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Anemia and its mechanism in goldfish *Carassius auratus* infected with *Trypanosoma danilewskyi*

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ABSTRACT: *Trypanosoma danilewskyi* Laveran & Mesnil, 1904 caused anemia in goldfish *Carassius auratus*. The severity of the anemia was associated with high parasitemia. At least 2 factors are responsible for the anemia; (1) hemolysin: a secretory/excretory product of living trypanosomes which lyses red blood cells (rbc) in the absence of antibody (in vitro study) and (2) hemodilution (in vivo study). No significant hemolysis was detected when rbc were added to sonicated trypanosomes. The hemolysis and hemodilution were correlated with numbers of parasites. Secretion/excretion of hemolysin by living trypanosomes was associated with declining pH in the incubation medium but the pH itself was not involved in hemolysis.

INTRODUCTION

Most piscine trypanosomes are not pathogenic, however *Trypanosoma danilewskyi* Laveran & Mesnil, 1904 causes mortality in experimentally infected goldfish *Carassius auratus* (Lom 1979, Woo 1981b). The parasite has been found in common carp *Cyprinus carpio*, crucian carp *Carassius auratus gibelio*, tench *Tinca tinca* and eel *Anguilla* sp. in Europe (Lom 1979). It is not host specific (Woo & Black 1984) and its development and multiplication in fish have been described (Robertson 1911, Woo 1981a).

Trypanosoma danilewskyi causes anemia in infected goldfish (Robertson 1911, Dykova & Lom 1979). Anemia is also associated with other piscine *Trypanosoma* (Becker 1977, Khan 1985, Lom 1979) and *Cryptobia* (Woo 1979, 1987) species. In a recent study, Thomas & Woo (1988) showed that the anemia in cryptobiosis is in part caused by hemolysin from the parasite and formation of an immune complex on red blood cells (rbc).

In the present study, in vivo and in vitro approaches were used to determine factors contributing to anemia in goldfish infected with *Trypanosoma danilewskyi*.

MATERIALS AND METHODS

The strain of *Trypanosoma danilewskyi* used in the present study was that used in earlier studies (Woo 1981a, b).

Goldfish were infected by intraperitoneal injection. Number of parasites was estimated using a hemocytometer (Archer 1965) and goldfish (average 7.35 g; 4.70 to 10.56 g) bought from a local supplier were maintained as before (Islam & Woo 1990). Blood from heavily infected fish was collected into a heparinized syringe, dispensed into microhematocrit tubes, sealed at one end and centrifuged for 4 min at 13 000 × *g*. The tubes were cut at the junction of the buffy coat and packed red cells (Woo 1969). Parasite suspensions, free of red cells, were washed 3 times in cold Ringer's solution and the number of parasites determined. The parasites in Ringer's solution (1.0 to 1.5 ml) were sonicated at 12 kHz for 15 min in an ice bath. A drop of sonicated material was examined under the microscope to ensure that there were no intact parasites. The antigen was used fresh or dispensed in 1 ml aliquots to tubes and stored in liquid nitrogen.

Fish rbc were washed thrice in Ringer's solution before they were used. Hemoglobin was assayed in the incubation medium after rbc were incubated with parasites in a tube. The tube was centrifuged for 10 min in

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the refrigerated centrifuge at 4000 × *g*; 0.02 ml (Eppendorf micropipette) of supernatant was dispensed into 5 ml of Drabkin's solution in a borosilicate glass tube. Light absorbance was read in a spectrophotometer and plotted on a standard curve to determine hemoglobin concentration. The standard curve was prepared by plotting absorbance reading against the concentration of a standard set of cyanmethemoglobin.

Experimental design

Anemia in infected goldfish. Each goldfish was infected with ca 19500 parasites. They were divided into 2 groups and were maintained at 20 ± 1°C and 10 ± 1°C. Five goldfish from each group were killed weekly; the number of rbc, the parasitemia and the packed cell volume (pcv) were determined for 7 wk.

The experiment was repeated with another group of 30 fish which were inoculated with 30000 parasites $fish^{-1}$. Three fish were bled at 0, 7, 12, 17, 22, 27, 32, and 38 d post-infection and the parasitemia and number of rbc was determined in each fish. The free-flowing blood was collected and the total blood volume measured using an Eppendorf micropipette. Fish were weighed and blood volume was expressed as $\mu l\ g^{-1}$ body weight of fish. The relationships between blood volumes and parasite numbers (log-transformed) were determined by fitting the data to a quadratic regression model.

In vitro study on hemolysis. The study was done aseptically. Two approaches were used after incubation of rbc with live or sonicated parasites on slides/tubes: (1) determine lysis by counting intact rbc, or (2) determine hemoglobin released and pH changes in the medium.

1. Reduction in number of rbc on exposure to live or sonicated trypanosomes: A known number of rbc were mixed with a known number of parasites. A drop of the mixture was dispensed onto a glass slide, covered with a vaseline-ringed coverslip and placed under a microscope. Before and after incubation for a specified period the number of rbc on the slide were determined either in a predetermined high power microscopic field or in a marked area on a slide. Alternatively, a known number of rbc and parasites were incubated in a tube. After incubation the remaining intact rbc were counted to determine the number of lysed rbc.

Incubation of rbc with live parasites: In this experiment 0.25% rbc was incubated with 4 million ml^{-1} live parasites at 20 ± 1°C for 7 h. In the control 0.25% rbc was incubated in Ringer's solution. The number of lysed rbc in a microscopic field on each slide was counted hourly. The experiment was repeated with 8 million parasites ml^{-1}.

Incubation of rbc with live and sonicated parasites: A suspension of 100 million washed parasites was suspended in 2 ml of Ringer's solution. One ml (50 million parasites) was placed into a tube and diluted serially by 2-fold dilutions (25, 12.5, and 6.25 million ml^{-1}). To each tube 0.5 ml of 2% rbc was added so that the final concentrations of parasites were 12.5, 6.25 and 3.125 million ml^{-1}. Three drops from each tube were dispensed on 3 different slides and covered with vaseline-ringed coverslips (22 × 22 mm). Each slide was set under a microscope for 10 h at room temperature (20 ± 1°C). The controls (no parasites) had a 1% rbc suspension. Intact rbc in a predetermined microscope field were counted every 2 h.

The other 50 million parasites (in 1 ml Ringer's solution) were sonicated and a similar set of 4 slides was prepared using sonicated parasites after appropriate antigen dilutions.

The number of lysed rbc was converted to percentage lysis. The data were transformed into arc-sine percentages before statistical analysis. Though the experiments were run for 10 h, results from the first 6 h were used in the analysis because there was 100% hemolysis at 6 h in the microscope field with 12.5 million parasites ml^{-1}. A strip-split-plot design was used to determine the difference between live and sonicated parasites and a split-plot design to determine the effects of number of parasites, incubation periods, and their interaction effects on lysis by live or sonicated parasites. Duncan's multiple range test (DMRT), which compared the mean of lysed rbc, was eventually used to compare the number of parasites and incubation periods.

Quadratic regression models are used to establish the relationship between incubation periods and lysis of rbc. In this case original percentage data were used and lysis of all incubation periods was included.

2. Hemoglobin released from rbc on incubation with live parasites and pH changes in medium: *Incubation of rbc with live parasites:* A 8.42 million ml^{-1} rbc suspension was incubated with 11.05 million ml^{-1} live parasites at 10 and 20°C. Another 2 sets of incubate (one for each temperature) were prepared with only 11.05 million ml^{-1} parasites in Ringer's solution. The controls contained only rbc. Hemoglobin released and pH changes were recorded after 6 h.

The experiment was repeated with 2% rbc in 200, 100, and 30 million ml^{-1} parasites at 20°C for 9 h. Released hemoglobin and pH changes were determined every 3 h.

The experiment was repeated again with 2% rbc and 30, 20, 10, and 0.00 (control) million parasites ml^{-1} at 20°C for 10 h. Hemoglobin and pH changes were recorded every 2 h.

Data were analysed using a split-plot design. DMRT

was used to compare the mean of released hemoglobin and the changes in pH. Regression analysis was carried out to determine the relationship between incubation period, the amount of hemoglobin, and the pH.

Incubation of rbc with live or sonicated parasites: Six ml of live parasites in Ringer's solution were divided into 2 equal parts. Three ml (100 million parasites ml^{-1}) were dispensed into a 13 × 100 mm sterile borosilicate glass tube and diluted serially by 2-fold dilutions such that the final concentrations in 4 tubes were 50, 25, 12.5, and 6.25 million ml^{-1}. Washed rbc (100 µl) were added to each tube to obtain a final 4 %. The content of each tube was dispensed (in 0.5 ml aliquots) into 6 sterile microcentrifuge tubes. The control was 100 µl of 4 % rbc in 3 ml Ringer's solution. Every 2 h one tube from each parasite concentration and one control were used to determine the released hemoglobin and pH changes. There were 3 replicates for each concentration of parasite. The incubation time was 10 h.

Another series was prepared using the remaining half of parasite suspension (same protocol) but sonicated parasite was used instead. This saline used was taken from a different stock (the initial pH was slightly different from the stock used in the experiment with live parasites).

A strip-split-plot design was used to determine the differences between live and sonicated parasite and a split-plot design was used to determine the effect of number of parasites, incubation periods, and their interaction on pH. DMRT, which compared the mean pH changes, was eventually used to compare the number of parasites and incubation periods. A correlation analysis was done to determine the relationship between pH and hemoglobin. Finally, data were fitted to a regression model to establish the relationship between incubation periods and pH.

RESULTS

Anemia in infected goldfish

Infected fish held at 20 °C were anemic (low numbers of rbc and pcv) during the course of the infections. The anemia was most severe in fish which had massive parasitemias. There was a negative correlation between numbers of rbc (and pcv) and numbers of parasites (Table 1). The numbers of rbc (and pcv) decreased significantly at high parasitemias. Although numbers of rbc and pcv declined at 10 °C there was no correlation with parasitemias (Table 1).

There was an increase in blood volume in infected goldfish when the parasitemia was high. However, the increase was not significant when compared with control fish. As a group there was a direct relationship between the blood volume and numbers of parasites. After a lag period the blood volume increased rapidly with increasing numbers of parasites and this is expressed in a quadratic regression model (Fig. 1).

Table 1. *Carassius auratus*. Correlation between blood parameters and parasite numbers in goldfish infected with *Trypanosoma danilewskyi*

Temp. group	Blood parameter	df	Calculated r value
20 °C	Rbc	31	-0.460^{**}
	Pcv	31	-0.416^{*}
10 °C	Rbc	33	-0.170^{ns}
	Pcv	33	-0.021^{ns}

** Significant at 1 % level
* Significant at 5 % level
ns Not significant

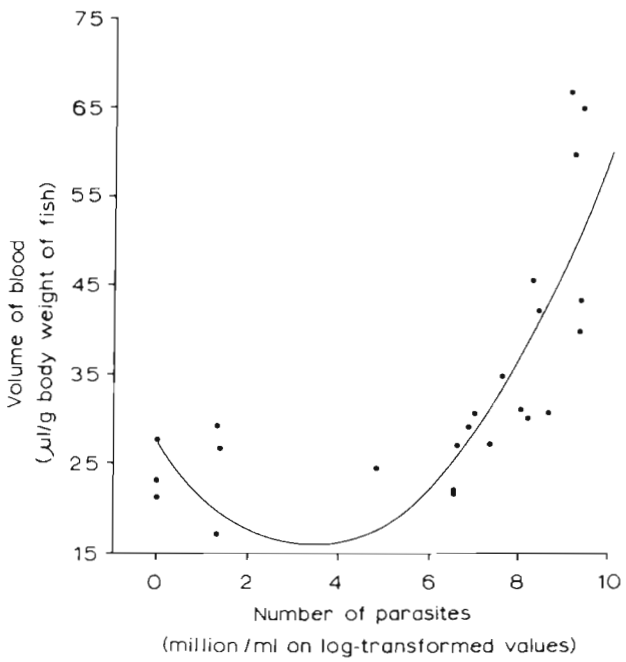

Fig. 1 *Carassius auratus*. Relationship between blood volume and number of parasites (data are log-transformed) in *Trypanosoma danilewskyi* infected goldfish maintained at 20 °C. $Y = 27.077 - 6.81X + 1.008X^2$; $R^2 = 0.70$; $p < 0.0001$ where Y = volume of blood, X = number of parasites

In vitro study on hemolysis

Reduction in number of rbc on exposure to live or sonicated trypanosomes

Incubation of rbc with live parasites. There was ca 56 % lysis of rbc after 7 h of incubation with 4 million

Table 2. Number of goldfish rbc (in 0.25 % suspension) lysed after incubation with live *Trypanosoma danilewskyi* in Ringer's solution at 20 °C. Values are no. of rbc lysed in a microscopic field (eyepiece × objective) with percentage lysis in parenthesis

No. of parasites ($\times 10^6$ ml^{-1})	Initial no. of rbc in microscopic field	Incubation period (h)							
		0	1	2	3	4	5	6	7
4.00	207	0 (0.00)	3 (1.45)	5 (2.42)	10 (4.83)	16 (7.73)	56 (27.05)	78 (37.68)	115 (55.56)
0.00	121	0 (0.00)	3 (2.48)	3 (2.48)	4 (3.31)	4 (3.31)	7 (5.79)	10 (8.27)	17 (14.05)
8.00	224	0 (0.00)	2 (0.89)	32 (14.29)	64 (28.57)	159 (70.98)	216 (96.43)	224 (100.00)	not done
0.00	122	0 (0.00)	6 (4.92)	10 (8.20)	13 (10.66)	14 (11.48)	15 (12.30)	16 (13.12)	not done

live parasites ml^{-1} (Table 2). This was about 400 % more than in the controls (without parasites).

The experiment was repeated and 100 % lysis was observed after 6 h when 8 million parasites ml^{-1} were used. In the control group there was 13 % hemolysis (Table 2). No significant differences were detected when rbc was incubated with or without sonicated parasite (at 20 °C or at 10 °C).

Incubation of rbc with live or sonicated parasites. A significantly higher number of rbc were lysed when they were incubated with live parasites than with sonicated parasites ($p < 0.002$). Hemolysis increased with the number of live parasites ($p < 0.0001$) and incubation period ($p < 0.0001$) (Table 3). It increased rapidly after a lag period and is described by a quadratic regression model (Fig. 2). Highest lysis (100 % after 6 h) was observed with 12.5 million ml^{-1} parasites and this was followed by 6.25, 3.125, and 0.0 (control) million ml^{-1} parasites respectively (Table 3).

There were some hemolysis when rbc were incubated with sonicated parasites. However, this did not increase with parasite number as observed with live parasites (Table 3). Hemolysis increased with incubation: this included the controls without parasite antigen.

Hemoglobin released from rbc on incubation with live parasites and pH changes

Incubation of rbc with live parasites. Hemoglobin was detected after 6 h incubation at 20 °C with live parasites but not in the controls (without parasites). No hemoglobin was detected at 10 °C. The pH declined in experimental (parasites and rbc) tubes and in tubes with parasites only. The decrease was greater at 20 °C than at 10 °C.

Results were similar when the experiments was repeated (Table 4). The amount of hemoglobin released was higher in tubes with larger numbers of parasites (0.45 mg dl^{-1} with 200 million ml^{-1} parasites). No hemoglobin was detected in the controls. The pH declined in all experimental groups (greatest decrease with 200 million ml^{-1} parasites). No decrease in pH was observed in the controls.

Table 3. Duncan's multiple range test (alpha = 5 % with 16 df) of percentage rbc lysed (mean of arc-sine-transformed percentage data) with different numbers of parasites and incubation periods. Total incubation period was 10 h (five 2 h incubation periods). As lysis of rbc reached 100 % with 12.5 million ml^{-1} parasites after 6 h of incubation, only data up to 6 h were included. Means with the same letter are not significantly different.

Group	Parameters			
	No. of parasites ($\times 10^6$ ml^{-1})	Rbc lysed (mean of percentages)	Incubation periods (h)	Rbc lysed (mean of percentages)
Live	0.00	11.930 d		
	3.125	16.438 c	2	4.676 c
	6.25	17.323 b	4	19.663 b
	12.50	41.956 a	6	41.397 a
Sonicated	0.00	12.598 c		
	3.125	17.003 a	2	9.272 c
	6.25	16.270 a	4	15.224 b
	12.50	15.195 b	6	21.304 a

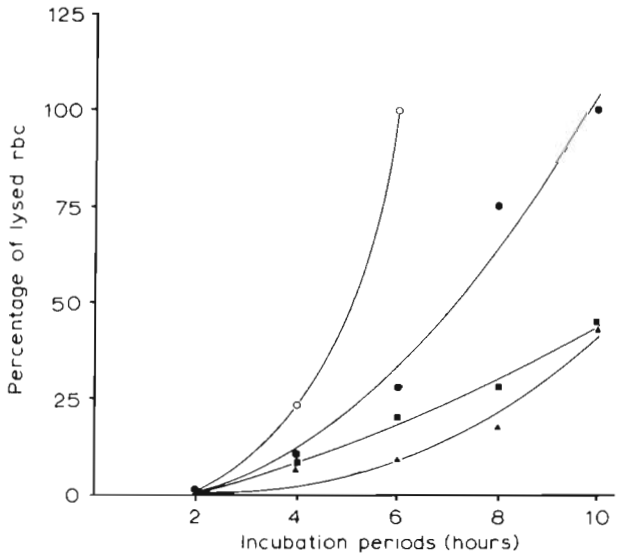

Fig. 2. *Carassius auratus*. In vitro lysis of goldfish rbc (%) when incubated with different numbers of live *Trypanosoma danilewskyi*. (▲) 0.0×10^6 parasites ml^{-1}: $Y = 8.937 - 4.903X + 0.813X^2$; $R^2 = 0.97$; $p < 0.0001$. (■) 3.125×10^6 parasites ml^{-1}: $Y = 3.874 + 1.987X + 0.282X^2$; $R^2 = 0.99$; $p < 0.0001$. (●) 6.25×10^6 parasites ml^{-1}: $Y = 5.737 + 1.100X^2$; $R^2 = 0.98$; $p < 0.0001$. (○) 12.5×10^6 parasites ml^{-1}: $Y = 33.680 - 29.634X + 6.781X^2$; $R^2 = 0.99$; $p < 0.0001$ where Y = percentage of lysed rbc, X = incubation period (h)

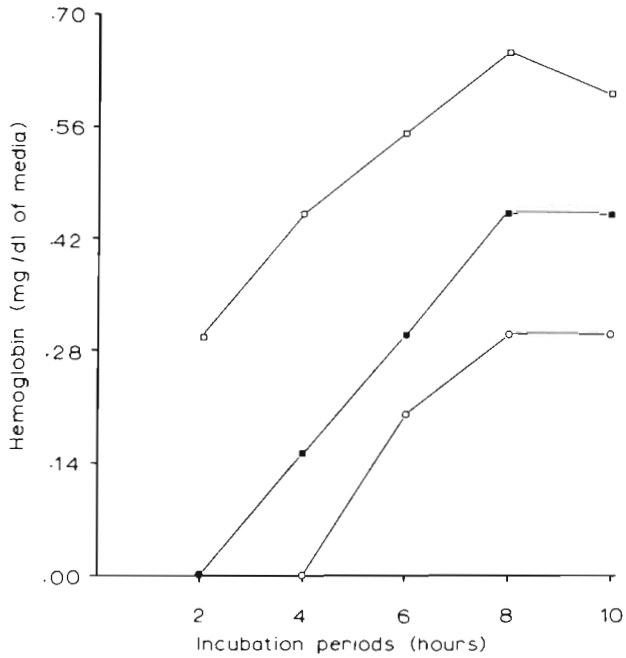

Fig. 3. *Carassius auratus*. In vitro release of hemoglobin during incubation of goldfish rbc with different numbers of live *Trypanosoma danilewskyi*; no hemoglobin was detected in controls where rbc were incubated with no parasites. (○) 10×10^6 parasites ml^{-1}; (■) 20×10^6 parasites ml^{-1}; (□) 30×10^6 parasites ml^{-1}

The experiment was repeated again with 0, 10, 20, and 30 million ml^{-1} live parasite and, again, results were similar (Fig. 3). Large amounts of hemoglobin were released when high numbers of parasites were used ($p < 0.0001$) and with prolonged incubation ($p < 0.0001$). Most parasites were dead in tubes after 8 h (30 million ml^{-1}) or 10 h (20 million ml^{-1}).

The pH declined with incubation ($p < 0.0001$) and numbers of parasites ($p < 0.0001$) (Fig. 4). The decline continued up to 4, 6, and 8 h with 30 million, 20 million, and 10 million ml^{-1} respectively. The changes in pH are expressed by a quadratic regression model (Fig. 4). The maximum decline in the pH (from 7.073 to 5.063) was after 4 h with 30 million ml^{-1}.

Incubation of rbc with live or sonicated parasites. No hemoglobin was detected when rbc were incubated with sonicated parasites. Hemoglobin was detected only when rbc were incubated with live parasites; the amount increased with incubation ($p < 0.0001$) and parasite numbers ($p < 0.0002$) (Table 5). The most hemoglobin released (0.60 mg dl^{-1}) was at 10 h incubation with 50 million ml^{-1} live parasites; no hemoglobin was detected with 0.0 to 6.25 million ml^{-1} parasites. Hemoglobin was first detected after 4 h of incubation in the media with 50 million ml^{-1} parasites and 8 and 10 h of incubation in media with 25 and 12.5 million ml^{-1} parasites respectively (Fig. 5).

Changes in pH were observed in the incubation media. These were significantly lower in preparations with live parasites than with sonicated parasites ($p < 0.0001$). Drop in pH was related to number of parasites and incubation period ($p < 0.0001$ in both)

Table 4. Released hemoglobin and changes in pH of Ringer's solution in which goldfish rbc (2 % suspension) were incubated (at 20 °C) with live *Trypanosoma danilewskyi*

No. of parasites ($\times 10^6$ ml^{-1})	Incubation periods (h) Hemoglobin (mg dl^{-1}) 0	3	6	9	pH 0	3	6	9
200.00	0.00	0.00	0.45	0.60	7.94	6.13	5.03	4.86
100.00	0.00	0.00	0.15	0.30	7.94	7.02	6.57	5.69
30.00	0.00	0.00	0.00	0.15	7.94	7.69	7.52	7.45
0.00	0.00	0.00	0.00	0.00	7.94	7.90	7.83	8.01

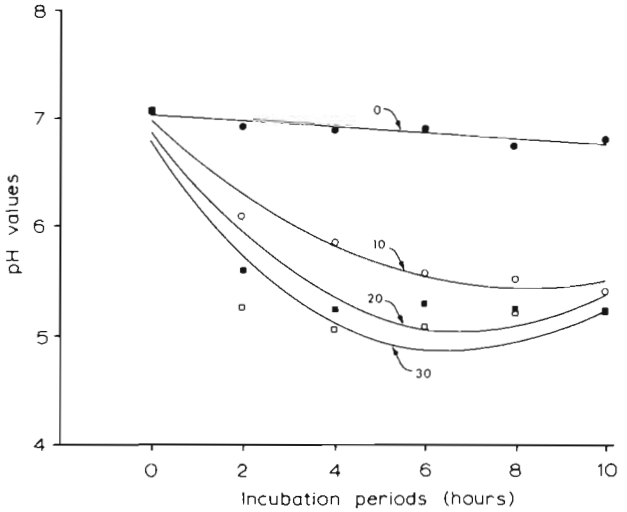

Fig. 4. *Carassius auratus*. pH changes during incubation of goldfish rbc with different numbers of *Trypanosoma danilewskyi*. (●) 0 × 10^6 parasites ml^{-1}: $Y=7.023 - 0.030X$; $R^2=0.81$; $p<0.0001$. (○) 10 × 10^6 parasites ml^{-1}: $Y=6.962 - 0.379X + 0.023X^2$; $R^2=0.96$; $p<0.0001$. (■) 20 × 10^6 parasites ml^{-1}: $Y=6.848 - 0.528X + 0.038X^2$; $R^2=0.89$; $p<0.0001$. (□) 30 × 10^6 parasites ml^{-1}: $Y=7.778 - 0.599X + 0.050X^2$; $R^2=0.84$; $p<0.0001$ where Y = pH, X = incubation period (h)

Table 5. Duncan's multiple range test (alpha = 5 % with 24 df) for released hemoglobin (mean) with different numbers of live parasite and incubation periods. No detectable hemoglobin was released with control (0 parasites) and 6.25 × 10^6 parasites ml^{-1} Means with the same letter are not significantly different

No. of parasites (× 10^6 ml^{-1})	Mean hemoglobin	Incubation period (h)	Mean hemoglobin
12.5	0.03 c	2	0.00 c
25.0	0.10 b	4	0.03 c
50.0	0.24 a	6	0.05 c
		8	0.20 b
		10	0.33 a

and the changes are expressed in a quadratic regression model. The lowest pH (5.41) was observed in the media with 50 million parasites ml^{-1} after 10 h (Fig. 6). In contrast the pH in control tubes did not change significantly. Most of the parasites were dead in the tubes with 50 million ml^{-1} after 8 h and were sluggish in tubes with 25, 12.5, and 6.25 million parasites ml^{-1}.

There was a slight decrease in pH with increasing number of sonicated parasites. Unlike with live parasites these changes were not dependent on length of incubation.

Finally, no hemoglobin was detected when rbc were incubated for 10 h in Ringer's solutions at pH 7.75, 7.25, 6.75, 6.25, and 5.75.

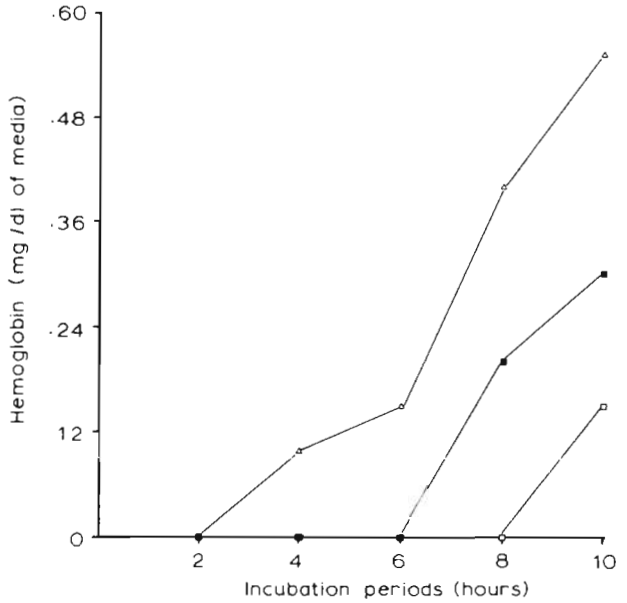

Fig. 5. *Carassius auratus*. In vitro release of hemoglobin by rbc with different numbers of *Trypanosoma danilewskyi*; no hemoglobin was detected when 0 parasites ml^{-1} (control) and 6.25 × 10^6 parasites ml^{-1} were incubated with rbc. (□) 12.5 × 10^6 parasites ml^{-1}; (■) 25.0 × 10^6 parasites ml^{-1}; (△) 50.0 × 10^6 parasites ml^{-1}

DISCUSSION

Trypanosoma danilewskyi cause a severe anemia in goldfish at, or following, peak parasitemias. The negative correlation between numbers of rbc (and pcv) and parasitemias indicates that lysis of rbc is dependent on parasitemia. Anemia is one of the clinical signs in piscine trypanosomiasis (Lom 1979, Khan 1985) and cryptobiosis (Woo 1979, 1987). Severity of the anemia in cryptobiosis is related to parasite numbers in the blood (Woo 1979, 1987, Thomas & Woo 1988).

The present study demonstrates that there are at least 2 factors which contribute to anemia in goldfish infected with *Trypanosoma danilewskyi*. The first is a 'hemolytic factor' (hemolysin) present in the secretory/excretory product of the trypanosome which causes direct hemolysis of rbc. In vitro incubation of rbc from naive goldfish and live trypanosomes indicated that the hemolysis increased with numbers of trypanosomes and incubation period. The second factor is 'hemodilution' (increased blood volume) which was at, or following, the peak parasitemia. Increased blood volume was correlated with the increased numbers of parasites.

Thomas & Woo (1988) showed that there are 2 basic components in *Cryptobia salmositica* that are responsible for the anemia in cryptobiosis. A lytic component, which is dosage dependent, causes hemolysis indepen-

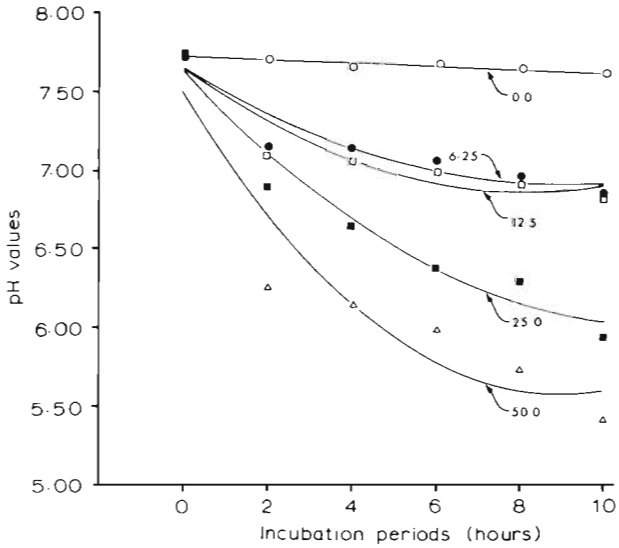

Fig. 6. *Carassius auratus*. pH change when goldfish rbc were incubated with different numbers of live *Trypanosoma danilewskiy*. (○) 0.0×10^6 parasites ml^{-1}: $Y = 7.720 - 0.010X$; $R^2 = 0.74$; $p < 0.0001$. (●) 6.25×10^6 parasites ml^{-1}: $Y = 7.631 - 0.166X + 0.009X^2$; $R^2 = 0.86$; $p < 0.0001$. (□) 12.50×10^6 parasites ml^{-1}: $Y = 7.638 - 0.198X + 0.012X^2$; $R^2 = 0.89$; $p < 0.0001$. (■) 25.00×10^6 parasites ml^{-1}: $Y = 7.625 - 0.291X + 0.013X^2$; $R^2 = 0.96$; $p < 0.0001$ (△) 50.00×10^6 parasites ml^{-1}: $Y = 7.479 - 0.431X + 0.024X^2$; $R^2 = 0.88$; $p > 0.0001$ where Y = pH, X = incubation period (h)

dent of antibody or complement, and a second immune-complex-forming component activates complement resulting in lysis. Hemodilution occurs in cryptobiosis as a result of generalized edema and may contribute to anemia (Woo 1979). In many respects the mechanism of anemia in trypanosomiasis is similar to that in cryptobiosis.

Dykova & Lom (1979) found severe histopathological changes in hemopoetic organs (i.e. spleen and kidney) in fish infected with *Trypanosoma danilewskyi*. Consequently, they suggested a relationship between the lesions and the blood profile, namely that anemia in infected goldfish is due to the malfunctioning of the spleen and insufficient compensatory hemopoetic capacity of the kidney interstitium.

The decline in pH during incubation of rbc with live parasites was probably due to excretory/secretory products of parasites. Similar declines in pH (pH 8.0 to 5.5) were observd in *Trypanosoma congolense* (Tizard & Holmes 1976). In the present study, as the parasites died because of changes in pH and depletion of nutrients, release of secretory/excretory product decreased. Hence there were no further declines in pH during the last hours of incubation (8 to 10 h).

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The slight initial decline in pH in preparations with sonicated parasites may be caused by the release of metabolic products by the trypanosomes (prior to sonication) and biological products of the cells (e.g. enzymes, cell fragments) which are released during sonication. There was little decrease in pH during the latter incubation periods.

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