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PROTECTION OF ANIMALS FROM TICKS BY INTERFERENCE IN THE FEEDING PROCESS OF TICKS

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INTRODUCTION

Ticks are blood-sucking arthropods which cause much damage to the health of man and his domestic animals throughout the world. Ticks cause irritation, blood-loss and transmit a large variety of pathogens. The main method for tick control has been treatment of domesticated animals with chemical acaricides. Recent emphasis on environmental protection and pure food lows, as well as the emergence of tick strains resistant to acaricides, indicate a need to develop alternative methods for tick control. Immuninzation of host animals with tissues and secretions of ticks has shown promise as a new method for tick control (Willadsen, 1987). When ticks are fed on immunized animals, they usually suffer from poor attachment, smaller blood-meal, and a direct damage to their gut and internal organs. A resistant host may display a combination of deleterious effects to ticks, this could be due to shared antigens in various tick tissues (Willadsen and McKenna, 1991). So far, immunization against ticks tissues provided only partial protection and in most cases ticks had to feed for some time before a significant damage was demonstrated. Thus, this method would not protect the immunized hosts from pathogens which are usually being transmitted during the early phase of tick's feeding. A better protection may be achieved by directing the host's immune response against the tick's gustatory receptors which are responsible for the initiation of blood feeding.

Compare with other blood-feeding arthropods, ticks are relatively slow feeders. Soft ticks (Argasidae) usually take 20-60 minutes to complete a blood-meal, while hard ticks (Ixodidae) require several days for feeding. The mouth parts of ticks had evolved for penetrating an animal skin and long term attachment to it. The mouth parts include two organs for cutting the host's

skin - the chelicera6e. The distal end of the chelicera has two movable digits, bearing a few sharp teeth. A few pores are found on the cheliceral digit. These pores are similar to cuticular openings of chemoreceptive sensillum in other arthropods. Indeed, histological and electrophysiological studies of the cheliceral digit in several tick species indicate that it bears the sensory receptors for initiation and maintenance of tick's feeding (Waladde and Rice, 1982). These investigators assumed that phagostimulating chemicals of the host's blood flow through the cheliceral digit pore toward a receptor membrane, where they induce feeding by binding to receptor protein(s). Galun and Kindler (1965, 1968) studied the feeding behavior of the soft tick Ornthodoros tholozani, using membrane feeding technique. They discovered that glutathion (GSH) or adenosine triphosphate (ATP), in combination with glucose, induce ingestion. Moreover, those ticks stop feeding within seconds following the removal of GSH from their diet (Galun, 1978). ATP also increased the frequency of sucking by the hard tick Boophilus microplus (Waladde and Rice, 1982). ATP stimulate feeding in the bug Rhodnius prolixus (Friend and Smith, 1982) and the mosquito Aedes aegypti (Galun et al.,1985). Some analogues of ATP had even greater affinity to the mosquito gustatory receptor than ATP, and novabiocin blocked ATP binding to the gustatory receptors of Aedes (Galun et al. 1985). In this research we evaluated the possibility of interfering with the feeding of ticks by blocking its cheliceral chemoreceptor with specific host's antibodies. If the tick's feeding response will not be blocked completely, anti-chemoreceptor antibodies will be imbibed during the blood meal and may block homologous receptors in the tick's gut or hemocoel.

Artificial feeding of soft ticks through various membranes had been used for laboratory mass rearing (Elkins and Lane, 1988; Jones et al., 1988; Mango and Galun, 1977; Osborne and Mellor,

1985), and in studies of disease transmission (Hokama et al., 1987; Wirtz and Barthold, 1986), nutrition (DeMeillon and Goldberg, 1947), and feeding behavior (Galun and Kindler, 1968; Tarshis, 1958). Waladde et al. (1979) partially fed the hard tick, B. microplus on artificial membrane. A unique system for a long term membrane feeding of hard ticks has been developed as part of this project (Waladde et al. 1991).

The evaluation of our proposed novel approach to control ticks was carried in several directions:

1. Studying the effect of known phagostimulants on the feeding response of various species of ticks.
2. Studying the effect of chemical analogues of known phagostimulants on ticks' feeding with the aim of finding an efficient ligand to be used for localization (see Sanser and Vanhouten, 1987) and isolation of cheliceral digit gustatory receptor protein(s).
3. Extraction and characterization of proteins from tick chelicerae.
4. Vaccination of cattle with R. appendiculatus cheliceral extract and evaluation of its anti-tick protection.
5. studying the effect of mechanical damage to ticks' chaliceral digit on their feeding response.
6. studying the potential damage to ticks internal organs from host's antibodies against the gustatory receptors.

METHODS AND MATERIALS

Artificial Feeding Apparatus

SOFT TICKS: A small glass beaker (25ml) was used to hold 5ml of diet solution and support the tick feeding chamber. The feeding chamber consisted of a hard plastic cylinder (r=1.2cm; h=5.5cm) with a membrane attached to its lower opening, and a soft plastic ring around its upper opening. The cylinder was inserted to the beaker and its level was adjusted to touch the diet by resting the plastic ring on the rim of the beaker. Ten beakers were held by a metal stand and their bottom was submerged (about 2cm) in a hot water bath. Because the ticks were tested after months of inactivity, we allowed 90 minutes for each feeding experiment. Ticks were considered "fed" if they more than doubled their unfed weight. Soft ticks were weighed immediately after repletion, before they excrete the coxal fluid.

For determining the optimal feeding conditions we studied the effects of the following factors while using defabrinated rabbit blood as diet:

a. **TYPE OF MEMBRANE**

Baudruche - this mambrane is made of ox intestine and supplied by Long & Long Co., Belleville, NJ, U.S.A.

modified Baudruche - the above mambrane was strengthened by applying 2 coats of diluted adhesive cement and rabbit hair was glued on one side (similar to the procedure reported by Waladde et al. 1991)

Parafilm - produced by American Can Co., Greenwich, CT, U.S.A.

silicone - produced in our laboratory using clear silicone sealer gel and polyester organdy cloth as described by Davis et al. (1983)

latex - a piece of disposable surgery gloves was used

b. **TICKS' AGE** nymphs stage II-IV

c. **HUNGER STATE** 1-7 months post feeding

d. **EFFECT OF GROUP FEEDING** individuals vs. 10 ticks per vial

e. **TEMPERATURE OF DIET** 25-40^{0}C

f. **VISCOSITY OF DIET** blood and plasma vs. aqueous solutions

g. **ADDITION OF HOST AND TICK STIMULI** rabbit and cattle hair and fresh ear wax; soft tick's coxal fluid and hard tick's fecal pellets.

HARD TICKS: similar to the equipment and procedure described by Waladde et al. 1991. Unfed R. appendiculatus and prefed R. sanguineus were fed on blood using this method.

Experimental Diet

Physiological saline (0.85% NaCl) served as the basis for all diet solutions and as a control diet. Test compounds (GSH, ATP, etc.) were diluted in saline containing glucose (1 mg/ml) and 10^{-3}M sodium bicarbonate (referred to as SGB).

Ticks

Ornithodoros tholozani- collected in a cave in Israel and kept in the laboratory for over 40 years. O. moubata - collected in Kenya National Park, Nairobi, and kept in the laboratory for over 15 years. Rhipicephalus sanguineus collected in Jerusalem and kept in the laboratory for one year. R. appendiculatus obtained from ICIPE colony in Kenya. All ticks were reared on rabbits and when not feeding, ticks were kept at 25^{0}C and 80% RH.

EXTRACTION AND CHARACTERIZATION OF PROTEINS FROM TICKS The distal part of the chelicerae from both male and female R. appendiculatus were cut in cold (4^0C) phosphate buffered saline (PBS, pH 7.2) containing 1 mM EDTA and 1 mM of the protease inhibitor PMSF. Several extraction methods (sonication, homoginization, addition of detergents) were compared as to the amount of protein extracted by each one. Protein extracted were studied using a 10% SDS-PAGE.

IMMUNIZATION STUDIES WITH CHELICERAL EXTRACT chelicerae from both male and female R. appendiculatus were cut and homogenized. A water soluble extract of this homogenate was used for immunizing 4 cows. The immunized animals were infested with larvae of ticks to asses the resistance level gained by immunization.

EFFECT OF MECHANICAL DAMAGE TO TICKS' CHALICERAL DIGIT ON THE FEEDING BEHAVIOR OF THE TICKS A scanning electron microscope was used for studying detailed morphology of the O. tholozani cheliceral digit. Female ticks of this species were immobilized (with the aid of Parafilm strips) and their chelicerae were exposed to 10-20 pulses of UV LASER beam. The LASER beam was guided and focused through a glass micropipette with the aid of stereomicroscope. The pulse was generated by EMG (model 500) excimer LASER (Lambda Physik) and it had energy density of 350 mj/cm2. Forty eight hours after ablation, the feeding behavior of treated ticks was compared to that of sham-treated ticks, using a live rabbit as a host. Details of the effect of ablation on cheliceral digit were studied with the aid of a scanning electron microscope.

PASSAGE OF SPECIFIC HOST'S ANTIBODIES INTO THE HEMOCOEL OF TICKS

A radioimmuassay was used to measure the concentration of specific (antiovalbumin) host IgG in the hemolymph of ticks following a blood meal on an immunized rabbit. The methods and materials used in this study had been described by Ben-Yakir (1989).

RESULTS

Artificial Feeding

Using silicone and modified Baudruche membranes gave the best and most uniform feeding of soft ticks. The original Baudruche membrane leaked, especially if several ticks fed together. When ticks fed on latex and dental gum they were unable to detach themselves upon repletion.

We decided to use nymphs of stages III because this group was large and most uniform in weight, and they were easy to handle.

The feeding response of both species of soft ticks was highest 1-2 months post feeding, then it decreased about 30% and stayed at this level from 3-5 months post feeding. At 6-7 months the feeding response increased again. In most of the feeding assays we used ticks that were 5-7 months post blood meal.

When ticks fed in groups of 10, usually they attached faster compared with ticks fed individually. More leaks occurred when ticks fed in groups. Therefore, in the second part of this project ticks were always assayed individually.

Figure 1 describes the feeding response of O. moubata at different diet temperatures. The optimal feeding response was obtained at diet temperature of 35^0C. At temperature of 40^0C ticks moved rapidly on the membrane without feeding, suggesting that the surface temperature was too high for them.

Table 1 describe the effect smearing ear wax on a silicone membrane on the attachment and feeding response of soft ticks. Cattle ear wax and tick feces worked together to enhance attachment and feeding of R. appendiculatus (Waladde et al., 1991). The hard ticks R. appendiculatus fed to repletion using the artificial feeding apparatus. This is the first reported success in feeding hard ticks artificially from flat stage to repleation. The ticks feeding behavior was very similar to natural feeding on animals and they laid down a cement cone. Prefed R. sanguineus attached and fed well for the 48 hrs they were followed.

EXPERIMENTAL DIET

The response of soft ticks to various diet components is given in **tables 2-4**. The effect of glucose on the feeding response of O. moubata in various diet solutions is given in **table 5**.

EXTRACTION AND CHARACTERIZATION OF PROTEINS FROM TICK CHELICERAE

Homoginization and sonication of cheliceral digits, in the presence of detergent, yielded the highest amount of proteins (**table 6**). Analysis of the extracted proteins by SDS-PAGE is given in **figure 2**.

IMMUNIZATION STUDIES WITH CHELICERAL EXTRACT Dr. Mongi and Prof. Dipiolo from ICIPE reported (personal communication with Prof. Galun) that tick larvae were reluctant to attach to 4 cows that had been immunized with cheliceral extracts. Based on these promising 8results further biochemical characterization and immunological studies of the cheliceral antigen were carried.

However, attempts to repeat the induction of tick resistance with this antigen were unsuccessful. Much effort was put into understanding the reasons for the inconsistent results, but to no avails. For that reason the Kenyan researchers were reluctant to report the details of this study. These studies went beyond the official date for termination of this project, and that was the main reason for the delay in sending the final report.

EFFECT OF MECHANICAL DAMAGE TO TICKS' CHALICERAL DIGIT

Results of the feeding assay following the treatment of ticks with the LASER beam are given in **table 7**. Treated ticks took twice as long to attach to the rabbit compare with the control. Less than half of the ticks that had been treated with 10 pulses fed successfully. However, at the end of the feeding session the treated ticks gained about one quarter more weight than the control. Examination of the treated ticks with scanning electron microscope revealed serious damage to the chelicerae that had been exposed to the sharply focused LASER beam (**figure 3**)

PASSAGE OF SPECIFIC HOST'S ANTIBODIES INTO THE HEMOCOEL OF TICKS

Only a very small fraction of the imbibed host's IgG was detected in the hemolymph of O. moubata and R. sanguineus (0.15 and 5.0 ug/ml, respectively). No host's IgG was detected in the hemolymph of O. tholozani. More details that resulted from this project are reported in the attached publication by Ben-Yakir (1989).

DISCUSSION

The addition of rabbit hair and ear wax to Parafilm increased the rate of attachment and feeding. In the modified Baudruche mambrane rabbit hair is glued in the process of making it. The rough surface of both silicone and modified Baudruche membranes (compare to Parafilm, and latex) enhanced tick attachment. These membranes also had fewer leaks than others. One of the maim achievements of this project was the successful long term feeding of hard ticks on an artificial membrane. This is the first time that unfed adult ticks are fed on a membrane to repletion. The feeding system for hard ticks developed by Waladde (Waladde et al., 1991) opens up new possibilities for studies of tick physiology and their disease transmission.

While O. moubata fed on saline (about 10%) and SGB (34% in group and 66% for individuals), O. tholozani feeding on those diets was negligible. The faster attachment rates of groups (10 ticks per vial) was probably due to stimulations by fast responding ticks. However, the feeding rates in groups was lower than for individual ticks fed on the same diet (table 2 vs. tables 3 and 4). This observation suggest that under our experimental conditions ticks interfere with the feeding of each other. It is interesting to note that when O. tholozani attached to the membrane they almost always fed as well. For O. moubata the feeding rates were significantly lower than the attachment rates. Another difference in the feeding behavior of these ticks was that O. tholozani always detached from the membrane upon repletion, while a significant number of O. moubata ticks remain attached. In the groups of O. tholozani ticks were sometimes feeding of each other. It is clear that attachment and feeding times and rates, as well as the weight gain, are positively related to the stimulatory level of the diet. The relatively quick attachment of ticks when blood was offered (Tables 3 and 4)

suggests that some volatile phagostimulants diffused through the membrane. The addition of surface stimulants like ear wax improved the rate of tick attachment and feeding, but had little effect on their weight gain (Table 1). GSH 10^{-3}M was the most stimulating compounds for the soft ticks (Tables 1-4). ATP at a concentration of 10^{-3}M was mildly stimulating for O. tholozani but seem to inhibit the feeding of O. moubata (feeding rate lower than SGB). When ATP was given at a concentration of 10^{-5}M, the inhibitory effect disappeared. For O. moubata, glucose (at 1mg/ml) acted as an independent phagostimulant (Table 5). However, almost no synergistic interaction was noted between glucose and the other compounds tested. The feeding response of O. moubata is very
different from the response of O. tholozani (Galun and Kindler 1965, 1968). The ability of some soft ticks, like O. moubata in our study, to feed on solutions of a variety of unrelated compounds, makes it less probable to inhibit tick feeding by blocking a GSH specific chemoreceptor.
Few proteins were extracted from the chelicerae of R. appendiculatus (**figure** 2). Unfortunately, further characterization of these proteins was not done, because Dr. Kamanga-Sollo had to leave the project in the middle. The good protection against ticks which had been induced in cattle by immunization with the cheliceral extract (Dr. Mongi and Prof. Dipiolo personal communication with Prof. Galun) brought about more attempts for biochemical characterization of these antigens by another group of researchers. Unfortunately, no additional information was released by this group for the final report.
When studying the effect of mechanical damage to the cheliceral digit receptors, despite the use of a highly focused LASER beam, it was difficult to avoid damaging other parts of the digit (**figure** 3). Thus, we can not be sure that a damage to the

receptor was responsible for the poor feeding response of the treated ticks (**table** 7). The feeding rate of ticks that had been treated with a less focused beam (20 pulses), was the same as in the untreated group. Although treated ticks took long time to attach, their final weight gain was significantly higher than the control group. It is not clear how the ablation of chelicerae caused an increase of blood intake. It is possible that the detachment process was slowed down by the cheliceral damage.
The passage of host's antibodies into the hemocoel of the tick species used in this study was very low (Ben-Yakir, 1989). Thus, it is unlikely that internal recptores of GSH and ATP will be blocked by specific antibodies that will be ambibed by the tick, while feeding on an immunized host.

In conclusion, the goal of preventing tick attachment and feeding by blocking their gorgment response is still very attractive. Protection of domestic animals using this approach will prevent disease transmission by the ticks. As the outcome of this approach is not lethal to the ticks, but rather they are directed to alternative wild hosts, adaptation (resistance) of the tick population to this control measure will be slow. One basic problem is still standing in the way of this control method. The response of ticks to a variety of phagostimulants which may act alone or synergies each other, makes it necessary to characterize all the various chemoreceptores on the chelicerae.
Recent advances in molecular biology enabling us today to characterize proteins derived from a single cell. This technique may allow to amplify the receptor proteins of the cheliceral digits chemosensory cells. In turn, these genrtically engeneered proteins may be used for immunizing hosts and induce resistance against ticks.

PUBLICATIONS

1. Ben-Yakir, D. (1989). Quantitative studies of host immunoglobulin G in the hemolymph of ticks (Acari). Journal of Medical Entomology, 26: 243-246

2. Wallade, S.M., Ochieng', S.A. and Gichuhi, P.M. (1991). Artificial-membrane feeding of the ixodid tick, Rhipicephalus appendiculatus, to repletion. Experimental and Applied Acarology, 11: 297-306

3. Ben-Yakir, D. and Galun, R. (in preparation) Comperative study of ticks' feeding response to phagostimulants.

SCIENTIFIC VISITS

August-september 1986 - Professor R. Galun and Dr. Ben-Yakir visited Nairobi, Kenya. Objectives and work plan for the three years project were discussed and defined.

September 1988 - Dr. David Ben-Yakir visited Nairobi, Kenya for meetings and consultations with collaborating scientists.

August 1989 - Dr. David Ben-Yakir visited Nairobi, Kenya for meetings and consultations with collaborating scientists.

April 1990 - Dr. E.I.P. Kamanga-Sollo visited Jerusalem, Israel for meetings and consultations with collaborating scientists.

July-August 1990 - Dr. S.M. Waladde visited Jerusalem, Israel for a study using the laser facilities at Hadassah Medical School.

TABLE 1. The effect of adding fresh rabbit ear wax to a silicone membrane on the feeding response of soft ticks

Tick sp.	Diet	n	% fed		Weight gain (mg) mean±S.D.		Time (min) for first repletion	
					ear wax			
			+	-	+	-	+	-
O. moubata*								
	SGB	20	28	30	16+8	17+2	24	33
	GSH 10^{-3}M	40	83	70	15+6	14+5	16	17
	GSH 10^{-4}M	40	70	47	15+6	16+8	14	19
	Blood***	40	85	73	22+3	24+6	12	17
O. tholozani**								
	GSH 10^{-3}M	40	72	75	24+11	19+10		
	ATP 10^{-3}M	50	50	30	32+20	35+19		
	Blood***	20	92	65	31+10	35+15		

* nymphs with unfed weight of 6 mg, 6 months post blood meal, fed in groups of 10 ticks per vial

** nymphs with unfed weight of 7-10 mg, 6 months post blood meal, fed in groups of 10 ticks per vial

*** fresh defabrinated rabbit blood

TABLE 2. Feeding response* of soft ticks** to various diets fed through silicone membrane.

Diet	Tick species			
	Ornithodoros moubata		Ornithodoros tholozani	
	n	response (%)	n	response (%)
saline	240	8	40	0
SGB	240	34	80	1
GSH 10^{-3}M	140	56	200	40
GSH 10^{-4}M	160	48	140	37
ATP 10^{-3}M	80	23	100	18
ATP 10^{-4}M	100	33	40	0
ATP 10^{-5}M	120	47	-	-

* response was to at least doubled its unfed weight during a 60 min. feeding session

** nymphs III, 1-4 months post blood meal, fed in groups of 10 ticks per vial

TABLE 3. Feeding response of O. moubata* to various diets offered through an artificial membrane**

Diet	n	Mean attachment time (min)	% attached	% fed***	Weight gain (mg) (mean+S.D.)	Mean rate of feeding (mg/min)
water	17	25	94	0		-
saline	57	22	80	30	15+4	0.8
SGB	64	12	95	66	16+6	0.7
GSH 10^{-3}M	28	12	100	86	14+5	0.5
GSH 10^{-4}M	43	12	100	67	15+5	0.7
ATP 10^{-3}M	18	24	78	44	11+4	0.7
ATP 10^{-4}M	27	20	89	63	13+4	0.7
ATP 10^{-5}M	23	15	91	70	15+5	0.9
Blood****	20	9	95	85	21+3	1.1
Rabbit	31	9	100	100	22+6	0.6

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* nymphs with 5 mg unfed weight, 5-7 months post blood meal, a single tick per vial

** modified Baudruche membrane with rabbit ear wax

*** response was to at least double its unfed weight during a 90 min. feeding session

**** fresh defabrinated rabbit blood

TABLE 4. Feeding response of O. tholozani* to various diets offered through an artificial membrane**

Diet	n	Mean attachment time (min)	% attached	% fed***	Weight gain (mg) (mean+S.D.)	Mean rate of feeding (mg/min)
SGB	10	20	10	10	8+4	1.1
GSH 10^{-3}M	105	10	78	77	25+10	2.2
GSH 10^{-4}M	15	11	33	33	26+8	2.5
GSH 10^{-5}M	15	32	13	13	12+3	1.2
ATP 10^{-3}M	41	27	52	44	22+10	1.8
ATP 10^{-4}M	34	23	27	24	17+8	0.9
ATP 10^{-5}M	34	40	21	21	18+15	1.3
Blood****	28	4	100	100	31+17	2.1
Rabbit	24	9	79	63	28+13	1.5

* nymphs with 5 mg unfed weight, 5-7 months post blood meal, a single tick per vial

** modified Baudruche membrane with rabbit ear wax

*** response was to at least double its unfed weight during a 90 min. feeding session

**** fresh defabrinated rabbit blood

TABLE 5. The feeding response of O. moubata* to GSA and ATP in the absence and presence of glucose**

	without glucose		with glucose	
Diet	n	% fed	n	% fed
SB	53	13	64	66
GSH 10^{-3}M	34	95	28	86
GSH 10^{-4}M	33	40	43	67
ATP 10^{-5}M	33	67	23	70

* nymphs with 5 mg unfed weight, fed individually on artificial membrane.

** 1 mg/ml

TABLE 6. Amount of protein* extracted from the cheliceral digits of Rhipicephalus appendiculatus using various extraction methods

	Extraction method**				
Source	H	S	HS	HD	HSD
Female	130	90	250	260	300
Male	110	70	200	250	250

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* ug/ml, detarmined by Bio-Rad protein assay

** 200 cheliceral digit per ml of diluent were used

H = homoginization

S = sonication at 300 watts pulses for 1 min.

HS = homoginization and sonication

HD = homoginization in presence of detergents

HSD = homoginization and sonication in presence of detergents

TABLE 7. Feeding response of **O. THOLOZANI** ticks ablated with Excimer laser

Treatment	n	% Feeding	% Wt. gain	Time taken to attach (hrs.)
Control	14	100	406	< 0.5
10 pulses	17	41	541	> 1.0
20 pulses*	19	100	570	> 1.0

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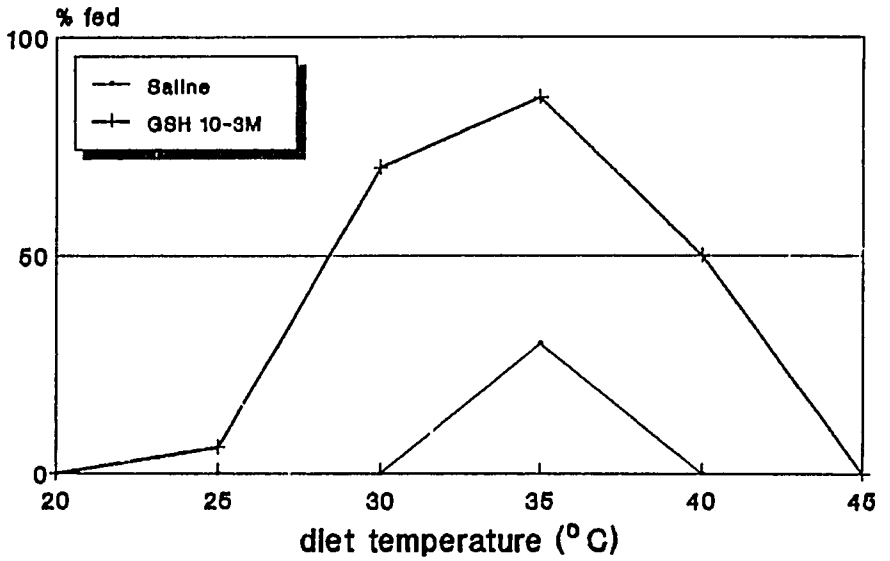
* Laser beam not sharply focussed, thus, ablation not as efficent as expected

Figure 1. The feeding response of *O. moubata* to diet solutions at various temperatures



nymphs, 6-8 mg unfed weight, one month post feeding

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