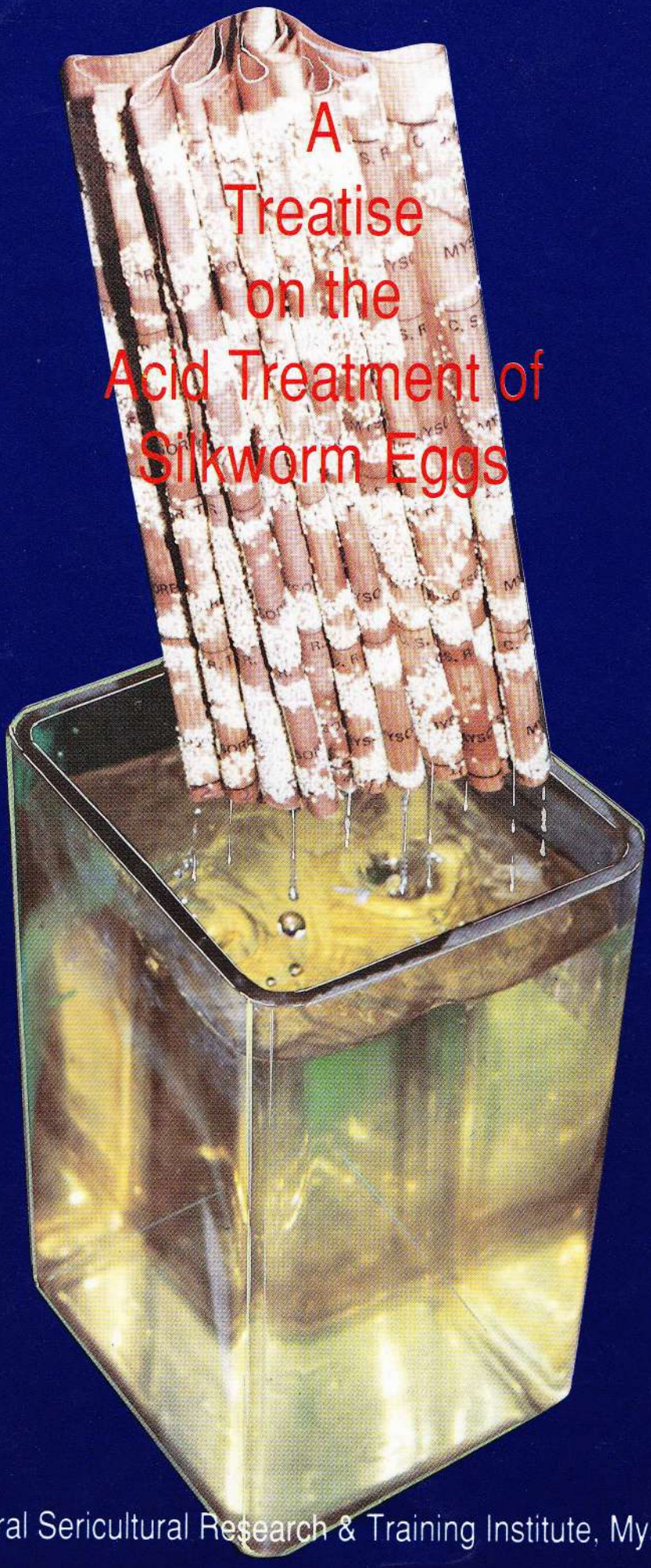
A Treatise on the Acid Treatment of Silkworm Eggs



Central Sericultural Research & Training Institute, Mysore

A Treatise on the Acid Treatment of Silkworm Eggs

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June, 1990
5,000 Copies

Published by

Dr. R.K. Datta
Director
Central Sericultural Research & Training Institute
Central silk Board - Govt. of India
Srirampura
MYSORE - 570 008

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Printed at

M/s. Jwalamukhi Job Press
Bangalore - 560 004

Preface

Acid treatment of silkworm eggs to block diapause in uni/bivoltine eggs for instant and any time hatching has gained great popularity due to its easy method of operation and extremely dependable results. Even then, failures take place because of the method not being understood/adopted properly.

So far, in India, mostly multi x bivoltine eggs were used for commercial rearing which involve no acid treatment. But the recent attempt to utilize bivoltine x multivoltine layings has broadened the scope of acid treatment. In addition, a large programme is being launched to increase the bivoltine cocoon production which will also involve the acid treatment of the eggs to be reared.

Until now, acid treatment was mostly restricted to Government Silk Farms and Grainages. But a time has come when private graineurs should also take up the acid treatment of eggs. It is in this context, this booklet is being published to bring home the correct methods of acid treatment with precautions to be taken at every step which are often overlooked leading to poor results.

Knowledge of acid treatment remains incomplete without understanding the developmental sequence in the eggs. So, in Part-II of this booklet, physiology of the egg before and after acid treatment has been discussed.

It is hoped that the booklet will be useful to all those connected with seed technology and commercial seed production.

Authors

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Introduction

The diapause, in insects, is a method of overcoming unfavourable period caused either by physiologically unfavourable conditions or non-availability of food. For the silkworms of the temperate region, both these conditions will prevail during winter and thus they have developed the diapausing (hibernating) character to overcome this period.

The phenomenon of diapause is environmentally, physiologically and genetically controlled. Acid treatment is just a method to change the physiology by blocking certain activities and inducing several new biochemical reactions for the continuous development of the eggs.

Diapause eggs are more customarily referred to as hibernating or bivoltine eggs, while the non-diapause ones as non-hibernating or multivoltine eggs.

Left to themselves, the diapausing eggs do not hatch on incubation. However, it is possible to make them hatch artificially by providing various physical or chemical stimuli. The various physical and chemical agents/methods by which it is possible to block or terminate the diapause are given in Table-1. Though several artificial methods have been devised, the immersion of diapausing eggs in Hydrochloric acid (HCl) has been the best choice and most widely adopted technique for immediate hatching, from the technical as well as practical points of view, both at the laboratory as well as commercial levels of bivoltine seed production.

To bring a shift from diapause to non-diapause type, changes in the incubation schedule like shorter exposure to light and low temperature during incubation, changes in the rearing conditions like continuous high temperature rearing, low temperature during early stages and high temperature during the late stages, cocoon preservation at high temperature, coupled with selection, are essential.

The scope of this booklet however has been restricted to methods of acid treatment of diapausing eggs for artificial hatching and related changes. This has been dealt with in two parts, Part - I dealing exclusively with the methods of Hydrochloric acid treatment and Part-II with the physiological changes associated with it.

Table 1 **Agents/Methods of Artificial Hatching**

Physical Stimulants	*Chemical Stimulants*
1. Artificial overwintering by subjecting to low temperature*	Hydrochloric acid *
2. Immersion into hot water	Nitric acid
3. Electrical stimulation using high voltage	Sulphuric acid
4. Friction by feather/hard brush	Aqua - regia
5. Enforcement of high atmospheric pressure	Acetic acid
6. Use of ultra high frequency vibrations	Sodium chloride
7. Use of radiated sunrays or exposure to sun-shine	Hydrogen peroxide
8. Use of ultra purple rays/ultra short waves	Enzyme treatment
9. Exposure to Oxygen	Ozone treatment

* *Most commonly used methods/agents*

Part I

Methods of Acid Treatment

Selection of the acid

For consistent good results, stronger inorganic acids have been found in general, better than organic acids. Amongst the inorganic acids again, Nitric acid, Sulphuric acid and Aqua regia (a combination of Nitric acid and Hydrochloric acid in a ratio of 1:3) have been found to be too strong for use and difficult to handle. Comparative figures with regard to concentration (percent by weight) and densities of different inorganic acids are given in Table 2.

TABLE 2 Specific gravity and concentration of different acids

Name of the acid	Concentration of acid at saturation (%)	Density at 20°C
Hydrochloric acid (HCl)	40	1.1980
Nitric acid (HNO_3)	100	1.5129
Sulphuric acid (H_2SO_4)	100	1.8305

Evidently, Nitric acid and Sulphuric acid are distinctly stronger in nature and pose handling problems. Thus, in practice, HCl alone is being exploited for the acid treatment of silkworm eggs both at commercial and laboratory levels. It has been found to be the safest and best medium for treating diapausing eggs to obtain maximum hatching.

Hydrochloric acid is a by-product obtained while manufacturing soda-ash and is subsequently purified to the required levels. Pure HCl is colourless and emanates strong and obnoxious fumes. The yellow colour of certain commercial HCl products is often caused by iron and other impurities. It fumes in moist air and is very easily soluble in water. It has strong acid taste with pungent irritating odour and attacks the mucous membrane upon inhalation.

HCl is marketed as a solution containing mostly 28 to 36% Hydrogen Chloride by weight and is commonly known as *concentrated HCl*. The concentration and specific gravity of HCl are correlated as indicated in Table 3.

TABLE 3 Relationship between Specific Gravity and Concentration of HCl

Specific gravity of acid at 20°C	Concentration of acid (%)
1.198	40
1.195	39
1.190	38
1.180	36
1.170	34
1.160	32
1.150	30
1.140	28
1.130	26
1.120	24
1.110	22
1.100	20
1.095	19
1.090	18
1.085	17
1.080	16
1.075	15
1.070	14

There are three grades of HCl available in the market namely Analytical , Laboratory and Commercial; one being cheaper than the other in descending order. For acid treatment of silkworm eggs the commercial acid is used, provided its properties conform to the specifications described above and the total impurities do not exceed one percent. Care should, however, be taken, to see that the acid is not too yellow which may either contain more impurities or is sufficiently old. The commercial grade HCl is generally available in a range of 1.150 to 1.180 specific gravity.

In view of its corrosive nature, while handling the acid, containers made of ceramic, glass, plastic or synthetic resins should be used.

Preparation of the HCl acid solution

Acid of required specific gravity is usually prepared by diluting the concentrated acid with the addition of approximate quantity of water. Subsequently, the resultant solution is verified with a hydrometer and corrected either by adding water or acid so as to obtain the required specific gravity. However, instead of resorting to this approximation procedure, which may lead to wastage, acid of desired volume and specific gravity can be comfortably prepared by using the following formula :

Quantity of concentrated HCl to be taken :

$$\frac{\text{(Desired Specific Gravity - 1.00) x (Volume of acid required in ml.)}}{\text{(Available Specific Gravity - 1.00)}}$$

(1.00 represents specific gravity of water)

The formula gives the quantity of concentrated acid (available acid), to which water has to be added to prepare the required volume of particular specific gravity.

Example : To prepare 15 litres of 1.075 Specific Gravity HCl from 1.160 Specific Gravity acid

$$= \frac{(1.075 - 1.00) \times 15{,}000}{(1.160 - 1.00)}$$

= 7030 ml. of acid + 7970 ml. of water together gives 15 litres of 1.075 specific gravity acid.

A ready reckoner to help in preparing one litre of 1.075, 1.100 and 1.110 specific gravity acid from concentrated acid of specific gravity ranging between 1.150 to 1.180 is provided in Table 4.

TABLE 4 **Ready reckoner for preparing one litre acid of desired specific gravity**

(Quantity in ml)

Specific Gravity of available HCl	1.075 HCl		1.100 HCl		1.110 HCl	
	Water	Acid	Water	Acid	Water	Acid
1.150	500	500	333	667	267	733
1.155	516	484	355	645	290	710
1.160	531	469	375	625	312	688
1.165	545	455	394	606	333	667
1.170	559	441	412	588	353	647
1.175	571	429	429	571	371	629
1.180	583	417	444	556	389	611

Generation of heat during acid preparation :

During the process of acid preparation, when water is added to the concentrated acid, due to an exothermic reaction, heat is liberated which significantly enhances the temperature of the prepared solution. The extent of the rise in temperature depends on the quantum of water added for dilution and may rise by 8°C to 10°C. To outmanoeuvre such variations, it is preferable to prepare the acid solution atleast 5 to 6 hours in advance or even cure it by keeping overnight so as to enable the acid to attain the room temperature and also for the exothermic reaction to subside. The acid solution thus obtained, is verified using a precision hydrometer of 1.050 to 1.100 range.

Relationship between temperature and specific gravity of HCl :

It is pertinent here to indicate that there exists a relationship between the temperature and the specific gravity of acids. As the temperature rises, the specific gravity decreases and vice-versa. Thus the specific gravity should be necessarily recorded at a lower temperature, preferably between 15°C to 25°C, where acids are more stable. Evidently, while recording the specific gravity it is but indispensable to consider the temperature of the acid solution, rather than room temperature. In view of this decisive relationship, a standardised (Table 5), indicating the acid specific gravity at a stable temperature of 15°C and the corresponding variations at different acid temperatures, is provided for accurate measurement. Unless otherwise mentioned, specific gravity of HCl relates to 15°C temperature.

Formalin treatment of eggs to enhance adhering efficiency :

The mother moth while ovipositing provides a thin film of gluey substance, secreted from accessory glands on the under surface of the eggs which enables them to stick to the egg card. If these egg cards are dipped as such into the acid, it tends to dissolve the gluey substance. As a result, large quantities of the eggs get detached during acid treatment and subsequent washing in water.

To overcome this problem, the egg cards, prior to acid treatment, are soaked in 2% formalin solution for 15 minutes. Formalin acts as a fixative agent which increases the adhering capacity of the eggs to the cards. Besides it helps in surface sterilization of eggs against different disease causing pathogens and in removing the wastes deposited on the cards.

The egg cards thus dipped in formalin solution are either directly taken for drying or subjected to washing in water. Washing in water helps in eliminating the irritating smell of formalin. In either case, the egg cards are dried in shade.

It is also a practical feasibility that formalin solution is used for dilution instead of water, at the time of acid preparation. In such a case egg sheets are directly dipped in the acid for the purpose of treatment. This is a simple method which is already in vogue in other countries. This method saves time, as separate soaking in formalin solution and subsequent drying process, are curtailed.

A note of precaution is that since formalin is very volatile in nature, a certain amount of formalin may be required to be supplemented after a few treatments judging from the condition of egg dropping.

TABLE 5 Relationship between temperature and specific gravity of HCl (Correction Table)

Temperature		Specific Gravity		
°F	°C	1.0750	1.100	1.110
59	15	1.0750	1.100	1.110
60.8	16	1.0746	1.0995	1.1095
62.6	17	1.0743	1.0990	1.1090
64.4	18	1.0739	1.0986	1.1086
66.2	19	1.0736	1.0982	1.1081
68.0	20	1.0732	1.0977	1.1076
69.8	21	1.0729	1.0973	1.1071
71.6	22	1.0725	1.0968	1.1066
73.4	23	1.0722	1.0964	1.1062
75.2	24	1.0718	1.0959	1.1057
77.0	25	1.0715	1.0955	1.1052
78.8	26	1.0711	1.0950	1.1047
80.6	27	1.0708	1.0946	1.1042
82.4	28	1.0704	1.0941	1.1038
84.2	29	1.0701	1.0937	1.1033
86.0	30	1.0697	1.0932	1.1028
87.8	31	1.0691	1.0928	1.1023
89.6	32	1.0690	1.0924	1.1018
91.4	33	1.0687	1.0920	1.1014
93.2	34	1.0683	1.0915	1.1009
95.0	35	1.0680	1.0910	1.1004
96.8	36	1.0676	1.0906	1.0999
98.6	37	1.0673	1.0901	1.0995
100.2	38	1.0670	1.0897	1.0990
102.2	39	1.0666	1.0892	1.0985
104.0	40	1.0663	1.0888	1.0981
105.8	41	1.0659	1.0883	1.0976
107.6	42	1.0656	1.0879	1.0971
109.4	43	1.0652	1.0875	1.0966
111.2	44	1.0649	1.0870	1.0962
113.0	45	1.0645	1.0866	1.0957
114.8	46	1.0642	1.0861	1.0952
116.6	47	1.0638	1.0857	1.0948
118.4	48	1.0635	1.0853	1.0943
120.2	49	1.0632	1.0848	1.0938
122.0	50	1.0628	1.0844	1.0934
59.0	15	1.0750	1.100	1.110

Note : The readings can be rounded off to the nearest third digit

Preparation of formalin solution :

Formalin under saturation is available at 40% while commercial grade formalin contains 36 to 38% formaldehyde. Formalin dilution to required levels is conducted by adopting any of the two following procedures :

a) No. of parts of water to be added for each part of formalin :

$$\frac{\text{Original Concentration of formalin - Required Concentration}}{\text{Concentration required}}$$

Example : To prepare 2% formalin solution where orginal concentration of formalin is 36%

$$= \frac{36 - 2}{2} = \frac{34}{2} = 1 : 17$$

i.e. one part of formalin has to be mixed with 17 parts of water

Thus, for 1 litre $= \frac{1000}{(17+1)} = 55.55$ ml. of formalin + 944.45 ml. of water have to be mixed.

b) To prepare desired volume of formalin of required strength:

$$\frac{\text{Concentration of formalin required} \times \text{Volume required in ml}}{\text{Original concentration of formalin}}$$

If the above mentioned example is worked out i.e. one litre of 2% formalin from 36% concentrated formalin

$$= \frac{2 \times 1000}{36} = \frac{2000}{36} = 55.55 \text{ ml}$$

To this 55.55 ml. of formalin, water is added to make-up to the desired volume of one litre i.e., 55.55 ml. of formalin + 944.45 ml. of water or 55 ml formaline and 945 ml. of water

Correct age of silkworm eggs for acid treatment

The sensitivity of HCl treatment is very much dependent on the age of the eggs. There is a critical period in the embryonic morphogenesis during which the treatment is most effective. The effectiveness of treatment is reflected in hatchability.

Fertilization is external in silkworm. The process of syngamy i.e. the fusion of sperm nucleus and egg nucleus takes place about 2 hours after the deposition of the eggs by the mother moth. The cleavage process occurs between 3 to 10 hours after oviposition with blastoderm appearing between 10th and 15th hour and the further embryonic differentiation from 15th hour onwards (at oviposition and preservation temperature of 25°C and RH 75 ± 5%).

One to ten hour old eggs are highly sensitive and if treated, the treatment turns out ineffective as many of them die at the early developmental stage leading to a poor hatching performance. If the treatment is conducted after 15 hrs. of oviposition, effective hatching can be obtained. However, the best time for acid treatment falls between 20 and 24 hrs. after oviposition, when the embryo has reached the *germband stage* and is in the process of becoming an independent embryo. Externally no symptoms are noticeable, as the eggs still present the original egg colour, with no morphological changes.

While these developmental stages are true to ideal situations, the pace of embryonic development is always regulated by the oviposition and preservation temperatures. Higher the temperature, faster is the rate of development and lower the temperature slower the embryonic development. In light of this precarious strategy, suitable time for conducting acid treatment has to be necessarily decided in relation to oviposition/preservation temperature. The oviposition temperature and appropriate time for acid treatment are given in Table 6.

TABLE 6 Oviposition and preservation temperature and suitable time for acid treatment

Temperature from oviposition to commencement of treatment	*Appropriate time for acid treatment (Age of the egg in hours)*
20 ± 1 °C	25 - 35
24 ± 1°C	20 - 24
27 ± 1°C	17 - 22
29 ± 1°C	15 - 20

The above information serves as a broad guideline to decide the time factor. However, in cases where violent temperature fluctuations are noticed during oviposition and subsequent handling operations, colour of the eggs may be taken into account to monitor the treatment time.

Freshly laid eggs are generally pale yellow or dark yellow in colour. In diapausing fertilized eggs, after a certain stage of development, the eggs begin to acquire a brownish colour. This is due to the appearance of a specific pigment known as *Ommochrome,* in the serosal cells, indicating the first phase of diapause initiation. It is always safe to carry out the treatment before this colour manifestation. However, if the eggs begin to present a faint brownish or pinkish tinge, they are immediately treated without any further delay. Such a practice has to be discouraged as delayed treatments may lead to irregular and ineffective hatching.

Eggs treated after 48 hours of oviposition result in bad and irregular hatching, while the eggs treated after 3 days after oviposition will have less than 10% hatching.

Determination of the age of eggs :

In any commercial seed production unit, as a matter of fact, 3 to 4 hours of mating duration is allowed to stimulate oviposition in the female moth. The ovipositing moths adopt a circadian rhythm of ovipositional pattern with two distinct peaks of egg laying - the first peak before and after every dusk and the second one between every midnight and dawn. Between the two peaks, the first one accounts for 80 - 90 % of the eggs deposited while a relatively small percentage is shared by the second peak. This being the oviposition pattern, 8.00 p.m. is universally regarded as the zero hour for the calculation of the age of the eggs, as majority of the eggs are deposited between 4 p.m. and 10 p.m. Basing on this, 20 hours or 24 hours developmental period is calculated.

The moths missing the first peak partly or completely will lay eggs more actively during 2nd peak (i.e., between midnight and dawn). In such layings, the age of eggs will not be correct for acid treatment. Hence, a few layings now and then behave irregularly, eventhough treated accurately.

It is relevant to mention that a pairing duration of six hours and above enables the moth to deposit maximum number of eggs within two or three hours of separation. The natural circadian rhythm is disturbed and the oviposition pattern is substantially altered wherein the first peak may result around 5 p.m. to 7 p.m. with little deviation in the second peak. In this case also, there will not be much difference if the zero hour is calculated from 8 p.m. However, in specific instances, the zero hour may be appropriately calculated depending on the degree of deviation from the normal pattern.

Common methods of acid treatment

There are two most popular methods of treating the diapausing type of silkworm eggs, to block their diapause and eventually make them to hatch like their non-diapausing counterparts. Such acid treated eggs are termed as *artificial non-diapause eggs.* The two methods are (1) Hot Acid Treatment (2) Cold Acid Treatment.

Hot Acid Treatment

It is so designated for the reason that treatment of eggs is performed while the acid is hot. It is conducted with HCl of specific gravity of 1.075 (recorded at 15°C) with 15% concentration and heated to 115°F (46°C) with a dipping duration ranging between 4 and 7 minutes.

a) Heating of acid :

Since the acid is corrosive in nature, only glassware or plasticware is used for handling it. It is heated indirectly by keeping the acid container inside the hot water, maintained in a hot water bath. However, if acid resistant heating coils are available, acid can be heated up directly.

A hot water bath made of fibre glass or acid resistant material is generally used for maintaining the hot water. It will have thermostatic control to effectively govern the temperature. Incorporation of a contact thermometer facility enables to have more precision and accuracy which automatically triggers off the heating device, whenever the temperature rises beyond the arranged level. A stirrer fabricated in the trough uniformly spreads the temperature of water. By manipulating the contact thermometer or the thermostatic control, a higher temperature of 125°F to 130°F (52°C to 55°C) is fixed so that the water acquires this particular temperature. The acid container is placed in the bath for indirect heating. The hot water radiates the heat through the acid container and maintains the desired temperature of 115°F constantly. Since the hot water bath is used for conducting the treatment, it is customarily called the *Acid Treatment Bath.*

b) Dipping duration

The eggs of different silkworm breeds respond to different dipping durations more effectively. For practical convenience, as such, three broad categories of the durations can be made :

Races	Immersion Time (in minutes)
European type	6 - 7
Japanese type	5 - 6
Chinese type	4 - 5

Though these broad categories serve as a guide line, it is always worthwhile to work out a specific dipping schedule to the individual silkworm breed to obtain best hatching results. This is accomplished by conducting a few trials effectively.

For the popular bivoltine races of South India such specific treatment schedules have been worked out which are given in Table 7.

TABLE 7 Acid treatment schedule for some of the popular bivoltine races of south India

Name of the breed	Type	Optimum range of dipping duration (in minutes)
KA	Chinese	4 - 5
NB7	Chinese	4 - 5
NN6D	Chinese	4 - 5
CC1	Chinese	4 - 5
CA2	Chinese	4 - 5
NB4D2	Japanese	5 - 6
NB18	Japanese	5 - 6
PCN	Yellow Bivoltine	4 - 5

While conducting the hot acid treatment the following precautions are to be essentially taken care of :

a Dipping duration is accurately followed as relevant to a particular breed.

b Specific gravity of the acid is precisely recorded.

c The temperature is accurately maintained.

d The heated acid is thoroughly mixed prior to the slipping of the egg cards into the container.

e For appropriate acid stimulus the egg cards/ loose eggs are frequently moved to enable uniform and proper exposure of the eggs to acid.

f Immediately after the treatment, the egg cards/loose eggs are thoroughly washed in running water leaving absolutely no acid trace.

g The treatment is resorted to only when the eggs have accomplished a minimum of 20 hours development i.e. around 4.00 p.m. of the next day of oviposition. Younger age eggs do not withstand the dual acid and temperature shock.

Advantages

1 Immersion period being less (only 4 to 7 minutes), a large quantity of eggs can be treated in a comparatively shorter period.

2 Since the specific gravity and concentration of the acid are lower and the dipping period is shorter, this treatment does not affect the non-diapausing eggs/layings.

3 Quantity of concentrated acid required is relatively less.

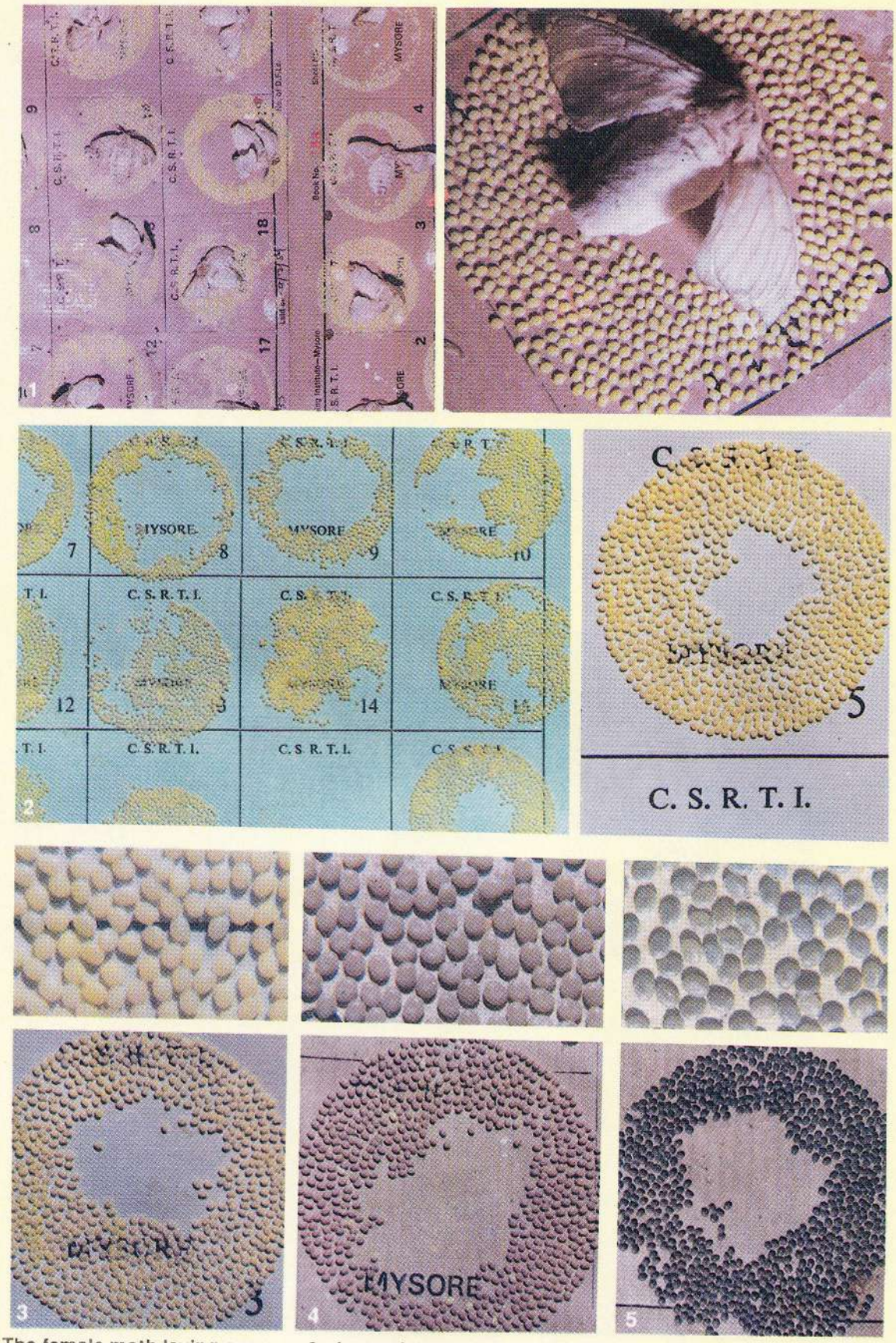
4 It is reported that the hot acid treatment controls Pebrine disease (Fujiwara & Kagawa-1984).

Disadvantages

1 It is not suitable for younger age eggs.

2 Demands additional infrastructural facilities for heating the acid.

3 Time of treatment being very short,one has to be quite alert and conduct the treatment with utmost precision and care.



1. The female moth laying eggs. 2. Just oviposited bivoltine (diapausing) eggs
3. 20 hours old diapausing eggs. 4. Acid treated eggs. 5. Hibernated (diapause established) eggs.

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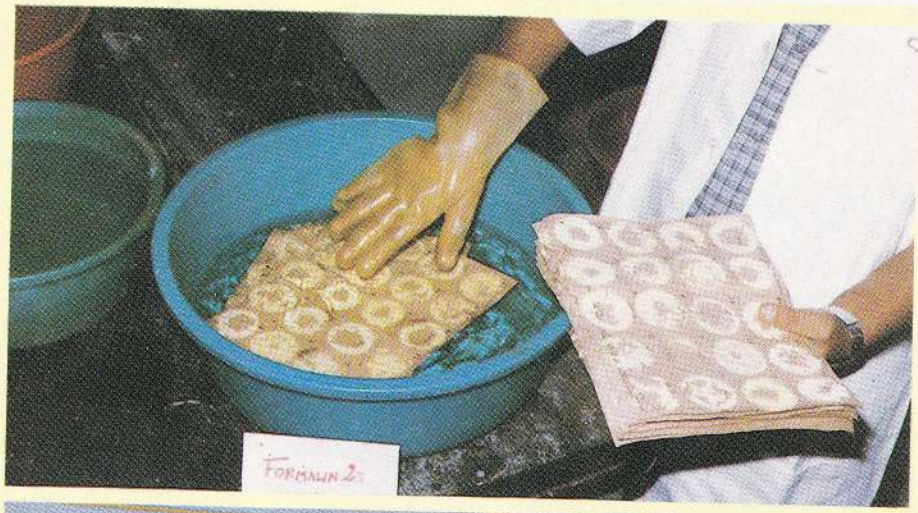

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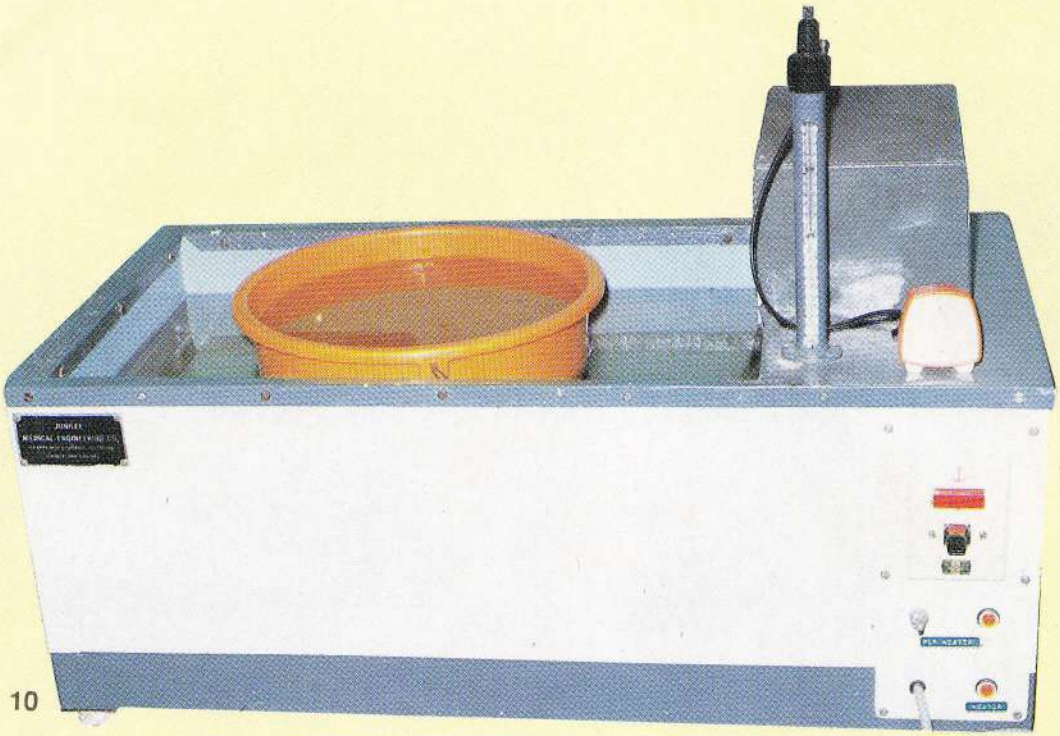
6. Hydrocloric acid (Fresh-colourless; old-yellow and used-turbid & brownish) 7. Preparation of HCl
8. Surface sterilization of silkworm eggs in 2% formalin 9. Drying of formalin sterilized egg sheets.



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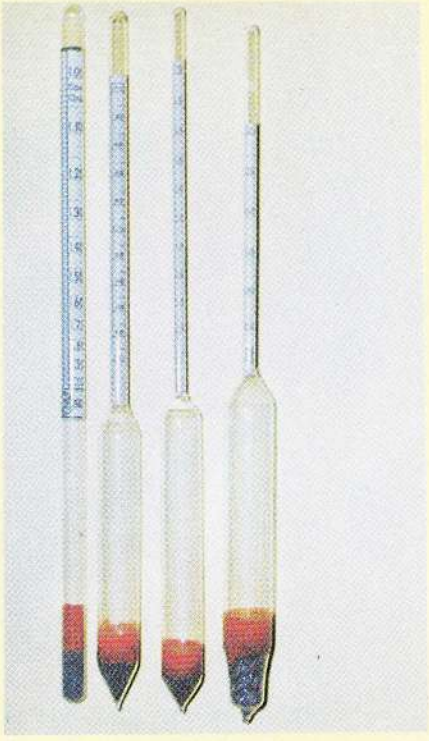

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10. The hot acid treatment bath.
11. Make-ready (stitching) of egg sheets for acid treatment.
12. Egg sheets ready for immersion.
13. Hydrometers of different ranges - (a) 1.000 to 2.000; (b) 1.000 - 1.200; (c) 1.000 - 1.100 and (d) 1.050 to 1.100.

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14. Verification of acid temperature prior to hot acid treatment.

15. Verification of acid temperature during the course of hot acid treatment.

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16. The cold acid treatment process - different steps.

17

18

17. The washing of egg sheets with running water in glaze tiled tanks in step-down arrangement.
18. Hot acid treatment of loose eggs.

19. The demonstration - cum - training for licensed silkworm seed producers.

4 Unfertilized eggs do not get crumbled, making their separation difficult during the loose egg preparation.

5 Continuous emanation of acid fumes may affect the health of the technicians conducting the treatment.

6 Liberated fumes may damage the equipments and fixtures of the chamber.

7 Acid treatment baths often go out of order and their maintenance is difficult.

Cold acid treatment

This is commonly referred to as *Room temperature acid treatment method* as the treatment is performed at room temperature. Since no heating of acid is involved, some authors have chosen to call it as *Non-heated acid treatment*. In this context, room temperature refers to a temperature range of 23°C to 30°C.

The specific gravity of HCl required is 1.10 (as measured at 15°C or 1.096 specific gravity at 25°C) with 20% concentration. In practice, the soaking time ranges between 40 to 90 minutes depending upon the prevailing acid temperature. (The acid temperature is governed by ambient temperature). The dipping duration in relation to different acid temperatures is indicated in Table 8.

TABLE 8 Dipping duration at different acid temperatures

Acid temperature ±1°C	Dipping duration (in minutes)
24°C	60 - 90
27°C	60 - 80
29°C	40 - 50

The treatment is conducted when the eggs have accomplished atleast 15 hours of development. However, it is more safe to treat the eggs between 20 and 24 hours of development.

Though the name of treatment implies that the room temperature is the primary factor, it is imperative to maintain the temperature of HCl constant during the course of treatment, either at 24°C ±1 or 27°C ±1 or 29°C ±1, to achieve best results.

Further, due to variation in seasons, there will be a lot of fluctuations in the temperature. In such a case, when the temperature is 20°C or slightly above or exceeds 30°C upto a level of 35°C, acid treatment may be performed as suggested in Table 9. However, in extreme cases when the temperature falls below 20°C, it is certainly not advisable to carry out the treatment, since at low temperature, various physiological activities of the eggs are sub-normal and they do not respond well to the treatment. It is desirable to warm up the acid to 25°C and perform the treatment.

Advantages

1 It is quite safe and reliable as the duration of soaking is relatively very long and the treatment is organised without any haste. Hence, there will be no confusion or pressure on the part of the person conducting the treatment. The errors are very much minimised.

2 It is even suitable for eggs of younger age.

3 Silkworm breeds relatively weak or sensitive to hot acid can easily be handled.

4 No heating of acid is involved and hence additional equipments and electricity are also not required.

5 In loose egg treatment, unfertilized eggs crumble and facilitate comfortable separation.

6 Quantity of fumes emanated is significantly less compared to hot acid treatment method where acid fumes are liberated continuously.

Disadvantages :

1 Needs sufficiently large quantities of concentrated acid.

2 Non-diapausing eggs do not withstand cold acid treatment and get damaged, whereas in hot acid treatment they will remain intact with very little damage. Hence silkworm breeds with low potency of hibernation cannot be handled in cold acid treatment.

3 The quality of the egg card should be good enough to withstand longer duration of dipping and subsequent washing in water.

Acid treatment of loose eggs

Treatment of loose eggs is comparatively an easier process, in both hot and cold acid. In contrast to that of egg cards, the acid requirement is significantly less and the acid strength is not disturbed much during successive treatments. Huge quantities say 1.0 to 1.5 kg. of eggs (4,000 to 6,000 dfls) can be handled at a time. Acid treatment is also foolproof as the entire egg surface gets the acid stimulus.

Loosened and dried eggs (after bleaching powder wash conducted for gum elimination and sterilisation) are gathered into a perforated plastic container specially designed for loose egg treatment. The container including the lid has numerous pores all around, for instant penetration and exchange of acid. It has a long plastic rod in the central axis which serves as a handle and enables the rotation of the equipment. The entire unit is dipped into the acid trough and rotated alternately in clockwise and anti-clockwise directions. Due to these slow and continuous movements, acid comes in to contact with all the eggs and the entire egg surface experiences the stimulus. The eggs also do not accumulate at one place. In the case of non-availability of such containers, mull cloth bags or nylon mesh bags, where acid penetration is ensured, are used. These can be handled either by a glass rod or by hands protected with gloves.

Soon after the specified dipping duration in the acid, the container/bag is extracted and held for a few seconds to drain off the acid and later slipped into a bucket, full of water. Later the container is rotated briskly to drive out the acid and the process is repeated under the running water for about of 15 to 20 minutes. This washing process ensures complete elimination of acid.

The washed eggs are collected into mull cloth bags and the moisture is removed using blotting papers. These eggs are later released into a laminated sheet affixed tray and dried under gentle breeze.

A new approach to cold acid treatment

The most common methods of acid treatment namely hot acid and cold acid methods demand certain amount of skill and precision to obtain effective hatching. Of these two, the cold acid method has been further simplified. A simplified ready reckoner for the treatment of eggs, for varied conditions of temperature in tropics, by using different specific gravities of HCl, varied dipping durations and acid temperatures, is provided in Table 9. This schedule of treatment has been proved to be as effective as the established ones.

TABLE 9 Ready reckoner for modified cold acid treatment method

(Dipping duration in minutes)

HCl Specific Gravity at 25°C	Acid Temperature						
	20°C	23°C	25°C	28°C	30°C	33°C	35°C
1.090	---	100	90-100	70-100	60-100	40-80	30-60
1.100	90-100	60-100	60-100	40-80	30-60	20-40	20-30
1.110	40-100	30-60	30-50	20-40	20	10-20	10

(Ref : Indian Silk, May, 1987, Page 35)

Postponement of acid treatment

Acid treatment, under normal conditions should not be postponed. However,under unavoidable circumstances postponement may be considered. Seed contemplated for postponement is cold stored,

for a maximum period of 5 days at 5°C to 7 days at 2.5°C, at a specific age. The optimum age for refrigeration has been found to be between 16 and 22 hours after oviposition. The eggs are essentially passed through an intermediate temperature of 15°C for 2 hours. Similarly at the time of release, the eggs are passed through 15°C and 25°C for a duration of 2 hours each before subjecting to acid treatment. This exercise prevents the eggs from experiencing shock due to sudden temperature change and helps in re-storing the normal physiology.

Postponement should never be resorted to for the eggs which have attained some degree of pigmentation. It may be implemented only when it is possible to maintain RH of 75% ± 5% and the different temperatures precisely to the requisite levels. It is advantageous when grainage operations of a particular lot with special relevance to moth examination are under progress. After verifying the moth examination results, the treatment can be conducted. Postponement can also be resorted to for the first and second day oviposited layings of a batch which are generally less in number.

Cold storage of acid treated eggs

The acid treated diapausing eggs, due to prevention of diapause, behave similar to non-diapausing eggs. Hence, they are also referred to as *artificial non-diapause eggs.* Such eggs can be stored at 5°C for a maximum duration of three weeks. Preservation beyond this may result in irregular hatching, as the embryo cannot withstand along span of cold storage. The preservation of eggs is arranged when the embryo has attained the longest embryonic stage which is generally accomplished between 50 and 60 hours of development.

Acid treatment for diapause eggs after chilling

The distinct advantage of diapausing eggs is that they can be made to hatch in any part of the year. This is accomplished by two different methods - (a) for immediate hatching by blocking the diapause and (b) for delayed hatching by its termination.

The hot and cold acid treatments, popularly referred to as *Common methods of acid treatment* are basically the tools for blocking or preventing diapause. The acid treatment of the eggs, before diapause initiation, inhibits certain activities and induces a score of biochemical reactions which ultimately transforms them into *artificial non-diapause* eggs.

On the other hand, where diapause is partially or totally established, it has to be eventually terminated. In silkworm eggs, diapause termination is achieved by exposing the eggs to low temperature of 5°C or 2.5°C *(artificial overwintering).* Effective termination of diapause can only be accomplished when the eggs experience low temperature stimulus for a minimum of 90 days. Such *chilled* eggs give good and uniform hatching. On the contrary, inadequate exposure cannot activate the diapause eggs completely and in such cases, hatching is irregular and poor. It is for such eggs that a specific acid treatment is supplemented to bring about unifrom and good hatching.

The eggs preserved at high temperature (25°C) for 30-35 hours and chilled at 5°C for 30-40 days (popularly referred to as *short term chilling*) or 40-50 hours old eggs chilled for 35-50 days (popularly known as *long term chilling*) are subjected to this acid treatment.

Under this method of treatment, HCl of specific gravity 1.10 (20% concentration) is heated to 47.8°C and the eggs are soaked for 4-5, 5-6 and 6-7 minutes for Chinese, Japanese and European races respectively. However, barring these parameters, the normal procedure described earlier, is followed to acid treat the chilled eggs.

Precautions :

1. While shifting the eggs from high temperature (25°C) to low temperature (5°C) or vice-versa, they have to be essentially passed through an intermediate temperature of 15°C for about 3 hours to avoid shock. Similarly, prior to acid treatment, eggs from 15°C are transferred to 25°C and kept for 3 hours.
2. The eggs released from 5°C have to be acid treated within six hours. This is so because the serosal pigment starts spreading uniformly in the sersoal cells. Once the spread of the pigment is complete, the impact of acid treatment gets nullified.

3. Acid treatment should not be performed immediately after relase from 5°C as it would give shock to the embryos and may result in *white rot eggs* (dead eggs).

Information on comparative requirements of different types of acid treatment is presented in Table 10, for immediate convenience.

Precautions to be taken during acid treatment

Quality of the acid

Commercial grade HCl which is generally marketed in a range of 1.150 to 1.180 specific gravity is suitable for acid treatment. It should be colourless emanating noxious fumes of highly irritating nature and these properties of the acid are ensured at the time of procurement.

Specific gravity of acid

There is a general misconception that if fresh concentrated acid is diluted to the desired level and treatment conducted, it results in burning of eggs. It is generally insisted that during the acid preparation some quantity of *used* acid has to be mixed to fresh acid to overcome this burning effect. Such a thing normally occurs when acid specific gravity is recorded independent of its temperature, thus failing to indicate the real specific gravity of the freshly prepared acid, being still warm. It is impressed upon again that the specific gravity of acid for treatment should invariably be measured in relation to its own temperature by referring to the correction table (Table 5).

Hydrochloric acid of specific gravity 1.075 at a temperature of 15°C, measures 1.0715, 1.0697 and 1.0642 at 25°C, 30°C and 46°C respectively. It is apparent that temperature and specific gravity parameters are inversely related. If one records specific gravity of 1.075 at 30°C or 46°C one gets significantly a higher specific gravity acid and the concentration is higher by 0.5 to 2%. This probably leads to burning of eggs. Hence, as a precaution specific gravity is measured either at 20°C or 25°C. It is indispensable to correct the specific gravity in relation to its own temperature. It is emphasized that room temperature has no relevance in recording specific gravity and that acid temperature alone is considered. Further while preparing acid solution, water has to be thoroughly mixed with acid and cured.

Re-use of acid

It is not necessary to change acid after each treatment. At the same time, with the progressive increase in the number of dips, the acid strength in terms of concentration is disturbed significantly. This is attributed to the exuberances drawn from the egg cards into the acid, absorption of acid by the egg cards, apart from the dissolving of detached eggs, evaporation of acid solution etc.

In light of this problem, if the quantity of layings to be treated is substantially high, (i.e., the number of dips are more), after ten or fifteen dips, the acid should be replaced with another trough of acid. For re-use, after the completion of treatment, the acid is allowed to settle and filtered. While filtering, only the supernatant is collected and the lower turbid acid is rejected. Acid if measured, after the completion of treatment, indicates either the same specific gravity or a higher one depending on the quantity of layings treated. However, its strength and physical status would have altered significantly which may affect the hatching results. Hence, the acid is filtered and concentrated acid is added for rectification. For the purpose of restoring the acid to the desired status, about two to three litres of concentrated acid is added to the filtered one and the entire acid solution is corrected by adding water so as to obtain the required specific gravity. This enables provision of appropriate acid stimulus to the eggs. The acid is rejected when it assumes a dark colour due to turbidity, after repeated use.

Temperature of the acid

When the acid treatment is under progress, it is but indispensable to watch the temperature at regular intervals, and its frequency depending on the method of treatment. In hot acid method of treatment, it is verified every minute and the dipping schedule is regulated in relation to the temperature as detailed in later paragraphs. In cold acid method, it is suggested to verify the temperature every ten minutes and the average is computed for formulating the immersion strategy. For example, if it is 25°C, 25°C, 26°C, 26°C, 27°C, 27°C, for every 10 minutes, in progression, the average works out to 26°C and immersion of egg cards is conducted for 60 to 80 minutes.

Table 10 **Comparative requirements of different types of acid treatments**

Characters	Common Hot Acid Method	Common Cold Acid Method	Acid Treatment for Chilled Eggs
Specific Gravity of HCl	1.075	1.10	1.10
Concentration of HCl	15%	20%	20%
Temperature of HCl during treatment	46°C	25°C/ 27°C/ 29°C	47.8°C
Immersion time (minutes)	i. Chinese Races 4-5 ii. Japanese Races 5-6 iii. European Races 6-7	60-90/60-80/40-50	i. Chinese Races 4-5 ii. Japanese Races 5-6 iii. European Races 6-7
Age of the eggs	20 - 24 hours	20 -24 hours	i. 30-35 hours old eggs chilled for 30-40 days ii. 40-50 hours old eggs chilled for 35-50 days
Colour of the eggs	Yellow (before the appearance of brownish colour)	Yellow (before the appearance of brownish colour)	i. Brownish black/Blackish brown ii. Before the dispersal of serosal pigment i.e. within six hours of release from cold storage.

Dipping duration

The dipping duration is precisely maintained as applicable to respective silkworm breeds under treatment. A difference of few seconds either way will have no deleterious effect. However, excessive soaking or insufficient dipping beyond a certain threshold point, is bound to cause an undesirable impact on the hatching behaviour of the eggs.

In the eggs treated normally, after a gap of 24 hours, the colour appears paler and colour formation is delayed in comparison to the untreated ones. Their upper surface will indicate slight depression or dimple. However, excessive dipping duration, higher temperature and sp. gr. of HCl, individually or in combination will pronounce a harmful effect on eggs. Such a symptom is detected by observing the treated eggs which would exhibit deep dimples at the centre and in acute cases the eggs collapse without acquiring any colouration and sometimes become crushed.

On the contrary, insufficient duration of dipping, treatment at low temperature and low specific gravity HCl, against the defined norms lead to improper and irregular hatching. This condition is attributed to the inadequate stimulus gained by the eggs. In this case, the symptoms are not distinctly expressive. The dimples appear rather late and would be very shallow and narrow. However, only a test hatching would reveal the defect precisely.

The treatment duration in respect of some of the parental races is given in Table 7. However, when a hybrid is to be treated, the female component of the hybrid combination is considered to fix the immersion time i.e. if NB_{18} x NB_7 hybrid layings are to be treated, NB18 is taken for deciding the immersion time. Further when different races comprising of pure and hybrids are to be treated, they are preferably treated breedwise separately.

Co-ordination

Duration of dipping in relation to breed, specific gravity of the acid and its temperature have to be co-ordinated. The hatching results are decisively dependent on the proper interaction of these factors. Age of the egg is also an important criterion. One should have comprehensive knowledge about these parameters so that if one factor is disturbed the other factors are appropriately regulated to overcome the effect. For example, during the course of treatment, if there is a fall or raise in temperature of acid by 0.5°C, for every minute of fall or rise, 5 seconds of immersion time is increased or decreased respectively. The acid temperature should be maintained between 45 to 47°C.

Use of separate cards

When acid treatment is contemplated, oviposition is allowed only for 24 hours. However, if a second day oviposition is desired, as is possible in case of loose eggs, separate cards are used for the purpose.

Use of timer

An electronic timer which sounds an alarm after a stipulated time is put to use for time accuracy. Ten seconds before the time, the egg cards are extracted from the trough; the acid is drained off for a few seconds and diverted for washing.

Use of dummy acid for treatment

The egg cards after formalin treatment are thoroughly dried. Improperly dried cards when soaked in acid, are likely to alter the specific gravity (concentration) as also the temperature. To overcome such a situation, it may be more practical to have dummy acid trough, maintained at a stipulated temperature. The egg cards are first dipped in the dummy container for ten seconds and later shifted to the regular acid trough. This avoids any possible dilution and facilitates maintenance of accurate temperature.

Maintenance of acid temperature

Even though the treatment baths are thermostatically controlled, water and acid temperature are verified after thorough mixing, before commencing the treatment. Very high water temperature will raise the acid temperature abruptly and significantly. Before the commencement of treatment the acid tempera-

ture is maintained at 46.5°C to 47°C so that when the egg cards are dipped, it acquires the optimum temperature. However, in case the acid temperature has raised too high, the temperature is lowered by keeping the acid container outside the hot water bath or by slipping a sealed polythene bag containing ice cubes into the container and the temperature is brought down to the required level. Otherwise the acid container is kept in a cold water basin.

To maintain uniformity in acid temperature, it is mixed well prior to dipping and gently when the treatment is under progress.

Quantum of egg cards for treatment

The number of egg cards to be dipped at one time is decided depending on the volume of acid. When 20 litres acid is used, about 30 to 35 cards are soaked at a time. To begin with, the volume of acid has to be substantially high as 15 to 20 ml. of acid would be lost for each egg card of 20 dfls, due to absorption at the time of treatment and retention by them when extracted for washing. The egg cards are fastened loosely together with a thread by folding each sheet with the eggs facing outside to facilitate free flow of acid between the cards. The egg cards should be completely submerged with ample space for their free movement inside the acid container. Further the acid level should be one to two inches above the level of the cards. The cards are constantly moved using a glass rod and the acid is regularly stirred for uniform maintenance of temperature in the trough. All the eggs should come in contact with acid to experience the stimulus uniformly.

Egg cards are preferably punched at the corners and moths are allowed for oviposition so that at the time of treatment, cards could be easily stitched without damaging the eggs.

Washing of eggs after treatment for removal of acid

After the treatment process, when egg cards are extracted from the acid container, they retain significant quantity of acid solution over their surface. This acid has to be completely eliminated and if the egg cards are unattended to immediately, it may have harmful effect and may even lead to burning of eggs. Hence thorough washing of eggs in flowing water for a duration of 15 to 20 minutes is indispensable to remove even the slightest acid traces, whatsoever. The water temperature should not exceed 35°C, as high temperature would impose instant burning effect. For practical convenience, the egg cards are taken out from the acid container 5 to 10 seconds ahead of the scheduled soaking time to drain off the acid. This precaution ensures easy washing of eggs.

Three or four tanks with glazed tiles (each tank of dimension : L 2' x W 2' and D 6 to 8") constructed in a downward gradation, are utilized for washing of eggs. A water tap is fixed atop the upper most tank for continuous water supply and provision is made that surplus fresh water falls from one tank to the next lower tank through the out-lets. Egg cards, after treatment, are immediately transferred to the lower most tank. After a few minutes wash, they are gradually shifted to the next upper tank. The egg cards after wash in the third tank are finally washed in the upper most tank. This progressive washing under running water facilitates complete and effective elimination of acid traces. In the absence of such facility, three to four big plastic basins are arranged in a slopy descending manner.

Presence of acid over the egg surface is confirmed either by using a blue litmus paper which turns red or verified with tongue which tastes sour. Where there is scarcity of water, use of washing soda helps in nullifying the acid effect. Acid, if not eliminated completely is likely to have a harmful effect on the eggs.

Use of hydrometers and thermometers

Hydrometers for the measurement of specific gravity, are available in different ranges. Use of precision or narrow range hydrometers would minimise the error. The range of hydrometers relevant to record specific gravity of HCl are : 1.000 to 1,200; 1.000 to 1.1000; 1.100 to 1.150; 1.050 to 1.100 and 1.060 to 1.110. A hydrometer with a range of 1.050 to 1.100 or 1.060 to 1.110 is apt for regular use, while a hydrometer of 1.00 to 1.200 range is required to measure the specific gravity of concentrated acid.

The hydrometers have to be essentially standardized by initially measuring the specific gravity of distilled water (1.000) and other standard laboratory grade acids of known specific gravity. The hydrometers are corrected accordingly and the corrections are imprinted on their stems. It is quite safe

to measure specific gravity by utilizing two standardized hydrometers in day-to-day operations, which necessarily should indicate similar reading. This exercise eliminates any possible risk involved. As is known, only the lower miniscus is considered while measuring specific gravity. The hydrometer must be static while recording the specific gravity. Similarly, precision and standard thermometers are used.

It may be categorically stated that defective hydrometers and thermometers would turn all efforts topsy-turvey and make the treatment a futile exercise.

Test hatching

Though every precaution is taken during treatment, to develop confidence in oneself, 10 dfls from every day's treatment are preserved and incubated to observe the hatching performance. It may be of more practical use, if layings for test hatching are drawn from the first and the last dip. This directly points out any mishandling in the treatment procedure and serves as a feed back to rectify such short-comings.

Colour of eggs as an index for treatment

It is always necessary to keep a close watch on the colour of eggs before the acid treatment. It is safe to treat the eggs before the manifestation of brownish colour. Only under unavoidable conditions, eggs which have attained a faint colour are treated. In such cases as for as hot acid treatment is concerned, dipping duration is preferably enhanced by 10 to 20 seconds over the normal schedule, depending on the colour intensity. During summer months due to high temperature, embryonic development gets accelerated and pigment formation takes place early. Hence, in such cases, treatment is conducted early between 15 and 20 hours, after oviposition.

Use of other materials for heating acid

At times, due to power failure or break down of hot water bath, it may not be possible to heat the acid. In such a situation, cold acid treatment can be resorted to. Alternately, hot water can be used for heating the acid and conducting the treatment.

Need for isolated acid treatment chamber

Acid fumes that emanate during the preparation and the actual process of treatment, escape and settle on various metallic structures and corrode them. Electrical switches, acid treatment baths etc., get corroded within a short period. These instruments should be protected by giving anti-corrosive painting coats. The fumes settled on electrical switches conduct electricity and make the premises insecure. To sort out and avoid such problems, the acid treatment room is maintained in isolation to other rooms and the requisite electrical connections are drawn from adjacent rooms. Exhaust fans, with blades made of fibre-glass/plastic, only are fixed in acid treatment chamber. The chamber should be independent with good ventilation to facilitate effective driving out of acid fumes. The floor is preferably made of porcelain tiles or any other acid resistant material.

Use of protective materials

As a precautionary measure to protect oneself from the acid hazards, one should use protective materials such as masks, aprons, gloves etc.

PART II

Physiological Changes Associated with Diapause and HCL Treatment

Initiation & Establishment of Diapause

Diapausing silkworm eggs are characterised by a considerably diminished metabolism (by switching over to glycolytic pathway), very low oxygen consumption, preservation of substrates and a marked resistance to desiccation and low temperature.

One of the major significant changes that could be observed in diapausing eggs is that the chorion which is permeable to air at the beginning of oviposition gradually becomes impermeable and forces the embryo to experience an oxygen deficient environment.

Other major events that take place with the progress of time upto the establishment of diapause are as under:

I ***ONE DAY AFTER OVIPOSITION***

a. Newly laid eggs which are yellowish in colour gradually transform into brown due to the appearance of the Ommochrome pigment in the serosal cells.

b. Glycogen level begins to decrease and progressively gets converted into antifreeze substances, Sorbitol and Glycerol.

c. Rate of oxygen permeation across the egg shell decreases rapidly while oxygen consumption is at the maximum level.

d. DNA content increases logarithmically.

II ***TWO DAYS AFTER OVIPOSITION***

a. Oxygen consumption declines steeply.

b. There is an abrupt elevation in glycogen phosphorylase-a activity followed by a gradual decrease.

c. The affinity of lysosomes in embryonic cells to acridine orange with supravital staining gradually declines and is lost during diapause.

d. The increase in DNA content gets arrested.

III. ***THREE DAYS AFTER OVIPOSITION***

a. There is an arrest of nucleic acid synthesis in the nuclei of embryonic cells and a gradual decrease in yolk cells.

b. Water loss from the diapausing eggs decreases to less than 0.1% per hour in contrast to their non-diapausing counterparts where in the water loss ranges between 0.25 and 0.3% per hour.

IV. ***FOUR DAYS AFTER OVIPOSITION***

a. Mitotic activity in the embryo gets arrested.

b. There is a continued steep decline in glycogen content and rapid and gradual accumulation of sorbitol and glycerol respectively.

c. The yolk cells stop synthesizing DNA and protein within about four days after oviposition. This stage is referred to as pre-diapause or young diapause.

Major Physiological Changes in HCl activated eggs

Hydrochloric acid treated eggs are regarded as artificial non-diapause eggs since they have little difference as regards morphogenesis in comparison to natural non-diapause eggs. It is still an open question and enigma as to how the acid acts in blocking diapause. Some of the theories are as under :

1. Miura (1929) first reported that activation and hatching of eggs by HCl is due to the electron charge of the chloride ion and concluded that the hatching possibility or impossibility of the eggs depends upon the eggs' ability to absorb negative ions.
2. Okada (1971) and Sonobe (1979) reported that soaking of 20 to 24 hours old eggs in HCl solution enhances oxygen permeability of the chorion which prevents the occurrence of diapause.
3. HCl treatment increases the rate of water loss abruptly, suggesting the prevention of diapause. (Sonobe, 1979).
4. HCl permeated through the egg shells during the course of treatment brings about a decline in pH values of egg contents (Yoshimi et al 1986).

The main physio-biochemical changes brought about by HCl treatment in diapausing eggs are discussed in detail.

CHANGES ASSOCIATED WITH PROTEIN METABOLISM

a. ***Impediment in protein synthesis activity***

The protein synthesis activity of the yolk cells is entirely dependent on certain coded information or an unknown material from the embryo.This material from the embryo may contribute to the protein synthesizing system from RNA to protein synthesis. These yolk proteins which are newly synthesized under the control of this embryonic information may be a particular enzyme necessary for the onset of diapause and contribute to the conversion of glycogen. This protein stays for a very brief period and is sufficient for the onset of diapause.

One day old pre-diapause eggs are extremely sensitive to HCl. All the eggs treated with HCl change to non-diapause ones and manifest good hatchability. On the other hand, three day old eggs are far less sensitive to HCl treatment and less than 10% of the eggs can be activated. It is evident that after 24 hours of oviposition certain changes in physiological processes concerned with diapause have progressed so far that they could not be reversed. As a result, the treatment turns out to be ineffective.

The protein synthesis activity of the yolk cells is arrested when the eggs are treated with HCl about 24 hours after oviposition. Acid treatment impedes embryonic role in the protein synthesizing system of the yolk cells which results in embryonic development like the non-diapausing embryo and the RNA synthesis of the yolk cells is not inhibited by acid treatment or cold storage or both.

b. ***Changes in Isozyme, Esterase-A***

The silkworm eggs contain a number of esterase isozymes of which esterase-A is closely related to the development of the embryo and is reported to be responsible for lysis of the yolk cells. The addition of esterase-A to the cultures of yolk cells causes lysis of the yolk cell membranes. It is possible that this enzyme may coordinate in mobilizing the yolk needed by the diapausing embryo to resume development.

The diapause hormone has been reported to exert an inhibitory effect on this isozyme. By blocking esterase-A, diapause hormone enforces the diapause by denying the embryo access to yolk.

Non-diapausing eggs of 20 hours age, after oviposition, exhibit a high esterase activity. On the contrary, diapausing eggs of the same age show very low activity. When acid treatment is performed on these eggs, the esterase-A activity rises abruptly about 30 minutes after treatment. The activity becomes more and more pronounced in one to two hours after treatment almost matching with that of non-diapausing eggs of the same age.

The instant response of diapausing eggs to acid treatment suggests the possibility that the esterase-A protein itself would be contained in a masked form and the acid treatment would activate the inert

esterase-A. This suggestion is likely, because as time duration of 30 minutes would be too short to synthesize *de novo* the enzyme protein to exhibit the enzyme activity. It is also possible due to an intramolecular modification of the enzyme itself.

Thus it can be summed up that acid treatment would cause a latent enzyme in diapause eggs to set in motion. It is quite obvious that esterase-A activity is correlated with an active resumption of morphogenesis.

c. *Changes in Amino Acid Pools*

The concentration of most amino acids in acid treated eggs will not change during development but the level of arginine and phosphoethanolamine (PEA) decreases quantitatively until the time of hatching. The fall in the level is very significant.

Ammonia concentration remains low during the entire period with only a slight increase after hatching. Aspargine remains at a constant low level, while aspartate decreases. Aspartate decreases abruptly after HCl treatment and then remains low. The developmental changes in aspargine and ammonium are similar to those in untreated diapause eggs, but there is a marked difference in the changes in Aspargine in HCl treated and untreated eggs.

The changes in glutamate, glutamine and proline in HCl treated eggs are very different from those in untreated eggs. The level of glutamate decreases slowly during development, while that of glutamine increases. The concentration of glutamine is increased by HCl treatment, but that of glutamate is not. Proline in the HCl treated eggs increases rapidly to a maximum by fifth day and then decreases rapidly, this change being quite unlike that in normal diapause eggs.

Developmental changes in the concentrations of free amino acids in HCl treated eggs are similar to those in untreated eggs after resumption of embryogenesis. It is interesting that in HCl treated eggs, proline accumulates just after HCl treatment and decreases at the time when the synthesis of cytochrome-C begins, suggesting that proline is an energy source with high energy efficiency of a lipid with easy transportation, for development of the silkworm embryos. The decrease in amino acid levels during late developmental stages of HCl treated eggs and of untreated eggs after diapause indicate active synthesis of protein during embryogenesis.

When embryogenesis proceeds in silkworm eggs i.e., after diapause of untreated eggs and in HCl treated eggs, the concentration of phospho-ethanolamine decreases rapidly suggesting the importance of phospholipid formation for embryogenesis or the importance of phosphoethanolamine either as a precursor of other metabolic products or as an energy source.

d. Pigment Formation

Diapause eggs immediately after oviposition and till the germband formation i.e. upto 24 hours have yellow colour. However, after a certain period of development, they gradually turn to brownish black or blackish brown colour due to the appearance of a specific pigment known as Ommochrome. This pigment spreads in the serosal cells and manifests the colour. Non-diapause eggs lack this pigment and hence, the light yellow colour.

Ommochrome is synthesized from Tryptophan through Kynurenine and 3-hydroxy-kynurenine (3-OHKyn). The pigment precursor 3-OH Kyn is synthesized in developing ovaries and other tissues such as fat body and the ovaries take up this substance from the haemolymph while also synthesizing it during pupal adult development.

Hence even after acid treatment, the pigment colour is manifested and the eggs acquire a colour. The colour is rather deep in diapause established eggs in comparison to the treated diapausing eggs.

CHANGES ASSOCIATED WITH CARBOHYDRATE METABOLOSM

Carbohydrate content is intimately related to the physiological event, diapause. Glycolysis as well as pentose phosphate pathway is understood to operate during glycogen conversion to polyols upon diapause initiation.

a. ***Glycogen Conversion***

At the onset of diapause, in Bombyx eggs, glycogen is reported to decrease rapidly as it is converted into sorbitol and glycerol which are basically anti-freeze in nature, (they increase the super-cooling ability, provide energy at diapause termination and stabilize the different enzymes even at low temperature). About 80% of the glycogen is converted into sorbitol during the first 10 days after oviposition whereas glycerol gets accumulated during the later stages of diapause. At the termination of diapause, these polyols are reconverted into a glycogen store, that is to be utilized by the developing embryo, as an energy source.

b. ***Changes in Glycogen Phosphorylase-A***

Glycogen Phosphorylase 'a' is known to play a key role in glycogen metaboloism. It is highly probable that the key enzyme controlling polyol formation from glycogen at the initiation of diapause is Phosphorylase 'a'.

HCl treatment of diapause eggs of 20 hours age after oviposition blocks diapause initiation immediately and causes a peculiar temporal increase in Phosphorylase 'a' activity. Enzyme activity returns to its initial level in about 5 hours and remains as that in non-diapause eggs without any further increase.

Some workers have also presented the view that soaking of 24 hours old eggs in HCl solution causes complete inhibition of the appearance of the enzyme, glycogen Phosphorylase 'a', activity at 50 hours after oviposition.

HCl treatment while preventing the diapause phenomenon to occur also helps in retention of glycogen except for some insignificant conversion initially. The storage glycogen is consumed during the course of embryogenesis.

c. ***Changes in Phosphofructokinase, Glucose-6-Phosphate Dehydrogenase and Fructose-1-6-Diphosphatase Enzyme Activities***

Glycolysis as well as pentose phosphate pathway are known to operate during the conversion of glycogen into glycerol on the initiation of diapause. Contrary to earlier reports, the activity of phosphofructokinase, a key enzyme of the glycolytic pathway, has been clearly demonstrated in prediapause, young diapause and developing eggs.

Phosphofructokinase activity, in HCl activated eggs, is found clearly on the first day of development and increases gradually and reaches maximal levels in the later stages of development. The enzyme activity in young diapause eggs would be operating for glycogen conversion to glycerol in diapause initiation while in HCl treated developing eggs for glycogen degradation involved in ATP production.

Glucose-6-phosphate dehydrogenase activity in HCl activated eggs is maintained at higher level throughout the embryonic development and drops suddenly just before hatching. Fructose-1-6-diphosphatase enzyme activity is hardly observable by 5 days of embryonic development but thereafter rises abruptly until the hatching.

CHANGES ASSOCIATED WITH LIPID METABOLISM

In diapause eggs the lipid concentration in egg contents is about 68 to 70 mg per gram eggs throughout, from 2 hours to 40 days after oviposition. On the other hand, in the HCl activated eggs, the lipid concentration decreases (68 to 32 mg/gm eggs), after oviposition until hatching especially in the later half of the development. It is interesting to note that the lipid concentration in egg shell does not change significantly during diapause stage and in HCl treated eggs.

Changes in pH levels

The pH value of the egg contents, after HCl treatment, is lower than that of the contents of untreated ones and this value also decreases with the duration of HCl treatment.

The results indicate that after HCl treatment the acid which permeates through the egg shells, brings about a change of the pH value in the egg contents.

Physiological changes in the diapuse eggs, treated with HCl after chilling

Chilling (cold storing) diapause eggs at 5°C for less than 30 days is known to be insufficient to terminate diapause. However, when HCl treatment is performed on these chilled eggs, diapause termination is completely achieved. This treatment brings about the following important physio-biochemical changes.

1. To begin with, the oxygen consumption increases and triggers the embryo for an active embryo genesis.
2. In HCl treated diapause eggs (after 30 days of chilling) the two polyols sorbitol and glycerol reconvert to glycogen. The level of glycogen increases upto 6 or 7 days of incubation with a subsequent decrease upto hatching. On the contrary, in the eggs treated with HCl after 20 hrs. of oviposition, glycogen level decreases continuously upto hatching.
3. The sorbitol content remains unchanged on the first day and then decreases dramatically during the next 2 days after HCl treatment, leaving only trace amounts.
4. The NAD-SDH (NAD dependent sorbitol dehydrogenase) activity increases rapidly reaching a maximum value seven days after the treatment and then decreases until one day before hatching. Thus there is a close correspondence between the time of the increase in NAD-SDH activity and the degradation of sorbitol during the termination of diapause.
5. The activities of mitochondrial Glutamate-oxaloacetate transaminase (GOT) and malate dehydrogenase (MDH) increase in diapause eggs which are chilled at 5°C for more than 20 days. Further, the HCl treatment brings about an abrupt increase in the activites of mitochondrial GOT and MDH with the maximum effect appearing, two days after the treatment.

The cumulative effect of these biochemical reactions ameliorates the hatching efficiency of the chilled and acid treated diapause eggs.

Conclusion

In conclusion, a question may arise whether the hot acid treatment is better or the cold acid treatment. Strange enough, no authentic literature is available to support the superiority of any of these methods. However, our clear answer is both. Both the methods when carried out properly have been found to be equally efficient and are bound to give excellent results. The ultimate choice is thus left to the user who should take a final decision considering the following points.

a. Type of eggs to be treated (purely uni/ bivoltine or mixed)
b. Age of the eggs for treatment
c. Season of treatment (during summer months there may be a tendency in the eggs to show some degree of polyvoltinism and vice versa in the winter)
d. Quantity of eggs to be treated and time at the disposal and
e. Availability of infrastructural facilities.

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