosin. ×67.

Abbreviations c, cortical parenchyma; co, cirrus opening; cs, cirrus sac; ga, genital atrium; gp, genital pore; lm, longitudinal muscle; m, medullary parenchyma; sv, seminal vesicle; t, tegument; tc, tegument cell layer; tm, transverse muscle; ts, testis; uo, uterine opening; vt, vitellaria; vo, vaginal opening.

れる。子宮内には大きさ 63.4～77.9×31.2～46.8 μ（平均71.7×36.8 μ）の虫卵がある。

考　　察

大複殖門条虫の人体症例は1971年までに55例が知られていた（Kamo *et al.*, 1971）。その後の症例を Table 2 に，主として発表順序に従って要約した。本論文の症例を日本における第85例として扱う。このほか静岡より１例が近く報告される筈で（影井，私信），これを加えると全 87 症例となる。高知からは1971年以前には岩田・安岡（1970）の１例があるのみで，本論文の症例は高知の第４例となる（高知の第 2，3 例は Table 2）。

本症例でみられた下痢，腹鳴，腹痛の症状は，従来もしばしば報告されている（Kamo *et al.*, 1971）が，これらの症状と飲酒との関連は，今回の症例の場合明らかでない。今回の症例でも，本条虫の寄生が貧血や炎症その他の症状をひきおこすという証拠はない。

大複殖門条虫はヒゲクジラ類寄生の *D. balaenopterae*（Lönnberg, 1981）と形態的に区別できないといわれる（Rausch, 1964; 岩田，1967）。鰭脚類やラッコ寄生の *D. tetrapterus*（v. Siebold, 1848）が人体寄生する可能性もある（Rausch, 1964）。今回の標本は虫体が大きいこと，卵黄腺の分布が子宮ループと重ならず，片節の前縁や後縁で連続しないこと，卵黄腺が大きいことなどの点で *D. tetrapterus* よりは *D. balaenopterae* に似る。人体寄生のものを後者と同一視することには未だ疑問があるので，今回の患者から得たものは大複殖門条虫 *D. guandis* と同定した。

条虫の成長は速く，たとえば広節裂頭条虫は犬体内で毎日，前日の約1.4倍の長さになり，人体内での絶対成長速度は犬でのそれの3倍を越えるといわれる（Wardle and Green, 1941）。このことから，大きな虫体断片が相次いで排出されたとしても，それらはすべて同一個体由来かもしれない。特に虫体断片 2, 3a, 3c は過剰生殖器の出現の様子が類似しているのでその可能性は十分に存する。一方，患者の嗜好から言って，複数寄生の可能性も全く否定はできない。

感染源については従来の各症例報告と同様に明らかではないが，本報告の患者が新鮮な海産類を常食していたという事実は，過去の大複殖門条虫症例が西南日本の海に面した府県に集中して分布していることと対応している（Figure 4）。感染源がカツオかアジなら初夏，サバかイワシなら秋から冬に感染する機会が多いものと思われる。感染から虫体排出までの期間は，広節裂頭条虫では

Table 2 Cases of *Diplogonoporus* infection, recognized after 1971.

no.	reference	patient locality	age	sex	worm length (cm)	width (mm)	scolex
56	7	Shizuoka	62	m	300	6	−
57	13	Nagasaki	33	f	154	13.5	+
58	13	Nagasaki	40	m	468	12.0	−
59	13	Nagasaki	38	m	458	18.0	−
60	2	Shimane	41	m	130	9	−
61	3	Ehime	41	m	130	7	−
62	16	Shimane	68	m	12	5	−
63	16	Tottori	38	m	370	13	−
64	16	Tottori	?	?	138	14	−
65	16	Tottori	36	m	56	11	+
66	16	Tottori	45	m	114	10	−
67	16	Tottori	38	m	16	8	−
68	16	Tottori	29	m	313	13	−
69	16	Shimane	40	m	470	21	−
70	16	Tottori	36	m	132	8	−
71	16	Tottori	53	m	237	5	−
72	16	Shimane	50	m	226	11	−
73	4	Ehime	72	m	60	7	−
74	11	Shizuoka	?	?	117	25.0	−
75	11	Shizuoka	52	f	492	18.5	−
76	11	Shizuoka	41	m	310	8	−
77	11	Shizuoka	46	m	550	20	−
78	11	Shizuoka	50	m	115	12	−
79	11	Shizuoka	42	m	96	7	−
80	11	Shizuoka	51	m	116	7	+
81	11	Shizuoka	31	m	215	17	+
82	15	Nagasaki	45	m	90	25	−
83	10	Kochi	70	f	85.1	5.5	−
84	10	Kochi	71	f	274.5	7.2	+
86	9	Oita	52	m	?	?	−

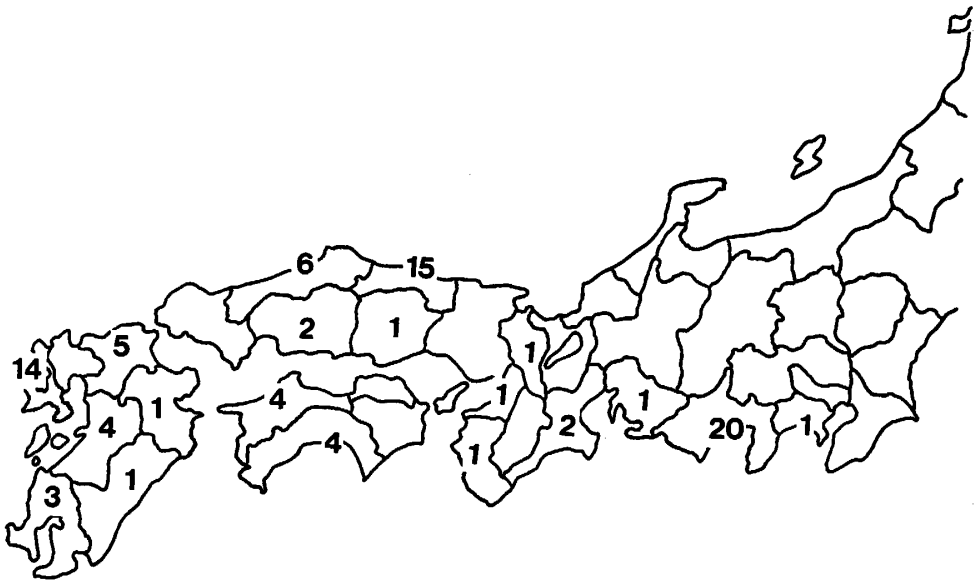

Figure 4 Distribution by prefecture incidence of diplogonoporiasis.

約80日という事例があり（吉田ら，1979），卵検出までの期間は条虫類一般にもっと短かい（Andersen, 1978）. Kamo *et al.* (1971) は大複殖門条虫の症例数のピークが南から北に次第に遅くなることと関連して，魚の消費量が秋から冬に最大となることに注目している。今後も，感染源を明らかにするために，個々の症例をさらに検討する努力が必要であろう。

要　　約

1．高知県安芸市在住の45歳男子より1980年1月29日，2月29日，3月18日の3回に亘り自然排出された4虫体断片を大複殖門条虫と同定した。

2．下痢腹鳴，腹痛のほかは，特別な症状はなかった。

3．虫体断片は長さがそれぞれ494 cm, 352 cm, 310 cm, 111 cm, 最大幅が19.5 mm, 20.5 mm, 21.5 mm, 13.0 mmで，いずれも2例に配列した完成した生殖器を有し，後3者の断片では1～3個の過剰生殖器を備えた片節があった。どの断片にも頭節はなかった。

4．本症例は高知県における第4例目である。

謝　　辞

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THE FOURTH CASE OF DIPLOGONOPORIASIS IN KOCHI PREFECTURE

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A total of four fragmented strobilae, evacuated spontaneously on January 29, February 29 and March 18 in 1980, from a 45 year-old man living in Kochi Prefecture, were

identified as *Diplogonoporus grandis* (Blanchard, 1894) Lühe, 1899. They were from 111 to 494 cm in length, and from 13.0 to 21.5 mm in the maximum width, all with two rows of complete genital organs, and three containing proglottides exhibiting supernumerary genital organs. The scolex was absent. The patient suffered from diarrhea, abdominal pain and borborygmus. Fresh raw fish was taken frequently by the patient. This is the record of the fourth case from Kochi Prefecture, Japan.

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EXAMINATIONS ON THE PROTECTIVE IMMUNITY TO THE COMMON LIVER FLUKE, *FASCIOLA* IN RATS, USING IMMUNOSUPPRESSANTS OR DIFFUSION CHAMBER TECHNIQUES

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Abstract: The current study was designed to obtain an information on the immune factors which could be underlying the induction of protective immunity to the genus *Fasciola* in rats, using immunosuppressive drugs and diffusion chamber technique. The rats became resistant to challenge with the Japanese species of *Fasciola* following an oral infection with metacercariae, indicating a significant degree of reduced worm burdens. This protective effect in the immunized rats, however, was abrogated by the administration of immunosuppressants, dexamethasone and prednisolone. In rats treated with these drugs, no antigen-antibody band in immunodiffusion plate was recognized throughout the infection, while many bands were observed in sera from the rats without treatment with the drugs. From the results obtained, the antigen-antibody bands in diffusion plate would be a good indicator to estimate the degree of protective immunity to the genus *Fasciola* in host animals. By intraperitoneally implanting the immature worms in diffusion chamber, the rats were able to induce a significantly high degree of protective immunity when compared to the natural-immunity control. However, the degree of protective effect was significantly high in rats which received free worm (without chamber) implantation. In the experimental rats, each fraction of their serum proteins was also examined by performing cellulose acetate electrophoresis. More detailed examination is required to investigate the immune factors in the protective immunity to the fluke in rats, using immunosuppressive drugs and/or diffusion chamber techniques in terms of host-parasite relationship.

INTRODUCTION

The Japanese common liver fluke, *Fasciola* sp. indicates a similarity to *F. hepatica* and *F. gigantica* in the migration and development in the final hosts (Ono and Isoda, 1952; Mimura, 1961), though its taxonomical status has not yet been established (Watanabe, 1965). So far as is known, little immunological study on the hosts infected with the Japanese *Fasciola* species has been carried out in terms of the protective immunity to the fluke. The protective immunity of rats against *F. hepatica*, on the other hand, has been widely recognized (Hayes *et al.*, 1972; Goose and MacGregor, 1973; Armour and Dargie, 1974; Rajasekariah and Howell, 1977, 1978). This immunity is induced not only by the oral infection of the normal or irradiated metacercariae but also by the implantation of the different ages of the liver fluke in

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rats (Thorpe and Broome, 1962; Eriksen and Flagstad, 1974; Anderson *et al.*, 1975; Rajasekariah and Howell, 1978). Moreover, the resistance of rats to reinfection with *F. hepatica* is also stimulated by the transfer to naive animals with the serum or lymphoid cells from infected ones (Corba *et al.*, 1971; Armour and Dargie, 1974; Hayes *et al.*, 1974a, b; Dargie *et al.*, 1974; Howell *et al.*, 1977; Rajasekariah and Howell, 1979). Thus, so far, the previous works have provided much important information on the immunological factors involved. The factors, however, should still be clarified to have a better understanding of the mechanisms which could be underlying the induction of protective immunity to *Fasciola* in host animals.

In the immunizing infection with the subcutaneous implantation of immature worms, 4 week-old, Rajasekariah and Howell (1978) have observed a significant degree of resistance to *F. hepatica* in rats. According to Lang *et al.* (1972), moreover, the immunization with different ages (8 and 16 day-old) of worms produced a significant reduction in challenge worm burdens when compared to natural immunity (challenge) controls in mice. By subcutaneously implanting adult worms, on the other hand, Eriksen and Flagstad (1974) and Anderson *et al.* (1975) also showed some degree of protective immunity to challenge infection in rats, although Rajasekariah and Howell (1978) could not recognize any protection following subcutaneous implantation with the adults. In this immunity, but, the duration of liver migration by young worms may be of greater importance in stimulating the protective immunity than is worm age (Lang, 1974). From these facts, anyway, there may be several factors to induce protective immunity in relation to the age or liver migration of immunizing worms in the host.

In the series of experiments reported hitherto, the observation seems to indicate a complexity of the protective immune factors involved. The present study, therefore, attempts to stimulate the hosts (rats) by introducing living parasite contained in a millipore diffusion chamber. In this method, a variety of soluble antigens would become effective, but the inability of the liver fluke to infect the host would reduce or eliminate all of the pathology usually associated with the infection, without liver and/or bile duct migration of the worms. In addition, the current work is designed to determine the effects of immunosuppressants on the induction of protective immunity to the Japanese common liver fluke in rats.

Materials and Methods

Animals. Male, around 250 g Wistar rats were used throughout the experiments. They were fed a commercially prepared diet and received water *ad libitum.*

Parasites. All the Japanese species of the genus *Fasciola* metacercariae were from the same batach. They were collected from experimentally infected *Austropeplea ollula* (*Lymnea ollula*) and administered *per os* to rats in normal saline, using an injection syringe with a slender vinyl tube.

Immunosuppressive drugs. Dexamethasone (Merck *Decadrone*) and prednisolone (Merck *Codelcortone*) were simultaneously administered into alternate thigh muscles of rats beginning one day before immunizing inoculation of the metacercariae, and continued every five days until necropsy. The dosage levels of these drugs are

1 mg/rat in *Decadrone* and 5 mg/rat in *Codelcortone*.

Preparation and implantation of diffusion chambers into rats. Diffusion chambers (Millipore Filter Corp., Bedford, Massachusetts) were made of diffusion chamber ring (Plexiglass U-100, diameter: 10 mm, thickness: 2 mm) and millipore filter (pore size: 14 μ). They were closed with millipore glue after introducing the flukes in sterile saline; the immature, 25 day-old, worms isolated by peptic digestion from stock-infected rats, were introduced aseptically into the chambers. Rats that were to receive diffusion chambers were anesthetized with ethyl ether during operation. The abdominal area of the rats was washed with 70% ethyl alcohol and a small incision was made either to the right or left of center. A single chamber including two worms was then placed intraperitoneally into each rat with sterile forceps. The incision was closed with a sterile surgical suture and then the area was washed again with 70% ethyl alcohol including iodine tincture. It was observed that 100% of the rats survived in these operations.

Experimental design. The experimental design consisted of three series. In the 1st series of the experiment, the degree of protective immunity to the Japanese species of *Fasciola* in rats was ascertained by the immunizing infection with the metacercariae *per os*. In the 2nd, an attempt to induce the protective immunity was made by intraperitoneally implanting the immature worms contained in diffusion chambers, and then the recipients were challenged with the metacercariae. In the 3rd, it was decided to assess the effects of immunosuppressants on the induction of protective immunity to the liver fluke in the animals infected experimentally. Student's "*t*" test was used to compare worm burdens between groups. Probability values larger than 0.05 were not considered significant. The standard deviation of the mean was calculated for mean worm burdens.

Autopsy. All rats were killed 2 weeks after challenge infection with the metacercariae, unless otherwise mentioned. In each examination the peritoneal cavity was opened, and the surface of the liver and other visceral organs was inspected for haemorrhages. The peritoneal surface was flushed with normal saline and the washings were examined for free worms under a dissecting microscope. All of the internal organs were removed separately, then washed with normal saline several times to recover free worms. The liver and bile ducts were examined for migrating immature worms and/or mature ones. The liver was minced with scissors in petri dishes. In order to obtain the penetrating worms, the minced liver in saline was incubated at 37 C for 2 hrs; the incubation revealed that the liver was free from any worms.

Serum examination. Blood samples were collected weekly intervals by cutting the tail part of rats. All sera were analysed with a cellulose acetate electrophoresis cell (Model SE-2, Toyo Kagaku Sangyo) supplying constant current. Serum samples were placed on a cellulose acetate membrane (Separax: Joko Sangyo Co. Ltd.), and then subjected to 0.8 mA per 1 cm membrane for 50 minutes The dried strips of cellulose acetate membranes were scanned in a Densitorol DMU-2 (Toyo Kagaku Sangyo), and the relative percentages of albumin, alpha-, beta- and gamma-globulins were determined.

Double immunodiffusion. Ouchterlony plates were prepared by flooding 5 by

11 cm slides with 9.5 ml of 0.9% agar. The central well was filled with the *Fasciola* antigen and the other wells with serum samples of rats from each experiment. The position of precipitin bands was recorded by drawings and photographs.

Worm antigen. Adult worms of the common liver fluke, recovered from the bile ducts of stock-infected rats and stored at −20 C, were added to 10 volumes (W/V) of phosphate buffer, pH 7.4. The worms were homogenized for 5 minutes at 4 C and centrifuged for 15 minutes at 3,000 rpm. The supernatant was stored at −20 C as the antigen for the agar double diffusion technique.

Results

Worm recovery from immunized and non-immunized rats 2 weeks after challenge. At 2 weeks after challenge all experimental rats had worms from immunizing infection in their bile ducts. In this series of the experiment, the immunized rats received challenge infection with the metacercariae 6, 7, 8, 9, 10 and 11 weeks after immunizing (1st) infection. As recognized in Table 1, the experimental rats harbored fewer challenge worms than the natural-immunity control, apart from the rats received challenge 11 weeks after immunization. The challenge (natural-immunity) controls harbored 3.6 worms in mean number, whereas the animals challenged during 6 to 9 weeks after immunizing infection harbored only 0.7 to 1.5 worms. The percentage immunity to the Japanese species of *Fasciola* in rats was calculated by the following formula, $\bar{C}-\bar{I}\times 100$, where $\bar{C}$ indicates a mean worm recovery of challenge control and $\bar{I}$ shows a mean recovery of worms from challenge infection in immunized rats. The challenge infection from 6 to 9 weeks after immunization, revealed a higher percentage immunity ranging from 58.3 to 80.6 than the natural-immunity control. These differences in worm burden are highly significant (Student's "t" test, $P<0.001$), but the challenge at 10 weeks later had trended to indicate a gradual decrease in the degree of protective immunity to the liver fluke in rats. Even in the rats which received both immunizing and challenging infections, there was no difficulty in the

Table 1 Summary of the percentage immunity to the Japanese *Fasciola* in the rats immunized with 4 metacercariae each *per os* before challenge infection.

Times (wks) of challenge after immunization	No. rats used	No. larvae challenged	No. worms recovered from challenge		Percentage immunity* (%)
			Total	Mean±s.d.	
6	5	25	4	0.8±0.40	77.8
7	5	25	4	0.8±0.75	77.8
8	4	20	6	1.5±1.12	58.3
9	3	15	2	0.7±0.47	80.6
10	3	15	8	2.7±0.47	25.0
11	3	15	13	4.3±1.25	0.0
Challenge control	5	25	18	3.6±0.49	—

* Percentage immunity was calculated by the formula, $\bar{C}-\bar{I}/\bar{C}\times 100$ (Miller and Smithers, 1980)

differentiation of the worms between the two infections, because of autopsy at the early phase (2 weeks) of challenge infection. Thus, the results mentioned above have demonstrated a significant degree of worm reduction from challenge, by the immunization with the Japanese *Fasciola* metacercariae *per os*.

Immunization by intraperitoneally implanting worms with and without diffusion chambers. The induction of protective immunity which was observed by the immunizing infection with the metacercariae *per os*, was examined using millipore diffusion chambers in relation to the liver migration and/or the ages of worms. The rat immunized by implanting worms was challenged 3 weeks after immunization, and autopsied 4 weeks after challenge. In the free worm implantation (without chambers), the differentiation of worms between the immunizing and challenging infections was performed according to the size of the worms recovered. In Table 2, the protective immunity to the Japanese species of *Fasciola* in rats is shown by performing implantation of the worms with or without chambers. In the experimental rats, the immunized groups indicated a significant degree of protective immunity by the immunizing infection (implantation) of the immature (25 day-old) worms in both with and without chambers. The percentage immunity obtained by the before mentioned formula, was 50.0% in the immunization with chambers and 88.2% in that without chambers, respectively. In the worm burden, again, the immunized rats harbored only 1.7 and 0.4 worms in the corresponding immunizations, whereas the rats in sham operation and challenge control showed the worm recovery of 4.2 and 3.4, respectively. The differences found in worm burdens between the immunized, and the non-immunized (sham) and challenge control are highly significant ($P<0.001$), but there is no significant difference between sham operation and challenge control. With implantation of the worms contained in diffusion chambers, the current study demonstrated a significant degree of protective immunity to the Japanese *Fasciola* species when compared to the natural-immunity control. The degree, however, was significantly higher ($P<0.001$) in the immunization with free worm implantation than that with enchambered worm; the implanted worms could

Table 2 Results of the induction of protective immunity by intraperitoneally implanting the immature worms, 25 day-old, with and without diffusion chambers in rats.

Group	Times (wks) of challenge aft. implantation	Times (wks) of autopsy aft. challenge	No. rats used	No. larvae challenged	No. worms from challenge		Percentage immunity (%)
					Total	Mean±s.d.	
*Immunized**							
With chamber	3	4	7	70	12	1.7±0.45	50.0
Without chamber (free worms)	3	4	5	50	2	0.4±0.49	88.2
Non-immunized							
Sham operation (chamber only)	3	4	5	50	21	4.2±0.75	0.0
Challenge control	—	4	7	70	24	3.4±0.49	—

* Viable 3 worms each were implanted intraperitoneally in rats.

survive 3 to 4 weeks or more in the diffusion chambers placed intraperitoneally in rats.

The results of serological analysis on serum samples from the rats that received worm implantation with and without chambers are shown in Figure 1, using cellulose acetate electrophoresis. In the case, the percentage value of each fraction in serum proteins was also determined in the samples from rats received sham operation, taking the blood 2 weeks after operation. The values obtained were $53.5 \pm 3.45\%$ in blbumin, $13.9 \pm 1.90\%$ in alpha-globulin, $6.8 \pm 0.25\%$ in beta-globulin and $19.5 \pm 0.65\%$ in gamma-globulin, respectively, and the average of albumin per globulin (A/G) ratio was 1.2 ± 0.15. In comparison with the percentage values in sham operation, the values in both groups showed a remarkably higher or lower value in each fraction, demonstrating a great fluctuation. The weekly changes in the values, however, were similar in the serum samples from the two experimental groups. It was noted that the percentage value of albumin indicated an increase

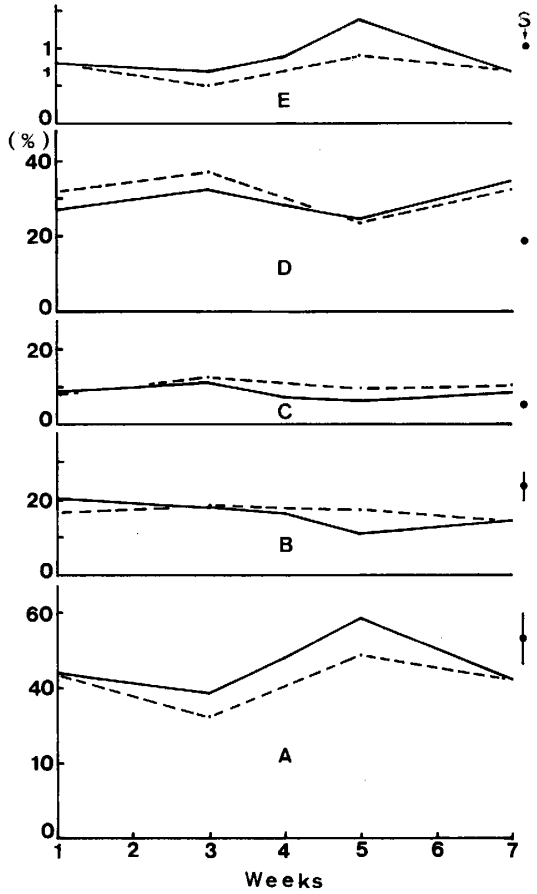

Figure 1 Weekly changes in each fraction of serum proteins in the samples from rats received immunizing implantation of the immature worms with (——) and without (----) chambers. A: albumin, B: alpha-globulin, C: betaglobulin, D: gamma-globulin, E: A/G ratio, S: mean value of sham operation with empty chamber 2 weeks later.

after 3 weeks when the rats were challenged, showing the peak at 5 weeks after immunizing infection. In agar double diffusion, almost all of the serum samples showed only one precipitin band in the rats received worm implantation with chambers, while 2 or 3 bands were observed in the samples from the animals implanted worms without chambers (Table 3). It was noted that the number of bands in the latter animals has increased gradually after challenge infection.

Table 3 Number of antigen-antibody bands appeared in agar double diffusion plate in serum samples from rats immunized by intraperitoneally implanting the immature worms with and without diffusion chambers.

Group	No. serum samples in each test	Weeks after immunizing implantation					
		1	2	4	5	6	7
*Immunized**							
With chamber	5	1, 1, 1, 1, 0**	1, 1, 1, 1, 1	1, 1, 1, 0, 0	1, 1, 1, 1, 1	1, 1, 1, 1, 1	2, 1, 1, 1, 1
Without chambers	5	1, 1, 1, 0, 0	1, 1, 1, 1, 0	2, 2, 1, 1, 0	3, 3, 2, 2, 1	3, 3, 2, 1, 1	2, 2, 2, 1, 1

* The rats were challenged with *Fasciola* metacercariae 3 weeks after immunizing implantation of 25 day-old worms with and without chambers.

** Number of precipitin bands appeared.

Effects of immunosuppressants on the induction of protective immunity. To determine if the protective immunity observed would be influenced by immunosuppressive drugs, the experimental rats which received immunizing infection with the metacercariae *per os*, were treated with dexamethasone and prednisolone throughout the experiment. The drugs were injected intramuscularly into the animals 5 day-intervals. The rats received the immunizing infection were challenged 6 weeks later with the metacercariae, and then necropsied 2 weeks after the challenge infection. At autopsy all the experimental rats contained worms from immunizing infection in the bile ducts. Table 4 shows the results of this experiment. In the immunizing infection, the non-treated rats (Group II) with immunosuppressive drugs harbored only 0.7 worms, whereas the animals treated (Group I) had 3.3 worms from challenge infection. The percentage immunity of rats in Group II was significantly high ($P<0.001$) when compared to both the Group I and natural-immunity control. No significant difference, however, was recognized between Group I and the control. These results demonstrated that the immunosuppressive drugs, dexamethasone and prednisolone, could greatly suppress the induction of protective immunity against the Japanese species of *Fasciola* in rats. In the immunizing infection (Groups I and II), antigen-antibody systems in the animals were examined by performing Ouchterlony technique. The formation of precipitin bands in agar double diffusion plates is shown in Table 5. Each test was carried out at weekly intervals from 1 to 8 weeks after immunizing infection with the metacercariae. All the serum samples in Group I showed no precipitin band throughout the experiment, while all serum

samples taken weekly intervals, apart from those 1 week later, in Group II produced 1 to 4 bands. In non-treated animals with immunosuppressive drugs, the bands were first found at 2 weeks and reached to the peak in numbers at 6 weeks when the challenge was performed, thereafter they had a tendency to decrease gradually in numbers. No stimulation was recognized in the activation of antigen-antibody systems in the rats by challenging infection during the period observed.

The results of serological analysis of the samples from rats treated with (Group I) and without (Group II) immunosuppressive drugs are depicted in Figure 2. In the examination of normal (non-infected) rats, the percentages of each fraction were $53.1\pm3.79\%$ in albumin, $18.7\pm2.00\%$ in alpha-globulin, $5.3\pm1.62\%$ in beta-globulin and $18.9\pm5.00\%$ in gamma-globulin, respectively, and the average of albumin per globulin (A/G) ratio was 1.1 ± 0.16. These average normal values are also shown in the same Figure. The gamma-globulin fraction in sera from treated animals (Group I) showed a consistently low percentage, while it represented a high value in the animals without treatment as copared with the average normal value. In other globulin fractions, there was a remarkable difference of alpha-globulin between both groups in the early time of immunizing infection during 1 to 4 weeks;

Table 4 Worm recovery from rats treated with (Group I) and without (Group II) immunosuppressive drugs; all the rats received immunizing infection with 4 metacercariae each *per os*, and then challenged with 10 or 20 larvae each 6 weeks later.

Group	No. rats used	No. larvae challenged	No. worms from challenge		Percentage immunity (%)
			Total	Mean±s.d.	
I*	7	80	23	3.3±0.70	0.0
II**	12	140	8	0.7±0.62	79.3
Challenge control	17	220	55	3.2±0.81	—

* Dexamethasone (1 mg/rat) and prednisolone (5 mg/rat) were simultaneously injected into alternate thigh muscles of rats beginning one day before immunizing infection with the metacercariae.

** Non-treated with any immunosuppressive drugs; the rats only received both immunizing and challenging infections with the metacercariae.

Table 5 Number of antigen-antibody bands appeared in agar double diffusion plate in serum samples from rats in each group treated with (Group I) and without (Group II) immunosuppressive drugs.

Group	No. serum sampler in each test	Weeks after immunizing infection							
		1	2	3	4	5	6	7	8
I	3	0, 0, 0*	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
II	5	0, 0, 0	3, 3, 3	3, 3, 2	3, 3, 3	3, 3, 3	4, 3, 3	3, 2, 2	2, 1, 1
		0, 0	2, 2	2, 2	2, 2	3, 3	3, 2	2, 1	1, 1

* Number of precipitin bands appeared in each sample.

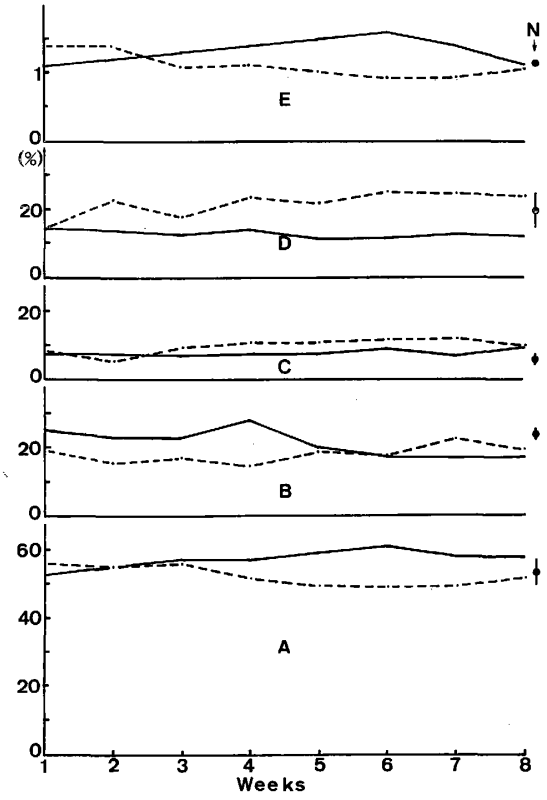

Figure 2 Weekly changes in each fraction of serum proteins in the samples from rats treated with (——) and without (----) immunosuppressive drugs; the rats in both groups immunized with the metacercariae *per os* and then challenged 6 weeks after the immunization. A: albumin, B: alphaglobulin, C: beta-globulin, D: gamma-globulin, E: A/G ratio, N: mean normal value of the non-infected and non-treated rats.

in the period the value in Group I was higher than those in Group II. The percentage value of beta-globulin in the two groups showed a similar trend indicating a slightly high rate of the value when compared to the normal value. In albumin fraction, on the other hand, a remarkable difference was found in the mean percentage between Groups I and II, during 4 to 8 weeks. A/G ratio in Group I had increased gradually up to 6 weeks, and then decreased, whereas it had decreased with the elapse of infection in Group II until necropsy.

Discussion

Kondo and Hashiguchi (1979) have observed, for the first time, on the influence of primary infection with Japanese species of *Fasciola* metacercariae to superinfection in rats. They demonstrated that the recovery rates of Japanese *Fasciola* from challenge infection were very low when compared to the natural-immunity control. The similar tendency, again, was also ascertained in the present study in rats, by

carring out challenge during 6 to 11 weeks following immunization. During the period, the percentage immunity (reduction) had a considerable variation among the experimental groups designed. From 6 to 9 weeks after immunization, the percentage reduction was significantly higher in the challenged groups than the control, indicating the ranges from 58.3 to 80.6 per cent. It was noted, however, that the ability of rats to induce the protective immunity to the fluke had trended to reduce gradually in the animals 10 weeks (25.0% immunity) after immunization; the immunity reduced to zero at 11 weeks.

In *F. hepatica*, this protective immunity following an oral infection has been amply documented as mentioned before. In most of these studies on the immunity to *F. hepatica* worm recoveries have been done after the flukes had matured. Hayes *et al.* (1972, 1973, 1974c), however, have performed a study on the ability of rats to resist a challenge infection with *F. hepatica*, recovering immature worms 3 to 4 weeks after challenge, and they have obtained a high rate of percentage reduction. This reduced worm burdens have also been recognizable during 24 to 48 hr after challenge infection of rats with *F. hepatica* (Hayes and Mitrovic, 1977; Hayes, 1978). In the current study, therefore, the markedly reduced worm burdens from challenge would be present at the early phase of challenge infection in rats. However, exactly when and where protective immunity is expressed still remains undetermined, though the immunity to reinfection with *F. hepatica* in rats is expressed by 48 hr after challenge (Hayes, 1978).

The protective immunity was examined in connection with the developmental ages of worms which were used in the immunization, and also with the liver migration of worms which might be related to induce the functional immunity in rats. Rajasekariah and Howell (1978) have examined the role of developmental stages of worms in the rat's resistance to challenge with *F. hepatica*. In their works the rats were sensitized by subcutaneous implantation of either metacercariae, 4 week-old juveniles, adult worms, or eggs of *F. hepatica*, and then challenged with the metacercariae 2 weeks later. All implanted stages, apart from adult worms, conferred a significant degree of protection on their recipient animals. With respect to the liver migration of worms, on the other hand, Lang *et al.* (1974) pointed out that the duration of liver migration by juveniles might be of greater importance in stimulating protective immunity in mice than was worm age. Thus, these observations suggest that there still remains undetermined factors in the mechanisms to induce protective immunity in the host animals against the genus *Fasciola*.

In order to study the immunological mechanisms which could underly the protective immunity, the immunizing infection was conducted by implanting the immature, 25 day-old, worms with and without diffusion chambers in the present experiment. The chamber allows for exchange of various materials between implanted parasites and their host (Hashiguchi *et al.*, 1976). Besides, with the use of diffusion chambers the inability of parasites to infect the host would reduce or eliminate all of the pathology associated with the infection (Despommier and Wostmann, 1968). The present study revealed that the rats immunized by the worms in chambers showed a significant degree of protective immunity when compared to the natural-immunity control or sham operation. The free worm implantation, however, stim-

ulated a higher degree of the immunity than the worm implantation with chambers. These results suggest that the liver migration by the immature worms implanted may be responsible, at some extent, for stimulating the induction of protective immunity to the Japanese *Fasciola* in rats. It is of great interest that the enchambered worms are able to induce the protective immunity by releasing a variety of soluble antigen through the millipore chambers placed intraperitoneally in rats. Thus, the diffusion chamber technique permits the host to come into direct contact with the living parasites, thereby exposing it to a great variety of antigenic stimuli (Despommier and Wostmann, 1968).

In the rats sensitized by worm implantation with and without diffusion chambers, serological analysis demonstrated that there was a remarkable difference in the antigen-antibody systems between both implantations. During 1 to 2 weeks after worm implantation, the precipitin bands in agar diffusion plate were nearly equal in numbers in the two groups. After challenge, however, larger numbers of the bands were recognized in serum samples from rats sensitized with free worms than in those with enchambered ones. Considering the relation between the markedly reduced worm burdens and the appearance of many bands in free worm implantation (without chambers), Ouchterlony technique may be effective as an indicator to estimate the degree of protective immunity to *Fasciola* in host animals. Each fraction of serum proteins has not indicated a remarkable difference between the two groups.

There have been many attempts to reduce or eliminate the natural immunity of hosts against various parasites; in most of these cases workers have treated the host animals with immuno-suppressants and/or X-irradiation. Recently, Hashiguchi and Hirai (1977) have examined the influence of various immuno-suppressants on the establishment of the lung fluke, *Paragonimus miyazakii* in rats. They demonstrated that combined treatment with dexamethasone and prednisolone or with hydrocortisone and dexamethasone had suppressed the host's immune responses, while the use of dexamethasone or prednisolone alone had a relatively little influence on the immunity of hosts. In consideration of these results, the present work was attempted to determine if the combined treatment of rats with dexamethasone and prednisolone would have an effect on the protective immunity to the Japanese *Fasciola* or not. The treatment greatly suppressed the induction of protective immunity in rats sensitized by oral infection with the metacercariae, and then challenged 6 weeks later. Both treated and control rats harbored 3.3 and 3.2 worms in mean recovery, while non-treated but immunized and challenged rats showed a significantly fewer worm burden indicating 79.3 per cent worm reduction. Hayes and Mitrovic (1977) have administered dexamethasone into immunized rats with the metacercariae *per os* starting 24 hr before challenge, and demonstrated that the drug used abrogated the protective effect of a previous infection. These facts indicate an important role of humoral immunity in the protective immunity to *Fasciola* in rats. No precipitin band in agar plate was observed in the rats given the immuno-suppressants throughout the experiment. The rats, on the other hand, theat were not given the drugs showed a considerable number of bands from 2 weeks after immunization. These results also suggest that antibody production was greatly suppressed in the animals given immunosuppressive drugs. Among fractions of serum proteins, gamma-globulin

in the non-treated rats with the drugs was remarkably high in an average after 2 weeks, when compared to that in the treated animals; the latter marked a consistently lower rate than average normal value. Once again, these differences may be caused by the suppression of the host's immune responses.

In the present study, the protective immunity to the common liver fluke, *Fasciola*, in rats was examined using immunosuppressants or millipore diffusion chambers. The use of drugs and the chamber techniques would provide a good measure for the examination on the mechanisms which could be underlying the induction of protective immunity to the parasites in hosts. For the genus *Fasciola*, more detailed experimental systems such as various ages of worms and the timing and sites of immunization, are needed to clarify the mechnisms using these measures in relation to the host-parasite relationships.

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免疫抑制剤およびDiffusion Chamber移植法によるラットの日本産カンテツに対する防御免疫効果の検討

橋　口　義　久

カンテツ *Fasciola Repatica* については，初回の感染によって，宿主動物の再感染に対する防御免疫が強く発現されることが知られている。しかし，分類学的位置づけが未だ不明確な日本産カンテツに対する宿主の防御免疫についての研究は極めて少ない。今回は，日本産カンテツによる初感染後6〜11週目にラットを再感染させたところ，6〜9週目の再感染群では，対照群（Challenge control）に比べて，有意の虫体排除が認められた。しかし，この防御免疫能は初感染から10週以降になると次第に低下し，11週後では対照群との間に差異を示さなかった。この防御免疫効果を調べるため，幼若虫体をDiffusion chamberに入れ，ラット腹腔内移植による感作を行い，移植3週後に再感染を試みたところ，防御効果が観察された。しかし，虫体のみの移植群に比べると，その効果は低い傾向を示した。このことは，虫体のみ移植群において，移植虫のラット肝臓内穿入による刺激が，何らかの形で強い防御免疫の発現に関与していることを示唆するもので興味深い。また，これらのラット血清をOuchterlong法で調べたところ，虫体のみ移植群ではChamber移植虫群より，多くの沈降線が出現し，抗体産生の面でも両群間に違いがみられた。一方，血清蛋白画像は両者間で大差なく，いずれも虫体移植，再感染を通して，かなりの変動を示した。次に，ラットの再感染防御免疫に対する免疫抑制剤（Dexamethazdne と Predonisolone の併用）の影響を調べた結果，免疫抑制群では，対照群と同様の虫体回収を示した。したがって，これらの抑制剤は今回の防御免の発現を著しく抑制することが明らかになった。また免疫抑制群では，Ouchterlongによる沈降線の出現は皆無であったのに対し，非免疫抑制群では，初感染から2週目以降に多数の沈降線が出現した。免疫抑制群の蛋白分画ではr-globulin値が著しく低下し，非抑制群との間に大差を示した。以上の結果は，ラットのカンテツ再感染防御機構を調べる上で，一つの手がかりを与えるものといえよう。

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EFFECT OF EXERCISE IN A HOT ENVIRONMENT FOR 7 SUCCESSIVE DAYS ON PHYSIOLOGICAL RESPONSES AND HEAT TOLERANCE

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Abstract: Eight young male university students took exercise for 7 successive days in a climatic chamber at 30°C, 70% relative humidity. Exercise was performed on a bicycle ergometer at a work load of 2 kp at a cycling rate of 50 r.p.m. for 60 min. Sweat tests were performed on the day before beginning of training and on the day after the end of training. Sweating was induced by immersion of both legs to just below the knees into circulating water of 42°C in a climatic chamber of 30°C, 70% relative humidity for 90 min. Body weight loss induced by a certain heat load increased after heat acclimatization, while a rise in rectal temperature and an increase in heat rate during sweat test decreased after heat acclimatization. Sodium concentration in sweat decreased slightly and sodium concentration at a given sweat rate decreased considerably after heat acclimatization. Heat tolerance, assessed by our numerical indices for assessment of heat tolerance, was improved by heat acclimatization. Improvement of heat tolerance was accompanied by great changes in physiological responses to heat and was induced at the cost of increase of physiological strain in terms of water metabolism.

INTRODUCTION

In work the metabolic heat produced in the body increases in proportion to the intensity of work, and body heat must be dissipated into the environment at the rate of its production to maintain a constant body temperature in a steady state. Since the amount of heat dissipation from the body decreases by increasing the ambient temperature, capacity for heat dissipation or tolerance to heat stress is one of the factors determining work capacity in a hot environment (Robinson, 1963; Piwonka et al., 1967). It is known that physiological responses to heat change as unacclimatized individuals are repeatedly exposed to a hot environment. After successive heat exposures it has always been found that subjects sweat more readily and more profusely and heat tolerance is improved by increased capacity of heat dissipation (Dill et al., 1938; Adolph, 1946; Belding and Hatch, 1963; Hori et al., 1975). It is known that the temperature regulatory responses of sweating and cardiovascular changes are involved during exercise (Robinson, 1963). Therefore, the acclimatization to heat may be facilitated by physical exercise in a hot environment. Thus it is of interest to study the effect of daily exercise in a hot environment on the physiological reponses to heat and heat tolerance of men. Recently we

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proposed a numerical index for evaluation of human heat tolerance (Hori et al., 1974). We reported that this index could follow improvement of heat tolerance during short-term heat acclimatization and long-term heat acclimatization such as superior heat tolerance of subtropical natives to that of temperate natives (Hori et al., 1975, 1978). In the present study, we attempt to compare the physiological responses of heat and heat tolerance of men before physical training with those after physical training to study adaptive changes induced by physical training in a hot environment for 7 successive days.

Materials and Methods

Eight young male Japanese, aged 18–23, were exposed to a combination of muscular exercise and environmental heat for 7 successive days in autumn. Muscular exercise was performed on a bicycle ergometer at a constant work load of 2 kp at the cycling rate of 50 r.p.m. for one hour in a climatic chamber of 30°C with 70% relative humidity. A sweat test was performed on the preceding day of beginning of physical training and on the first day after physical training for 7 successive days. The sweat test was performed as follows. Each subject dressed in shorts only and sat in a chair at rest in the climatic chamber of 30°C with 70% relative humidity for 30 min. Then the subjects, sitting in chairs, dipped their legs to just below the knees into a stirring water bath of 42°C and stayed there for 90 min. Rectal temperature was measured continuously by copper-constantan thermocouples before and during exercise, and heart rate was recorded continuously by electrocardiography. Body weight was measured with an accuracy of ± 5 g before and after the experiment and the net body weight was calculated by subtracting the weight of the dry and wet shorts from body weight measured before and after experiment respectively. Local sweat samples from the chest and back were collected by the filter paper method during each 15 min. period (Ohara, 1966). The skin areas for sampling of sweat were washed with distilled water and an area of the skin covering 12.6 cm^2 was covered with a sweat capsule by using collodion solution. Sweat was absorbed by a prewashed filter paper, local sweat rate was determined by the difference between weight of filter paper after absorption of sweat and that of dry filter paper. The filter paper was diluted with distilled water and sodium concentration in the sweat was determined by flame photometry. The magnitude of physiological strain induced in the body by heat exposure was expressed by the combination of relative rise in rectal temperature, relative water loss and relative salt loss. Heat tolerance indices I and S are calculated as follows:

$$\text{Heat tolerance index } I=\sqrt{A^2+B^2+C^2}$$

$$\text{Heat tolerance index } S=\frac{B}{\sqrt{A^2+C^2}}$$

$$A=\frac{\Delta W}{0.07\times W},\qquad C=\frac{\Delta T}{40.6-T},\qquad B=\frac{Q}{0.75\times W}$$

where W = Body weight before the experiment (Kg)

ΔW = Weight loss at the end of the experiment (Kg)

T = Rectal temperature before the experiment (°C)

ΔT=Rise in rectal temperature at the end of the experiment (°C)

$Q=0.058\times\Delta W\times\bar{C}$

C=The mean sodium concentration in local sweat (mEq/L)

Smaller value of heat tolerance index I indicates small physiological strain induced by a certain heat load and superior heat tolerance.

Results

Table 1 shows body weight, body weight loss, mean Na concentration in local sweat, rise in rectal temperature and increase in heart rate observed in sweat test before and after physical training in a hot environment for 7 successive days. The mean value of body weight loss after training was considerably greater than that before training, while mean Na concentration in sweat decreased slightly after physical training. However, these differences were statistically not significant. The mean value of rise in rectal temperature after training was significantly smaller than that before training, and that of increases in heart rate after training was considerably less than that before training though this difference was statistically not significant. Thus the ratio of sweat volume (body weight loss) to rise in rectal temperature increased after training. Changes in heat tolerance indices and their three components during physical training in a hot environment are shown in Table 2. The mean value of component A after physical training was greater than that before training. The mean values of component B, component C, heat tolerance indices I and S decreased after physical training. Among these differences, differences of component B and heat tolerance index S were statistically significant. Contri-

Table 1 Changes in physiological responses to heat induced by daily exposure to a moderate work and environmental heat.

Day	Weight (kg)	Weight loss (kg)	Mean Na conc. (mEq/*l*)	Rise in rectal temp. (°C)	Increase in H.R. (beats/min)
0	61.7±4.1	0.588±0.141	51.2±15.4	0.63±0.10*	15.5±4.9
8	61.2±4.0	0.658±0.152	49.9±15.8	0.50±0.10	12.6±4.6

Mean values are given with their standard deviations.

* Significant difference between days of experiment at 1% level.

Table 2 Changes of heat tolerance indices and their components induced by acclimatization to heat.

Day	A	B	C	I	S
0	0.139±0.038	0.193±0.030**	0.045±0.015	0.244±0.039	1.48±0.50*
8	0.155±0.041	0.153±0.029	0.042±0.016	0.226±0.039	1.07±0.41

Mean values are given with their standard deviations.

* Significant difference between days of experiment,

* at 5% level, ** at 1% level.

bution of component A to heat tolerance indices I and S was the greatest after training, while that of component B was the greatest before training. Since the values of components A and B represent the magnitude of strain induced by sweat volume and rise in core temperature respectively these results indicate that the pattern of physiological responses to heat after training differs greatly from that before training.

Discussion

Considerably greater mean value of sweat volume and slightly smaller mean value of Na concentration in sweat after physical training in a hot environment for 7 successive days than those before training are shown in Table 1. Many investigators have already reported that unacclimatized subjects tend to sweat more profusely as they are repeatedly exposed to hot environments (Adolph, 1946; Bass, 1963; Dill et al., 1938; Hori et al., 1976). It is known that the Na concentration in sweat increases progressively as the sweat rate increases (Dill et al., 1938; Kuno, 1956). Thus, changes in sweating reaction induced by acclimatization to heat were characterized by a higher sweat rate and lower Na concentration in sweat at a given sweat rate (Dill et al., 1938; Kuno, 1956). As shown in Table 1, rise in rectal temperature during exposure to a hot environment decreased significantly and increase in heart rate during heat exposure tended to decrease after heat acclimatization. Thus it may be assumed that direction of change in correlation between rise in rectal temperature and sweat volume during heat acclimatization differed from that in correlation between rise in rectal temperature and increase in

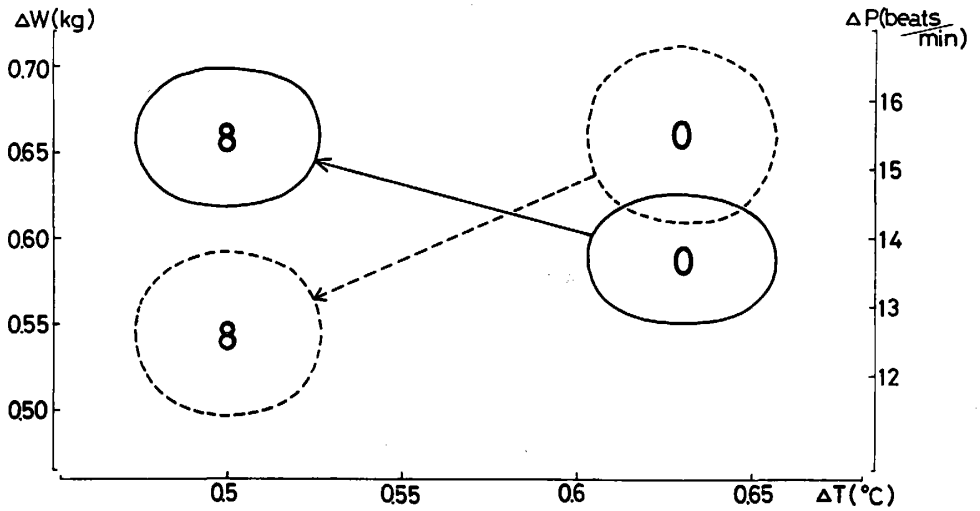

Figure 1 Changes in correlation between body weight loss, increase in heart rate and rise in rectal temperature.

ΔW: Body weight loss, ΔP: Increase in heart rate, ΔT: Rise in rectal temperature, ○: Correlation between ΔW and ΔT, ○: Correlation between ΔP and ΔT, Circles: Drawn around the means with radiuses of standard errors. Arrows indicate direction of changes induced by physical training. Figures in circles indicate time of experiment in day.

heart rate (Fig. 1). This figure indicates a decrease in rise of rectal temperature under a certain heat load induced by heat acclimatization tended to be accompanied by an increase in sweat volume and a lessening of increase in heart rate. Since rectal temperature is a dominant factor in determining sweat volume and heart rate at rest (Benzinger at el., 1963; Newburgh, 1949) the sweat center became more responsive, and sensitivity of the vasoregulatory center against rise in core temperature changed slightly during heat acclimatization. As shown in Table 2, values of B, relative rise in rectal temperature, and index I, the magnitude of physiological strain induced in the body, decreased after heat acclimatization, while value of A, relative water loss increased after heat acclimatization. A smaller value of B (rise in rectal temperature) after heat acclimatization might be due to increased heat dissipation by profuse sweating. A smaller value of index I indicates superior heat tolerance (Hori et al., 1974). Thus, it can be said that heat tolerance was improved at the cost of increase of strain in terms of water metabolism. The value of index S indicates the ratio of relative rise in rectal temperature to relative water loss and salt loss i.e. pattern of physiological responses to heat. As shown in Fig. 2, the magnitude of decrease in value of index S was greater than that in value of index I after heat acclimatization when compared with those before heat acclimatization. These results indicate the pattern of physiological responses to heat changed greater than the magnitude of physiological responses during short-term heat acclimatization. Changes in relationship between indices I and S are shown in Fig. 3. In this figure, circles are drawn around the mean values of indices I and S with radiuses of their

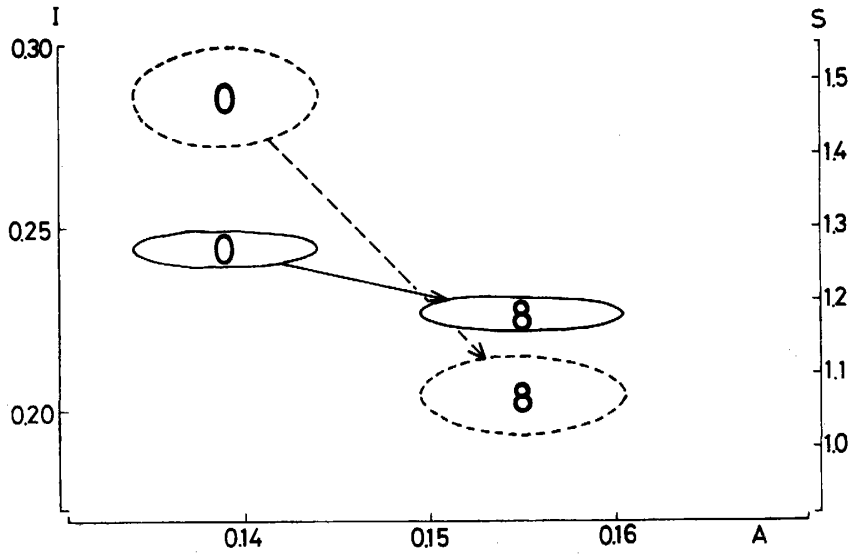

Figure 2 Changes in correlation between heat tolerance indices and relative water loss during physical training.

I and S: Heat tolerance indices, A: Relative water loss, ○: Correlation between I and A, ◌: Correlation between S and A, Circles: Drawn around the means with radiuses of half value of standard errors, Arrows indicate direction of changes induced by physical training. Figures in circles indicate time of experiment in day.

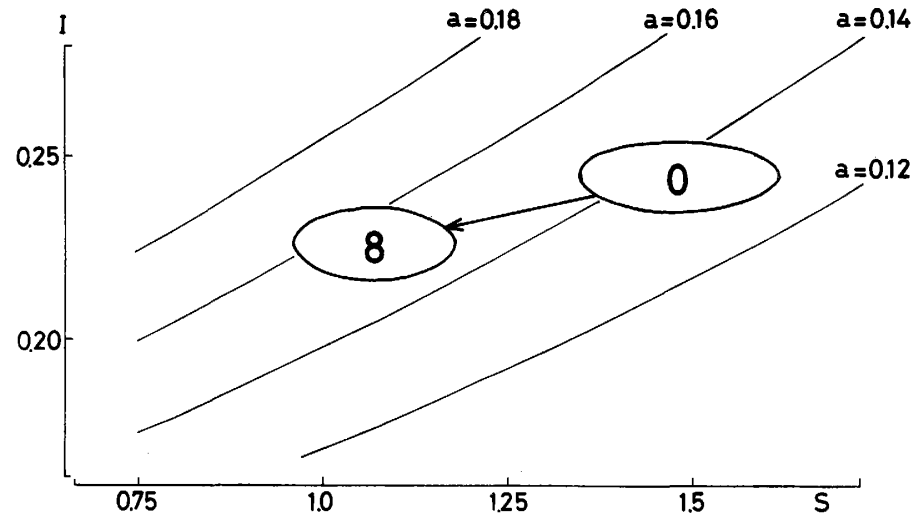

Figure 3 Changes in correlation between heat tolerance indices I and S during physical training in a hot environment for 7 successive days.
a: Parameter indicating the points of same value of $A\sqrt{1+\frac{C^2}{A^2}}$, Circles: Drawn around the means with radiuses of standard errors, Arrow indicates direction of change induced by physical training. Figures in circles indicate time of experiment in day.

standard deviations and the family of iso-sweating lines are drawn by connecting the points of the same values of parameter "a". By calculation and transformation, Eq. $I=a(1+S^2)^{\frac{1}{2}}$ can be derived as follows (Hori et al., 1974):

$$I=A\left(1+\frac{C^2}{A^2}\right)^{\frac{1}{2}}\times(1+S^2)^{\frac{1}{2}}=a(1+S^2)^{\frac{1}{2}} \quad \text{where} \quad a=A\left(1+\frac{C^2}{A^2}\right)^{\frac{1}{2}}$$

Thus, the value of parameter "a" runs parallel to change in sweating reaction. Mechanism of changes in physiological response to heat induced by physical training for 7 successive days might be as follows. The increases in sweat rate after repeated heat exposure is attributable to an increase in the activity of the sweat glands and increased excitability of the sweating center (Kuno, 1956). Lower Na concentration at a given sweat rate after short term heat acclimatization may be caused by increased reabsorption from precursor sweat in the duct of sweat glands. The cooling power of sweat is dependent on the degree of wetness of the skin and the difference in vapor pressure between the skin surface and the air. Lower Na concentration in sweat increases difference of vapor pressure between the skin surface and the surrounding air (Hori et al., 1978). Therefore, less rise in body temperature after short-term heat acclimatization might reflect increased capacity of evaporative heat loss due to profuse sweating with a lower Na concentration.

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高温環境下における連続7日間の運動が高温に対する生理的反応および耐熱性に及ぼす影響

堀　　清　　記

8名の男子大学生に7日間毎日 30°C, 湿度70% の室内で労作を行わせた。労作は負荷 2 kg, 毎分50回のペダル踏み運動を60分間行わせた。発汗テストを訓練開始前日と訓練終了の翌日に行った。発汗テストは 30°C, 湿度70% の室内で両下腿を 42°C の水槽に90分間温浴させて行った。高温環境下の労働の馴化により発汗量の増加，発汗テスト中の直腸温上昇の減少と心拍数の減少が認められた。高温馴化によって同じ発汗量に対する汗の Na 濃度はやや減少した。我々の開発した半定量的耐熱性の指標で判定すると，耐熱性は高温馴化で向上したことが示された。耐熱性の向上は高温曝露時の生理的変化を著しく変えて水分損失による生体内に生ずる歪を犠牲にしてもたらされている。

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FLUORESCENT FOCUS SIZE REDUCTION BY SPLEEN CELLS OF MICE IMMUNIZED WITH RABIES VIRUS

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Abstract: The spread of rabies virus between adjacent mouse neuroblastoma cells was inhibited in the presence of spleen cells from mice sensitized to rabies virus as measured by the size of fluorescent focus formation. This activity of focus size reduction was appeared to be closely related to the development of cell-mediated cytotoxic activity. Rabies virus inactivated by beta-propiolactone was also capable of generating such an activity as live virus.

The role of cell-mediated immunity either in the recovery from primary rabies infection or in the postexposure immunoprophylaxis of rabies is poorly understood. Miller *et al.* (1978) have suggested the contribution of cell-mediated immunity in the recovery from rabies by demonstrating the potentiation of HEP rabies infection both in immunosuppressed and thymectomized mice. Kaplan *et al.* (1975) described similar observations. Recently, we have established the mouse model system by which the protection mechanisms of postexposure vaccination against rabies might be studied. The study has demonstrated that although interferon (IF), which is induced early after vaccination, is important, the postexposure prophylaxis of rabies cannot be elucidated simply by the IF nor neutralizing (NT) antibody produced by the vaccine and suggested the requirement of some other mechanisms including cell-mediated immunity (Mifune *et al.*, 1980).

Evidences indicating the development of cell-mediated immunity in rabies immunized animals have also been demonstrated by several *in vitro* and *in vivo* studies (Wiktor *et al.*, 1974; Tignor *et al.*, 1976; Wiktor *et al.*, 1977; Lagrange *et al.*, 1978; Tsiang & Lagrange, 1980). However, whether these cell-mediated immune responses really play a role in the recovery or in the postexposure prophylaxis has not clearly been shown. Simmons *et al.* (1974) demonstrated that the *in vitro* control of the growth of herpes virus was quantified by determining plaque size in monolayers to which sensitized spleen cells were added as opposed to nonsensitized spleen cells and this method was a sensitive and more direct parameter to measure a cell-mediated immunity.

Present paper briefly describes that this technique is applicable to rabies virus and immune spleen cells are capable of limiting the spread of virus in the infected cell cultures.

C3H/He strain mice were immunized by intraperitoneal inoculation with ap-

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proximately 5×10^7 focus forming units(FFU) of live or the corresponding amount of beta-propiolactone(BPL) inactivated HEP-Flury strain of rabies virus (Kondo, 1965; Mifune *et al.*, 1980). Spleens were harvested from the mice at various intervals after immunization. They were pressed gently through stainless steel mesh (#200) and single cell suspensions were prepared in RPMI 1640 medium supplemented with 10 per cent heat-inactivated fetal calf serum(FCS). The single cell suspensions were then fractionated into the cells nonadherent to plastic plate and the effluent cells from nylon wool column as described by Julius *et al.* (1973). Cloned N–18 strain of mouse neuroblastome(MNB) cells were grown in Dulbecco's modified Eagle's medium supplemented with 5 per cent FCS, 5 per cent calf serum, 3 g/*l* of NaHCO; and antibiotics and was plated into Lab-Tek 4-chamber slides (Miles lab.). When the cells became confluent, they were infected with 100 FFU of plaque purified CVS strain of rabies virus (Buckley & Tignor, 1975) and incubated at 37°C for 4 hrs. Whole or fractionated spleen cells from nonimmune or immune mice were carefully overlayed onto the infected MNB cells at a spleen cell to monolayer cell ratio of 50: 1, and further incubated for 26 hrs at 37°C. Preliminary experiments indicated that during the 30 hrs of incubation period, secondary fluorescent focus was not formed by the progeny virus from the primary infected focus. At the end of the incubation period, the culture media were harvested and the supernatants after low speed centrifufation were assayed for the activities of antiviral NT antibody, IF and lymphotoxin(LT) which might be produced in the co-cultivated culture. NT antibody was assayed as described by Smith *et al.* (1973) using CER cells (Smith *et al.*, 1977), IF was assayed in L cells with vesicular stomatitis virus and LT was assayed as described by Shimizu *et al.* (1977) using L cells and MNB cells. On the other hand, infected MNB cells on the chamber slides were washed in phosphate buffered saline, fixed with cold acetone and then stained with FITC-conjugated anti-rabies globulin (BBL, #40604). The number of cells in each immunofluorescent focus was conuted under a fluorescent microscope. At least, one hundred fluorescent foci were examined and the statistical significance of the focus size reduction was estimated by the analysis of variance.

Infected MNB cells co-cultivated with non-immune spleen cells (Fig. 1A) showed large fluorescent foci, usually consisting of 5–20 cells, whereas when co-cultivated with immune spleen cells (Fig. 1B), fluorescent foci were small, usually consisting of 1–5 cells.

The effect of immune spleen cells harvested on day 7 after immunization on the focus size of rabies virus was shown in Table 1. The average focus size of the cultures co-cultivated with non-immune whole spleen cells was 6.2 cells, while that with immune whole spleen cells was 3.2 cells and the reduction rate of the focus size was 48.4 per cent which was statistically significant (Exp. 1). Depletion of plastic plate-adherent cells from immune spleen cells did not influence the activity of immune spleen cells to limit focus size (Exp. 2). Effluent cells from nylon wool column consisted mostly of T cells as tested by rosette formation and this T cell-rich fraction also suppressed the virus spread by 47.3 per cent (Exp. 3). In contrast, pre-treatment of immune nonadherent spleen cells with anti-Thy 1.2 serum (OLAC, clone F7D5) plus complement before co-cultivation resulted in the abrogation of the activity

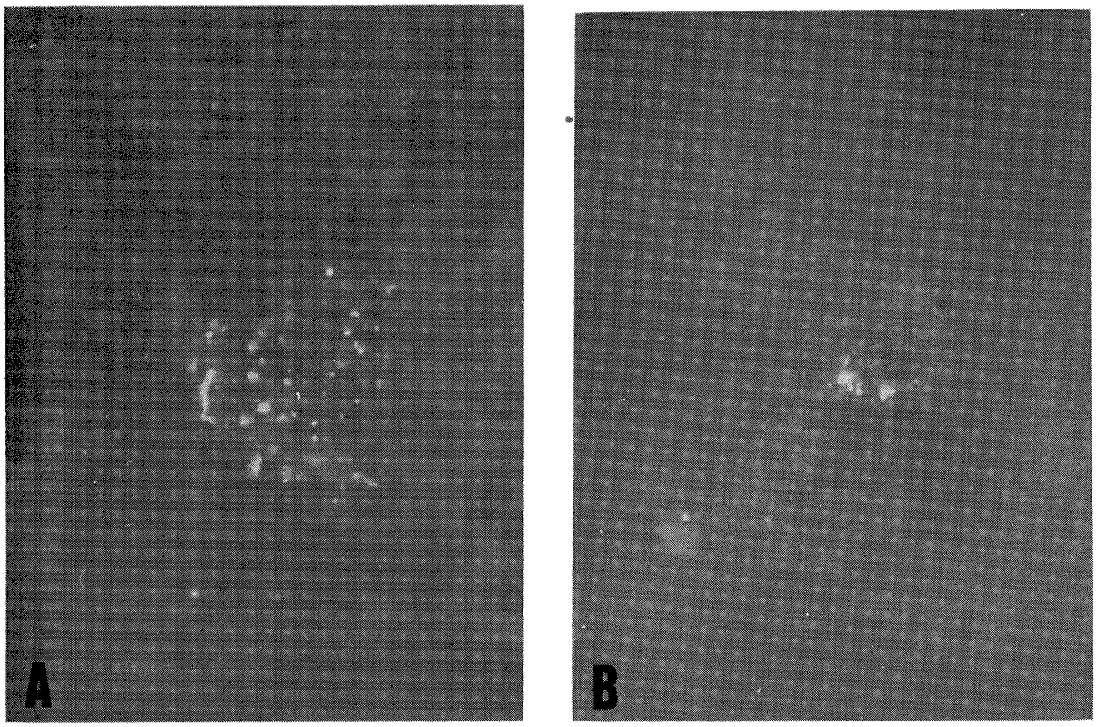

Figure 1 Immunofluorescent focus of rabies virus-infected murine neuroblastoma cells. The cells in the 4-chamber slides were infected with 100 FFU per chamber of plaque purified CVS strain of rabies virus. Four hours later, the cells were carefully overlayed with (A) non-immune spleen cells or (B) rabies immune spleen cells and further incubated at 37 C for 26 hours. Aftert he incubation period, the cell monolayers were washed with PSB, fixed with cold acetone and stained with FITC-conjugated anti-rabies gamma-globulin.

(Exp. 4). No significant inhibition of focus size was observed in the cultures co-cultivated with spleen cells from mice immunized with Sindbis virus (Exp. 5). In addition, neither NT antibody, IF nor LT was detected in each of the co-cultivated MNB cell culture medium. These results appear to suggest that the suppression of virus spread by immune spleen cells is mediated by virus specific immune T cells and not by the NT antibody, IF nor LT.

The kinetics of the development of focus size limiting activity after immunization is shown in Table 2. High activity was observed in the spleen cells obtained on day 6 after immunization. However, such high activity was no longer observed in the spleen cells harvested on day 14. In parallel with this experiment, cell-mediated cytotoxic activities of spleen cell suspensions prepared at intervals after immunization were examined against virus infected MNB cells by ^{51}Cr-release method. The results showed that the cytotoxic activity was first detected on day 4 and reached a maximum level on day 7 and then declined to a low level by day 14 (Data not shown) as already described by us and other investigators (Tignor *et al.*, 1977; Mifune *et* al., 1979).

These results seemed to suggest that the suppression of virus spread by immune spleen cells is closely related with the development of the cytotoxic activity of immune

Table 1 Fluorescent focus size reduction by immune spleen cells in rabies virus-infected murine neuroblastoma cells.

Expt.	Effector cell population	Fluorescent focus size[a]	Reduction rate (%)	Ab, IF and LT assay[b]
1	Normal whole spleen cells	6.2		Ab (−) IF (−)
	Rabies immune whole spleen cells	3.2	48.4 (SS)[c]	LT (−)
2	Normal non-adherent spleen cells	9.3		Ab (−) IF (−)
	Rabies immune non-adherent spleen cells	4.9	47.3 (SS)	LT (−)
3	Normal nylon wool column effluent cells	11.0		Ab (−) IF (−)
	Rabies immune nylon wool column effluent cells	5.8	47.3 (SS)	LT (−)
4	Rabies immune non-adherent spleen cells treated with anti-Thy 1.2+complement	6.6		Ab (−) IF (−)
	Rabies immune non-adherent spleen cells treated with medium+complement	2.1	68.2 (SS)	LT (−)
5	Normal non-adherent spleen cells	7.9		
	Sindbis virus immune non-adherent spleen cells	5.6	29.1 (NSS)[d]	NT[e]

a) Average number of cells an fluorescent focus consisted.
b) Ab (NT antibody), IF (Interferon) and LT (Lymphotoxin) in cocultivated culture media were assayed as described in the text.
c) Statistically significant, $P<0.01$ by analysis of variance. d) Not statistically significant.
e) Not tested.

Table 2 Development of fluorescent focus size reduction activity of spleen cells from mice immunized with live or BPL-inactivated rabies virus.

Days spleen cells harvested after immunization	Effector cells	Stimulant	Fluorescent focus size[a] with non-immune effector	with rabies immune effector	Reduction rate (%)
6	nylon wool column effluent cells	live	14.1	6.0	57.4 (SS)[b]
14	nylon wool column effluent cells	live	14.1	10.1	28.4 (NSS)[c]
6	non-adherent spleen cells	BPL-inactivated	19.8	7.6	61.6 (SS)
14	non-adherent spleen cells	BPL-inactivated	19.8	12.3	37.8 (SS)

a) Average number of cells an fluorescent focus consisted.
b) Statistically significant, $P<0.01$ by analysis of variance.
c) Not statistically significant.

spleen cells. It should be noted that BPL-inactivated rabies virus is also capable of generating the activity of spleen cells to limit the virus spread as live virus. This observation might be of interest in terms of the possible role of cell-mediated immunity in the postexposure treatment of rabies by BPL-inactivated vaccine.

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狂犬病ウイルス免疫マウス脾臓細胞によるウイルス螢光フォーカス形成抑制

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狂犬病ウイルス（HHp-Flury 株）で免疫されたマウス脾細胞は，マウス神経芽細胞腫（MNB）細胞上でのウイルスの螢光フォーカス形成を有意に抑制した。脾細胞のこのウイルス伝播抑制作用は，脾細胞からプラスチック面付着細胞を除去しても活性が消失しないこと，ナイロン綿カラム通過細胞に認められること，脾細胞を予め，抗 Thy-1.2 血清と補体で処理すると活性が消失すること，MNB 細胞と免疫脾細胞の混合培養液中に，ウイルス中和抗体，インターフェロン或いはリンホトキシンがが検出されないこと等から，T リンパ球によることが明らかであった。また，この活性は，同時に行なった免疫脾細胞のウイルス感染 MNB 細胞に対する細胞障害活性の発現と時期を同じくして発現し，脾細胞の細胞障害活性と密接な関係があることが示された。また，β-プロピオラクトンで不活化されたウイルスで免疫されたマウス脾細胞にも，生ウイルスと同様にこのウイルス伝播抑制作用が認められ，不活化ワクチンによる狂犬病のいわゆる感染後投与による発症防御機構に，細胞性免疫が一つの役割を果たしている可能性を示唆するものとして興味深い。

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CULEX TRITAENIORHYNCHUS GILES: SOME EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON LARVAL DEVELOPMENT AND SELECTED ADULT ATTRIBUTES

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Abstract: A Pakistan strain of *Culex tritaeniorhynchus* was exposed to conditions of 18, 22, 28 and 34°C at 16L: 8D and 18 and 22°C at 9L: 15D in an incubator. Cooler temperatures reduced immature survival from eclosion to emergence and slowed the rate of development resulting in larger and heavier adults at emergence with increased energy reserves in the form of triglycerides. The rate of mating, blood feeding and the synthesis of triglycerides from sucrose increased as a function of temperature. Short day length further slowed the rate of development increasing the size and weight, but not triglyceride content, of adults at emergence. The insemination rate also decreased under shorter day length. These results suggested that cold-induced quiescence, but not necessarily diapause, was induced by the present experimental regimens.

The climate of Punjab Province, Pakistan, presents an extremely varied temporal series of ecological regimens to its mosquito fauna. To persist, species must withstand temperatures ranging from 45°C in midsummer to 0°C in mid-winter, seek more ameliorated microhabitats or evolve physiological mechanisms to alter metabolism during periods of stress. Although aestivation has yet to be reported, several *Culex* species, including *Culex tritaeniorhynchus* Giles, appear to undergo a cessation of reproductive activity in response to shorter days and/or cooler temperatures and overwinter as inseminated, unfed and nulliparous females; males and larvae are not collected during mid-winter (Reisen, 1978 and unpubl.). However, on warm days during winter, *Cx. tritaeniorhynchus* females occasionally are collected biting bovid baits (Reisen, 1978), suggesting that if a diapausing state is indeed realized, it must not be obligatorily governed by photoperiod or require an elongated period of cold exposure prior to reactivation. Under slightly colder winter conditions in Japan, overwintering diapause is induced during autumn by a combination of cooler temperatures and shorter day lengths (e.g., Omori, *et al.*, 1965a, b; Wada,

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et al., 1973) and persists through the first warm days of spring. Some gonotrophic dissociation occurs in autumn (Oda and Wada, 1973); however, the accrual of fat reserves prior to diapause seems to be accomplished mainly through sugar feeding.

The purpose of the present study was to examine the effects of different temperatures and long and short day photoperiods on immature development and adult characteristics under controlled laboratory conditions. Emphasis was placed on studying those attributes related to overwintering behavior to determine if diapause could be induced in strains of *Cx. tritaeniorhynchus* collected from Punjab Province, Pakistan.

Methods and Materials

Strain: The Balloki strain from Punjab Province, Pakistan, was used throughout, and was well-adapted to mating in 3.8 liter carton cages (Reisen, *et al.*, 1979) and feeding on restrained laboratory mice. The females used to produce the cohorts for experimentation were held under normal insectary conditions of 28°C, 60% R. H. and 16L: 8D photoperiod with 1.5 hr crepuscular periods simulated by gradually changing the illumination produced by 60 watt incandescent bulbs from 18 to 0 Lux.

Biological methods: Egg rafts, within 12 hrs of oviposition, were placed in an incubator to hatch under the test temperature and photoperiod regimens. On the morning of eclosion, 216 1st instar larvae were counted into each of 4 replicate, 16×27 cm enameled pans (density=0.5 larvae/cm^2 of pan surface area) filled with well-water to a depth of 1.5 cm. and were fed a total of 1.075 gm of finely sifted liver powder (Siddiqui, *et al.*, 1976). The addition rate of the food was adjusted for the larval development rate at each temperature. Pupae were counted daily into plastic jars and the numbers of males and females successfully emerging recorded. Adults unable to leave the water surface were considered dead. The length of the wing, from its insertion at the mesothorax to its most distal margin excluding wing scales, and dry weight were determined for the first and last 5 females and 5 males emerging from each of the four replicate pans. The triglyceride content of the weighed females was determined using the colorimetric method described by Shahid, *et al.* (1980).

Ten females per pan were released into plastic jars (vol. =1200 cm^3) where they were continuously offered a 10% sucrose solution in vials with sponge wicks. After four days, the females were killed by freezing, dried for 2 days at room temperature in a dessicator, weighted and the triglyceride content determined. The remaining males and females were pooled by date of emergence and released into 3.8 liter carton cages, in which they were continuously offered 3% sucrose in vials with sponge wicks, and, at night, a restrained laboratory mouse as a blood source. Each morning all blood engorged females were isolated individually in vials with dilute straw-infusion water as an oviposition substrate (Reisen and Siddiqui, 1978). Dying females as well as some unfed females were dissected to determine if they were inseminated, and during the short-day photoperiod, if oögenesis had progressed beyond Stage I (Clements, 1963). Females ovipositing hatching egg rafts were released into a separate cage and were offered a mouse as a blood source. Those

refeeding were re-isolated for a second oviposition. The number of eggs in each raft was counted under a microscope to determine fecundity.

The entire experiment was done in an incubator set at 18, 22, 28 and 34°C, with a 16L: 8D photoperiod and at 18 and 22°C with a 9L: 15D photoperiod. The lowest temperature at which restrained mice survived was 18°C. The photoperiod was established by turning the incubator fluorescent lights off and on, with a 1 to 2 hr dusk simulated at light-off by allowing the used cells of a flashlight to dim gradually from about 100 to 0 lux. *Cx. tritaeniorhynchus* had been found to mate readily at 16L: 8D and 28°C in 3.8 liter cages under these illumination regimes (Reisen, *et al.*, 1979).

Statistical methods: Median developmental time (E_{50}) was calculated by regressing the cumulative proportion of adults emerging, transformed to probits, as a function of time in days from eclosion, transformed to $\log_e$. Temperatures within the 16L: 8D photoperiod were compared by model I analysis of variance (ANOVA) with individual means compared by *posteriori* least significant range tests (Sokal and Rohlf, 1969). Measurements of the adults subsampled from each pan were considered to be nested within pans, and the among-pan mean square was used to test the main effects and first and second order interaction terms. The effect of shorter photoperiod was tested individually at each temperature using Student's t-tests or ANOVA. Observed sex ratios were compared using 2×2 contingency Chi Square adjusted by Yates' correction factor (Sokal and Rohlf, 1969). Non-replicated observations were tested over temperature using a Model I regression analysis with the regression coefficients tested for significant departure from 0 by ANOVA (Sokal and Rohlf, 1969).

Results

Effects of temperature at 16L: 8D Survival from eclosion to pupation did not significantly vary among temperatures, but pupal to adult survival was significantly lower at 18°C when many of the adults, especially the males, did not successfully shed the pupal integument and/or leave the water surface (Table 1). Overall survival from eclosion to emergence was highest at 28°C. The sex ratio at emergence (males/total) was lowest at 18°C and significantly departed from 1:1 at 18 and 28°C ($p<0.05$).

Median developmental time (E_{50}) varied inversely with water temperature (Table 1). Males developed significantly faster than females, and this disparity varied disproportionately over temperature, since the sex by temperature interaction term in the ANOVA was significant ($p<0.01$). The relation of temperature (t) to the velocity of immature development ($V=1/E_{50}$) was expressed by the law of heat summation, $V=(t-t_0)/K$, where t_0 is the developmental point and k=a thermal summation constant (Mogi, 1978). V varied linearly as a function of temperature for both males and females, with $t_0=8.3$ and 9.2°C for males and females, respectively (Fig. 1). The thermal summation constant of 158.7 for females and 164.2 for males implied the number of degree days above t_0 required for the completion of immature development.

Table 1 The effects of temperature and photoperiod on selected immature attributes.

Attribute	Photoperiod:	16L: 8D[1]					9L: 15D[2]	
	Temperature (°C):	18	22	28	34	group x̄	18	22
Survivorship[3]								
L to P		.813[a]	.767[a]	.866[a]	.647[a]		.598*	.899[ns]
P to A		.600[b]	.831[a]	.913[a]	.806[a]		.629[ns]	.886[ns]
L to A		.488[b]	.646	.791[a]	.521[b]		.380[ns]	.796[ns]
Sex ratio (♂/total)		.356[b]	.520	.448[a]	.486[a]		.442*	.493[ns]
E_{50} (days)[4]	♀♀	17.67	13.08	7.59	6.85	11.30[a]	18.62*	13.82*
	♂♂	17.51	11.97	7.55	6.85	10.97[b]	18.03[ns]	13.25*
	group x̄	17.59[a]	12.53[b]	7.57[c]	6.85[d]			

[1] Means compared within 16L: 8D; means in each row or group means followed by the same least significant range test ($P>0.05$).

[2] Means compared between photoperiods at the same temperature, ns: $P>0.05$; *: $P<0.05$; **: $P<0.01$.

[3] L=Larval stages I to IV; P=pupae; A=adult.

[4] E_{50}=median developmental time to adult emergence in days.

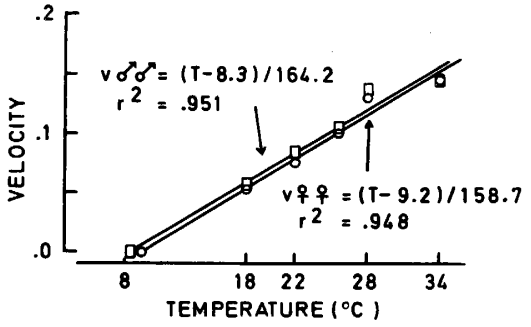

Figure 1 The velocity of immature development (velocity, $v=E_{50}^{-1}$, where E_{50} is median emergence in days) for males (squares) and females (circles) plotted as a function of water temperature, T, in $C^0 \cdot r^2$ =the coefficient of determination indicating the goodness of fit.

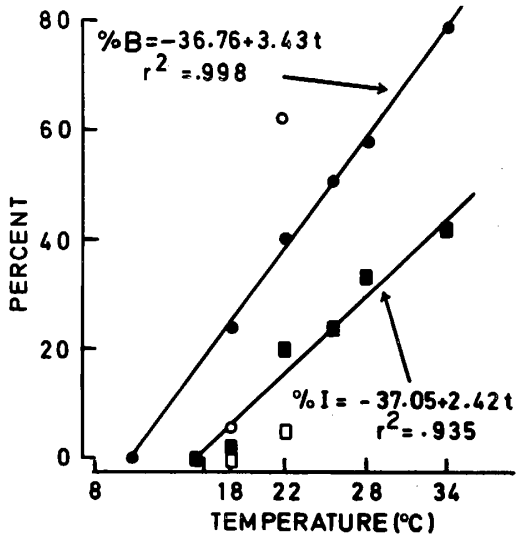

Figure 2 The percentage of emerging females imbibing a blood meal (B) and mating (I) plotted as a function of holding temperature, t, in °C for long day (16L: 8D=filled squares and circles) and short day (9L: 15D=open squares and circles) photoperiods. Regression functions calculated from long day photoperiod data; r^2=coefficient of determination indicating the goodness of fit.

Table 2. The effects of temperature and photoperiod on selected adult attributes.

Attribute \ Photoperiod:	16L: 8D[1]					9L: 15D[2]	
Temperature (°C):	18	22	28	34	group $\bar{x}$	18	22
♀♀ inseminated (%)	2.08	20.0	34.41	41.73		0.0^{ns}	5.40**
♀♀ blood fed (%)	23.81	40.47	58.80	79.68		6.58**	62.57**
Fecundity (eggs/♀)[3]: GC1	62.3(14)	101.6(9)	125.3(57)	124.0(84)	117.94(164)^{a}	65.0(3)^{ns}	112.8(9)^{ns}
GC2	0(0)	45.0(2)	125.9(28)	111.7(53)	115.29(83)^{a}	0(0)	98.3(4)^{ns}
group $\bar{x}$	62.3(14)^{b}	91.3(11)^{b}	125.5(85)^{a}	119.3(137)^{a}		65.0(3)	108.3(13)
Length gc (days)[3]: GC1	17.5(18)^{a}	14.6(22)^{b}	7.6(115)^{c}	6.8(153)^{d}		16.8(3)^{ns}	12.2(45)*
GC2	NE	4.3(3)^{a}	3.0(29)^{a}	2.9(101)^{a}		NE	3.3(4)*
Wing length (mm) T1[4]: ♀♀	3.25	3.13	2.80	2.74	2.85^{a}	3.49	3.26
♂♂	2.95	2.85	2.62	2.50		3.22	3.03
T2[4]: ♀♀	3.22	3.20	2.81	2.62	2.82^{a}	3.39	3.32
♂♂	2.90	2.90	2.47	2.46		3.16	2.91
group $\bar{x}$	3.08^{a}	3.02^{a}	2.67^{b}	2.58^{b}		3.32**	3.13**
Dry weight (mg) T1[4]: ♀♀	.383	.435	.349	.410	.351^{a}	.464	.360
♂♂	.321	.352	.286	.275		.407	.319
T2[4]: ♀♀	.391	.365	.363	.316	.325^{b}	.433	.408
♂♂	.320	.322	.266	.259		.405	.329
group $\bar{x}$	.353^{a}	.368^{a}	.316^{a}	.315^{b}		.427**	.354^{ns}
Dry weight after sucrose (mg)	.522^{c}	.582^{bc}	.664^{b}	.782^{a}		.529^{ns}	.590^{ns}
gain[5] (%)	135.3^{b}	145.8^{b}	188.3^{a}	216.0^{a}		118.0^{ns}	153.7^{ns}
Triglyceride (mg/♀) T1[4]	.131	.062	.086	.072	.088^{a}	.070	.040
T2[4]	.154	.029	.029	.029	.060^{b}	.075	.072
$\bar{x}$	.142^{a}	.046^{b}	.057^{b}	.050^{b}		.072**	.056^{ns}
Triglyceride after sucrose gain[5] (%)	.206^{b}	.133^{c}	.218^{b}	.251^{a}		.107**	.169**
	146.2^{c}	306.4^{b}	398.5^{ab}	504.2^{a}		143.8^{ns}	323.9^{ns}

Footnotes 1–2 see Table 1.

[3] GC1 and GC2 refer to the first and second gonotrophic cycles, respectively; (No. hatching egg rafts and ovipositing females in parentheses).

[4] T1 and T2 refer to the first and last five adults of each sex emerging, respectively.

[5] gain (%) $=(T_4-T_0)/T_0\times 100$, where T_0 is the estimate at emergence and T_4 is the estimate after feeding for 4 days on 10% sucrose solution.

The proportion of females imbibing a blood meal and mating (i.e., the number of dissected females that were inseminated or that oviposited hatching egg rafts) decreased as a linear function of decreasing temperature (Table 2 and Fig. 2). The extrapolated temperatures at which no blood feeding or mating would occur were 10.7 and 15.3°C, respectively.

There was no significant difference ($p>0.05$) between female fecundity (egg/female) in the first and second gonotrophic cycles (Table 2). Fecundity at 18 and 22°C was significantly lower than fecundity at 28 and 34°C. The mean duration from emergence to oviposition (GC 1) significantly decreased with increasing temperature; however, the length of the second gonotrophic cycle, the night of re-feeding to oviposition 16 (2), did not differ significantly among temperatures. At 18°C, no female laid more than 1 egg raft, and none of the rafts hatched or contained embryonated eggs.

Mosquitoes reared at 18 and 22°C had longer wings than those reared at 28 and 34°C (Table 2). Females had longer wings than males and this difference varied disproportionately over temperature, since the sex-by-temperature interaction term in the ANOVA was significant ($p<0.05$). Adults emerging first did not have significantly longer wings than those emerging last ($p>0.05$). Similarly, the dry weight of adults reared at 18 and 22°C was heavier at emergence than the dry weight of adults reared at 28 and 34°C (Table 2). Females were heavier than males, and the difference remained proportionate, since the sex-by-temperature interaction term was not significant ($p>0.05$). The mean weight of adults emerging first was significantly greater than adults emerging last, suggesting some form of larval intraspecific competition for food as described previously by Siddiqui, et al. (1976). The significantly greater weight of females emerging first was attributed to fat reserves at emergence, since the triglyceride content was also significantly greater (Table 2). Adults reared at 18°C had significantly more triglycerides at emergence than did those reared at 22, 28 and 34°C.

The dry weight of females offered 10% sucrose solution for four days increased markedly from estimates made at emergence and the percent gain ranged from 135.3 to 216.0% (Table 2). The mg of dry weight (dw) and the percent gain was significantly higher at 28 and 34°C than at 18 and 22°C and increased as a linear function of temperature (t), ($dw\% = 35.75 + 5.32\,t$, $r^2 = 0.982$). Weight increases were attributed to triglyceride synthesis from the imbibed sucrose, since the greatest percent triglyceride increases were also realized by females held at 34 and 28°C. The rate of triglyceride synthesis (ts) was temperature dependent, i.e., the percent of triglyceride gain increased as a linear function of temperature (t): $\%ts = -201.19 + 21.18t$, $r^2 = 0.955$.

Effects of photoperiod at 18 *and* 22°*C*: Under a "short day" illumination, there was no difference in survival from larval eclosion to adult emergence; however, the median developmental time was generally slower (Table 1). The sex ratio of the immatures reared at 18°C increased significantly, but the proportion of males remained lower than the expected 1:1 ratio when tested by Chi square ($p<0.05$).

None of the females held at 18° and significantly fewer females held at 22° mated under the shorter photoperiod (Table 2). In addition, significantly less

and more females at 18 and 22°C, respectively, imbibed blood meals. Females taking blood meals matured eggs at all photoperiods and temperatures studied, although under 9L: 15D, 1♀/16♀♀ dissected (3.2%) at 22° exhibited gonotrophic disassociation, i.e., took a blood meal, but after death 2 or more days later, ovarian development had not progressed beyond Stage Ib.

Fecundity at 9L: 14D did not differ significantly at either 18 or 22°C (Table 2). The duration of the first gonotrophic cycle at 18° did not differ significantly, although at 22°C the duration of both the first and second gonotrophic cycles were significantly shorter.

At 9L: 14D the wing lengths of adults reared at 18° and 22°C and the dry weight at 18°C, but not 22°C, were significantly longer and heavier than adults reared at 16L: 8D, respectively (Table 2). Females were agian larger and heavier than males. At 18°, but not 22°C, the wing lengths of the adults emerging first were significantly longer; no significant differences in the dry weights of the adults emerging first and last was found. At 22°C, there was significant among-rearing pan variability ($P<0.05$) in both the wing length and dry weight ANOVAs which contributed to the inability to detect significant differences in the time of emergence effects. The triglyceride content of the emerging females was significantly less at the 9L: 15D photoperiod and 18°C, while no significant difference was detected at 22°C. This was unexpected, since at 18°C the dry weights were significantly greater at emergence and usually the triglyceride content was significantly correlated with dry weight (Shahid, *et al.* 1980).

The dry weight of females after being offered 10% sucrose for 4 days increased markedly from the values estimated at emergence, but the weight attained and the percent weight gain did not vary between photoperiods (Table 2). Unexpectedly, the mg of triglyceride accrued at 9L: 15D was significantly less and greater than the mg of triglyceride accrued at 16L: 8D and 18 and 22°C, respectively; however, there were no significant differences in the percentage of triglyceride gained. Thus, although the actual values of triglyceride at emergence and after 4 days of being offered 10% sucrose were significantly lower at 18° under the short day photoperiod, the relative quantity of triglycerides synthesized was comparable. Discrepancies in the absolute values were attributed to variability among the groups of mosquitoes measured.

Discussion

As defined by Clements (1963), "Quiescence is a state of inactivity induced by an unfavorable environment and it ceases on amelioration of the environment. Diapause is terminated only after reactivation, i.e., exposure to a factor such as cold for a period of time." Faculative diapause in the adults of multivoltine species is characterized by the accrual of fat reserves usually in response to an exogeneous cue such as photoperiod. The influence of photoperiod in some mosquitoes is mediated by temperature which reduces the rate of development, so that a critical number of short-day photoperiods are experienced and diapause is induced (e.g. Beach, 1978).

In Punjab, differences between the parity rate of biting and resting *Cx. tritae-*

niorhynchus females during autumn suggested that the population bifurcates into gonotrophically inactive overwintering and active non-overwintering segments (Baker, *et al.* 1979; Reisen, *et al.* 1980). Parallel observations have been recorded in Japan by Fujimiya and Yajima (1976). In Punjab, the rate of insemination typically decreases in November, as indicated by an accrual of uninseminated resting females; however, by January all females were inseminated (Reisen, *et al.*, 1977), suggesting that the uniseminated females either mated or did not overwinter. Females collected during December and January are typically without blood in the gut, nulliparous and have Stage I ovaries, although blood fed and gravid females may be collected during every month of the year. These data suggest that an overwintering state is perhaps achieved, but loosely maintained due to mild winter temperature regimens.

In the present study, cooler temperatures and shorter day length elongated larval development and resulted in the emergence of larger adults with more energy reserves. These results agreed with studies in Japan by Yoshida, *et al.* (1974) who found that cooler temperatures and shorter photoperiods elongated immature development, and by Nakamura, *et al.* (1968) and Fujimiya and Yajima (1976) who found that the diapausing generation was always significantly larger in size than females that remained gonotrophically active during autumn.

The mating and blood feeding rates were slower and the time from emergence to initial oviposition was greatly elongated at cooler temperatures, which, through time, could result in an accrual of uninseminated, unfed nullipars under field conditions. At 18° the shorter photoperiod decreased the proportion of females feeding, while at 22° the proportion feeding actually increased. These observations contrasted with the findings of Eldridge (1962) who reported that a Japanese strain of *Cx. tritaeniorhynchus* was reluctant to feed on a shaved chick at 28° and 8L: 16D. In addition, in the present study, little gonotrophic dissociation was observed at 9L: 15D and 22°C, whereas this phenomenon was commonly reported under similar experimental conditions with strains of *Cx. tritaeniorhynchus* from Japan, although rarely observed under field conditions (e.g. Harada, *et al.*, 1968, Takahashi, 1970, and Oda and Wada, 1973). The rate of triglyceride synthesis from sucrose did not increase under the shorter photoperiod, but rather decreased with temperature, as was expected from similar experiments with other species of mosquitoes (Van Handel, 1966a and b).

Survivorship and longevity did not appear to be greatly enhanced, and adults at all temperatures and photoperiods were dead 6 weeks after emergence. In contrast, Omori *et al.* (1965a), using semi-natural conditions in Japan, induced *Cx. tritaeniorhychus* females to hibernate in October and more than 20% of females fed with sugar survived for more than 20 weeks, November through March. In Pakistan, females would have to survive at least 15 weeks to overwinter from November through late February or early March, when the first overwintering females can be collected at bovid baits. These data suggest that a true diapause state was probably not induced during the present study.

The results of the present study indicated that some of the attributes characteristic of diapausing *Cx. tritaeniorhynchus* were induced, although these attributes were

not separable from quiescence, or cold-induced physiological changes, and did not impart marked longevity. Speculative explanations included:

1. Incubator conditions were not condusive for diapause; i.e., metabolic processes were not slowed sufficiently.
2. The photoperiod regimens were too extreme and "not recognized" as the diapause induction cue.
3. The exposure of the immature stages to a critical number of "short day" photoperiods does not induce diapause.

In Japan, Omori, *et al.* (1965a) were able to induce diapause experimentally and keep females alive throughout the winter. They found that survival was enchanced in hibernacula that were continuously dark, had little air movement and were thermally stable at about 11°C. Conditions in the present study retained a diel photoperiod, were warmer (min. temp. =18°C), but had reduced air movement in paper carton cages with a wet cotton pad on the gauze top to increase humidity. However, the habitats of diapausing females appear to differ considerably. In Japan, *Cx. tritaeniorhynchus* females have been collected from brush piles and caves (Bullock, 1959), but crevices and rodent burrows in the stone walls supporting permanent terraced rice fields seemed to be the preferred microhabitat (e.g. Omori, *et al.* 1965a, Wada, *et al.* 1973). In Punjab overwintering females can be recovered throughout the winter in closely spaced agricultural crops such as clover (*Trifolium* spp.) and in dense clumps of elephant grass (*Imperlata* spp.). Hibernacula in the Lahore area of Punjab are thus more subject to diel temperature changes, which ranged in 1978, for example, from a minimum of 2°C in January to a maximum of 25°C in February, and are exposed to diel illumination changes. Perhaps, the constant incubator temperatures of 18°C may have been to warm.

Natural photoperiods in Lahore area range from 14: 17L: 9: 43D on 21 June to 10: 09L: 13: 51D on 21 December, with 11: 00L: 13: 00D occurring on 1 November. Photoperiods in the incubator were markedly longer and shorter than the natural condition. Some "long day" insects such as *Acronycta* enter diapause only after exposure to critical photoperiods; days which are either too long or too short fail to induce diapause (Lees, 1955). Possibly, the 9L: 15D photoperiod presented in the present study was not recognized as diapause induction stimulus by *Cx. tritaeniorhynchus.*

Bifurcation of overwintering and non-overwintering populations may begin as early as October in Punjab. At this time, afternoon water temperatures at breeding sites were 25°C, dropping to 15 or 16°C by early morning; however, developmental time in nature remained at 6 to 7 days (Reisen and Siddiqui, 1979). In the present study at 9L: 15D and 22°C, E_{50} exceeded 13 days, and thus if the immature stages were receptive to the induction cue, they should have experienced a sufficient number of "short-day" photoperiods to induce diapause. Our results suggest that the exposure of the immature stages to a given number of short-day photoperiods was not the triggering stimulus responsible for diapause induction. In agreement, Omori, *et al.* (1965b) used adults reared from their laboratory colony (no details given) for their diapause experiments, and Eldridge (1962) found that

blood feeding activity of females reared at 14L: 11D decreased after exposure to 2 to 4 "short-day" (8L: 16D) photoperiods.

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