STUDIES ON THE HEAT RESISTANCE OF
TIGRIOPUS JAPONICUS[1]

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With Plates XVI–XVII and 16 Text-figures

The copepod, *Tigriopus japonicus* MORI, is a microscopical marine organism which inhabits small pools on rocks exposed above the sea. Since its habitat is situated well above the high tide mark, this species is always subjected to rapid and drastic changes of environmental conditions. Such pools with limited quantities of water are very susceptible to the daily and annual fluctuations of solar radiation and air temperature. Water temperature may rise above 40° in summer and fall down toward 0°C in winter. Salinity is liable to be changed steeply by concentration due to evaporation and by dilution with rain water. Oxygen concentration may also vary considerably because of the smallness of water volume in comparison to the abundance of organisms.

Such being the habitat, *Trigriopus* is expected to possess a high degree of capacity to adapt itself in response to changes in the environment. TAKEDA (1954), studying the temperature acclimatization in this species, found that its heat tolerance was affected markedly by its thermal history. This noteworthy study was discontinued by his regrettable death. The present writer was much interested in the findings of TAKEDA and has undertaken the present investigation to trace his experiments and further to analyse the mechanism underlying the temperature acclimatization of this species.

The phenomenon of temperature acclimatization in poikilotherms has long attracted the attention of many biologists and it has become of recent years an increasingly fascinating object of inquiry in the field of animal physiology and ecology. There is an enormous mass of information available on this phenomenon (*e.g.*, LOEB and WASTENEYS 1912, BRETT 1944, 1946, DOUDOROFF 1942, 1946), which was summarized and reviewed by some authors, such as PRECHT (1958) and

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Fisher (1958). As for the mechanism by which temperature acclimatization is effected, however, still there is a disagreements among opinions and the problem has not yet been settled.

The effect of ambient temperature is no doubt more direct on the protoplasm of every part of the body in poikilotherms than in homoiotherms. Thus, one of the orthodox ways to approach this problem is to push the analysis further down the levels of organization, namely, from the individual level to the organ, tissue and cellular levels. This direction of analysis has lately been growing in popularity. Another important way of approach is to keep under various conditions large numbers of animals, which are relatively small in size and easy to culture in the laboratory, and to observe precisely the influences of these conditions on the process of temperature acclimatization. Based on the results obtained, the mechanism of acclimatization is to be inferred. *Tigriopus* is certainly one of the most suitable experimental materials for the latter way of approach. In addition, the results of experiments with this material will perhaps be very interesting from the ecological point of view when they are considered in comparison to the peculiarities of its natural environment.

The present paper deals mainly with the processes of loss and gain of heat resistance in *Tigriopus* acclimated to different temperatures. The experimental results were partly published (Matutani 1960 a, b).

Materials and Culture Method

Experimental materials were all obtained from our laboratory stock cultures of *Tigriopus japonicus* Mori, which had originated from masses of individuals collected from the small pools near the Seto Marine Biological Laboratory, Sirahama, Wakayama Prefecture (Plate XVI) and had been maintained in our laboratory for at least 5 generations at 19°–20°C. *Tigriopus*, from hatching to maturity, passes through eleven stages which are divided into six nauplius stages and five copepodid stages. The 5th copepodid stage is followed by the adult stage. The distinction between sexes, which is very clear in the adult stage, appears first in the 4th copepodid stage (Takeda 1939).

The stock lines were cultured under fluorescent tubes in natural sea water which was carried from the Seto Marine Biological Laboratory. They received as food the marine alga, *Plectonema terebrans*, cultured in the laboratory and, in small amounts, powdered dry shrimp.

All the experiments were made with adult animals, 0.81–0.88 mm in body length, sampled at random from the stock cultures (Plate XVII 3 and 4). When they were exposed to a high lethal temperature, they became immobile at last bending upward their abdomens (Plate XVII 5 and 6). These individuals never recovered if returned to the normal temperature. Hence this response was adopted as the criterion of death for discriminating the dead from the alive in the experiments.

Experiment 1.

Change of heat resistance according to acclimation
temperature in animals reared at 20°C

To begin with, the mode of change of heat resistance was investigated in animals which were transferred from a rearing temperature of 20°C to four different acclimation temperatures.

Materials and methods

From the stock of *Tigriopus japonicus* MORI which had been maintained in the laboratory for about 4 years after collection from Sirahama, adult animals were gathered and reared at 20°C (rearing temperature) for 45 days in glass

Rearing   Acclimation
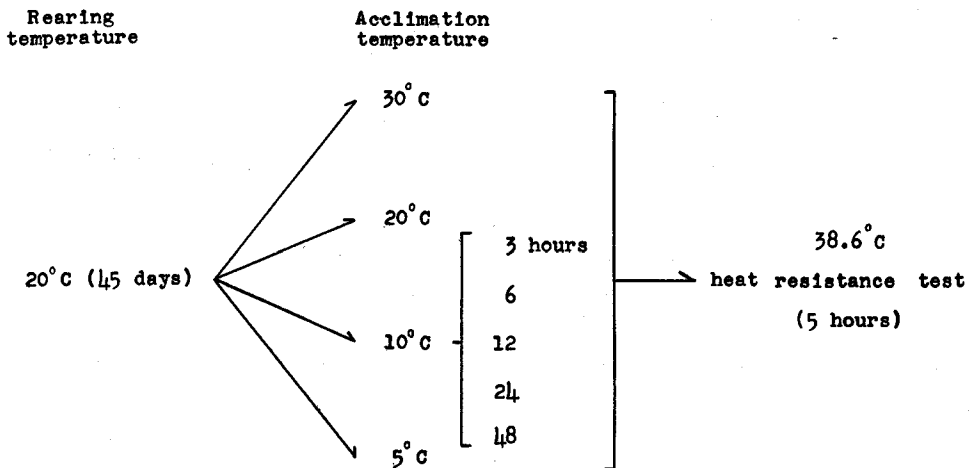temperature   temperature

Fig. 1. Procedure of experiment 1.

cylinders, 93 mm in diameter by 50 mm high, containing 170 ml of sea water and placed under a 20 W fluorescent tube. They received the food once a day. From this 20° culture, adult animals were transferred to four different acclimation temperatures of 5°, 10°, 20° and 30°C, at which they were maintained in hemispherical glass bowls, 55 mm in diameter, containing 22 ml of sea water for definite lengths of time of 3, 6, 12, 24 and 48 hours before they were tested for their heat resistance (Fig. 1). They received no food in the acclimation temperatures.

The heat resistance test was conducted in the following way: Four test tubes, 15 mm in diameter by 160 mm long, containing 20 ml of sea water were immersed fully in a water bath controlled thermostatically at 38.6°C. After thermal equilibrium was established between the sea water in the test tubes and the water bath, about 100 individuals sampled at random from each temperature acclimation

group were put in each test tube. The tubes were examined every hour till the 5th hour and the numbers of surviving individuals were recorded, picking out dead individuals every time.

In this way, about 2000 individuals were used for one series of tests. The experiment was repeated 5 times, and average values were calculated from the results of these experiments.

The temperature of 38.6°C at which heat resistance was tested was determined by a number of preliminary experiments. Takeda (1954) used 38°C for his heat resistance test on the same species. But this temperature was too low to conduct the heat resistance test efficiently and took so long time to detect differences among the acclimation groups that there was an increasing probability that the results of test might involve some side effects, such as the secondary temperature acclimatization and microbial interference during the test. The temperature of 38.6°C was found most appropriate, being neither too high nor too low to evaluate efficiently as well as exactly the net effect of acclimation.

Results and consideration

The results obtained are illustrated in Fig. 2 A–E. Fig. 2 A gives the time-survival curves at 38.6°C of animals acclimated from the same rearing temperature of 20° to four different temperatures for 3 hours. In both sexes, 30°–acclimated animals show the greatest heat resistance. Difference in resistance is slight among 5°–, 10°– and 20°–acclimated animals, their per cent survival curves sometimes crossing one another.

It is within 6 hours in acclimation (Fig. 2 B) that the differences in heat resistance among the four temperature groups become distinct and consistent. These differences grow larger with the duration of time of acclimation and the heat resistance of each group appears to reach a stationary state in 12 hours, though that of 20° group remains almost unchanged for 48 hours. The groups other than the 20°–acclimated show more or less marked gains in heat resistance. Thus they are eventually resistant to heat in the order: $30° > 5° > 10° > 20°$ of acclimation temperature, in both sexes.

These time-survival curves are very implicative representing the extents of individual variation in heat resistance. For the convenience to understand the mode of change of heat resistance with acclimation time, however, it is required to express the resistance of each acclimation group in terms of single index. For this reason, the sum of the survival percentages for 5 hours, i.e. the 5-hour cumulative per cent survival, was calculated and used as index of heat resistance. In such a case as the present, the median or 50% survival time is usually adopted. But this value was found to show a much larger extent of experimental fluctuation than the cumulative value. So the writer preferred the latter index to the former.
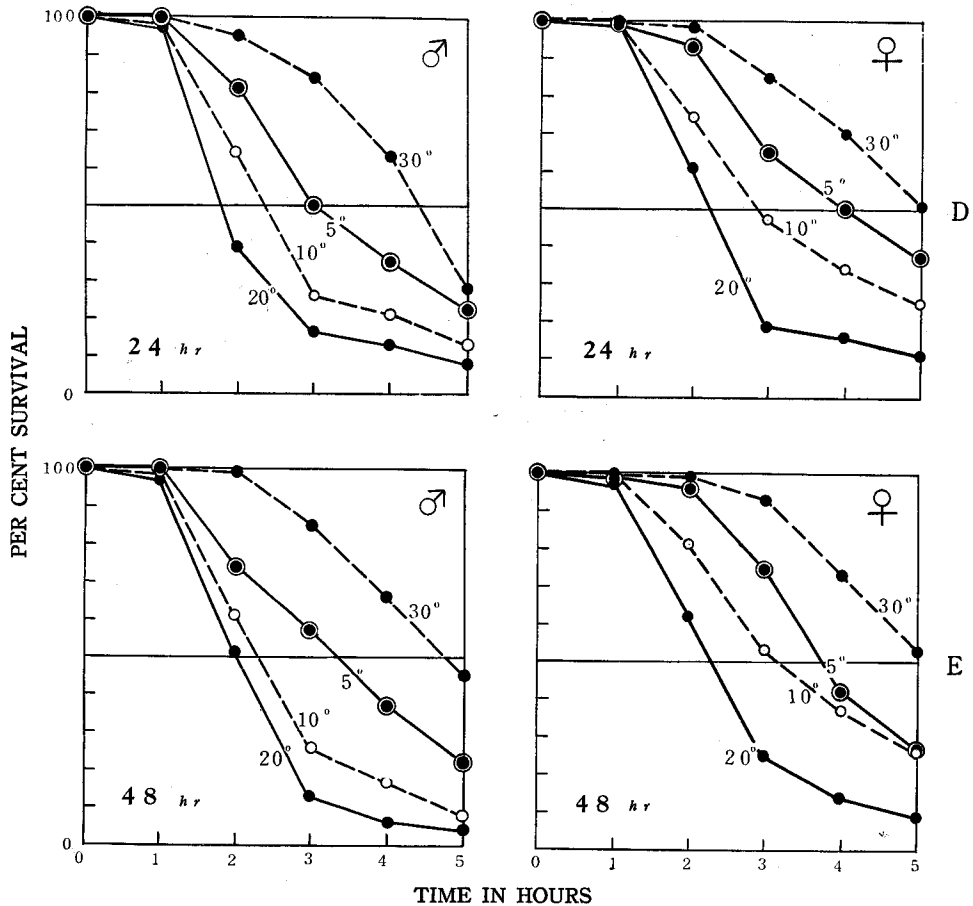
PER CENT SURVIVAL

TIME IN HOURS
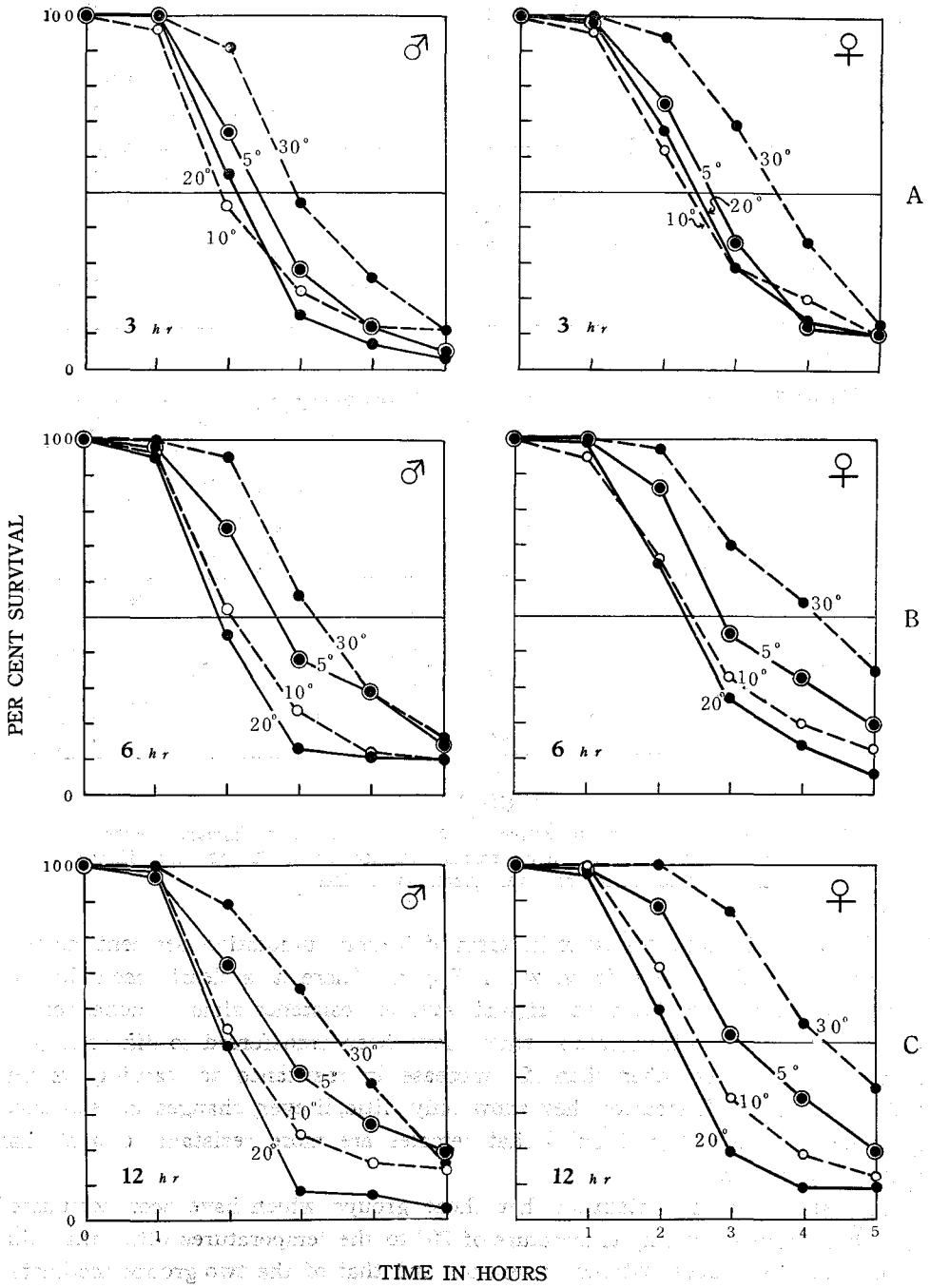
Fig. 2 (A–E). Time-survival curves at 38.6°C of adults of *Tigriopus japonicus*
acclimated from the same rearing temperature of 20° to four different
temperatures stated for given durations of time.

Change of the heat resistance in terms of 5-hour cumulative per cent survival
in each acclimation group is shown in Fig. 3. There it is clearly seen that the
control 20° group maintains its original level of resistance almost unchanged for
48 hours while the other groups which have been transferred to different accli-
mation temperatures other than 20° increase in resistance to varying extents
within 24 hours. Thereafter they show only little, if ever, changes in resistance.
It is evident from Figs. 2 and 3 that females are more resistant to heat than
males in every case.

It deserves special attention that those groups which have been acclimated
from their original rearing temperature of 20° to the temperatures other than this
acquire heat resistance without exception, and that of the two groups acclimated
to the lower temperatures the 5°-acclimated gains decidedly greater heat resistance

than the 10°-acclimated does. This phenomenon is inconsistent with the general
rule of temperature acclimatization acknowledged extensively among poikilotherms
that a rise in acclimation temperature entails a gain in heat resistance and a loss
in cold resistance while a fall in acclimation temperature results in the reverse
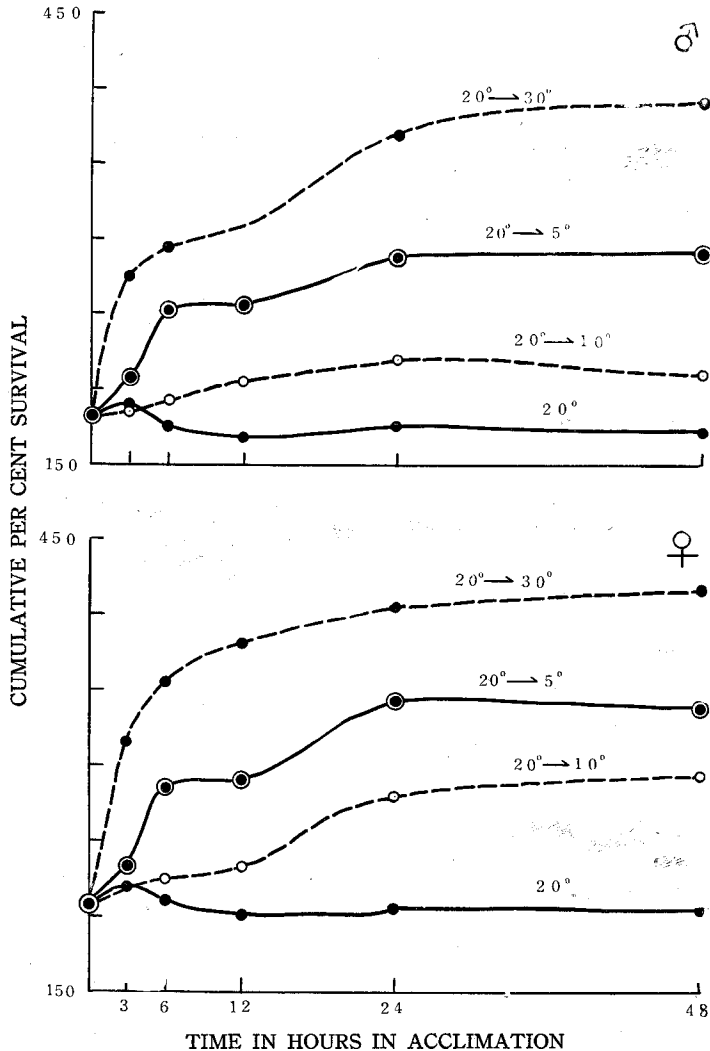

Fig. 3. Change of the heat resistance of adults of *Tigriopus japonicus*,
as indicated by 5-hour cumulative per cent survivals at 38.6°C,
with the duration of time after transfer from a rearing tem-
perature of 20°C to four different acclimation temperatures.

(for crustaceans, SPOOR 1955, RANADE 1957). This general rule is also found true
in many cases of geographical and seasonal adaptation in heat resistance (for
crustaceans, *e.g.* BOVJERG 1952, KROG 1954).

In plants and poikilotherms other than crustaceans, however, there are a few examples of such a peculiar relation as has been shown in the present experiment (*cf.* Precht 1958). Sapper (1935) has observed that cold-acclimated plants have not only high cold resistance but also high heat resistance. Dehydration appears to be involved in this case as one of the important factors. Tsukuda and Ohsawa (1959) have shown in the fresh-water red snail, *Physa sp.*, that the heart rate of animals acclimated to 20° drops at higher temperatures than that of 25°-acclimated animals. Hart (1952) has found in the fresh-water fish, *Micropterus salmoides*, that the northern race withstands higher temperatures better than the southern race. A similar phenomenon has been reported in the periwinkle, *Nodilittorina granularis* (Ohsawa and Tsukuda 1956).

The phenomenon that *Tigriopus* gains heat resistance when it is acclimated from 20° to 30° may have some underlying mechanism in common with the phenomenon of temperature acclimatization generally observed in poikilotherms. It is, however, impossible to apply the same principle to the acquirement of heat resistance by *Tigriopus* acclimated from 20° to the lower temperatures. Here arise two important questions that have to be answered by further researches: Can a mere change in the environmental temperature, irrespective of its direction, induce an increase in heat resistance?; and, is the cause of the increased heat resistance the same in quality whether the environmental temperature is raised or lowered? The former appears improbable since, as has been reported (Matutani 1959), a respective higher level of resistance once acquired by *Tigriopus* that has been acclimated from 20° to another temperature is maintained as such as long as 240 hours. If heat resistance were gained through a stimulus which is nothing but a fall in the environmental temperature, it would be lost within a much shorter duration of time. The latter question requires a physiological approach.

Experiment 2.

Change of heat resistance according to acclimation temperature in animals reared at four different temperatures

The foregoing experiment has revealed a peculiar phenomenon that *Tigriopus* reared at 20° gains heat resistance when it is acclimated to 10° and 5° as well as when it is acclimated to 30°, giving a U-shaped acclimation temperature-heat resistance curve with a minimum resistance value at 20°. This phenomenon appeared to point to a possibility that the heat resistance of *Tigriopus* might increase, whether it was warmed or cooled, in function of, so to say, the absolute value of the difference between the rearing temperature and an acclimation temperature. To examine this possibility, animals reared at four different temperatures were acclimated respectively to four temperatures and the change of heat resistance was traced.

Materials and methods

Tigriopus was reared at 5°, 10° and 20° for 180 days and at 30°C for 30 days. It was found impossible to keep animals at 30° for a longer period than 30 days without any significant effect of selection. From these cultures adult animals were transferred to four acclimation temperatures of 5°, 10°, 20° and 30°C, at which they were maintained for 24 and 48 hours before they were tested for their heat resistance (Fig. 4). The culture of animals and the heat resistance test were made in the same way as described above.

About 3200 individuals were used for a series of tests, which was repeated 11 times, and average values were calculated from the results.
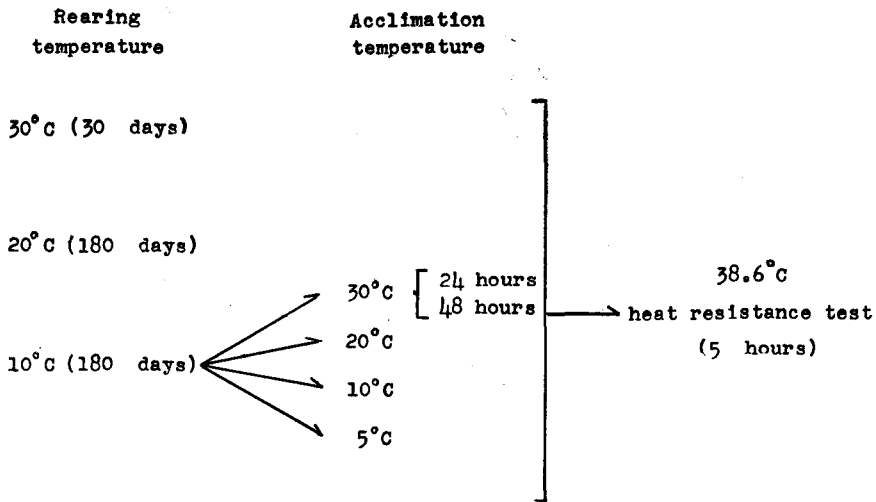
Rearing temperature   Acclimation temperature

30°c (30 days)

20°c (180 days)

10°c (180 days)
30°c   24 hours   48 hours
20°c
10°c
5°c

38.6°c
heat resistance test
(5 hours)

Fig. 4. Procedure of experiment 2.

Results and consideration

The results of the heat resistance test at 38.6°C are shown in Figs. 5 and 6. These time–survival curves show complicated relationships between the rearing temperatures and the acclimation temperatures. In 30°–reared animals, the original high level of heat resistance is well preserved for 24 hours after transfer to the lower temperatures, but in 48 hours they show some variation due to the acclimation to the altered temperatures. In 20°–reared animals, on the contrary, the change of heat resistance is most pronounced, the resistance rising rapidly when animals are transferred to the lower temperatures as well as when they are acclimated to the higher temperatures. The increase in resistance is rapid in the order of 30°, 5° and 10° acclimation. Among both 10°– and 5°–reared animals, the
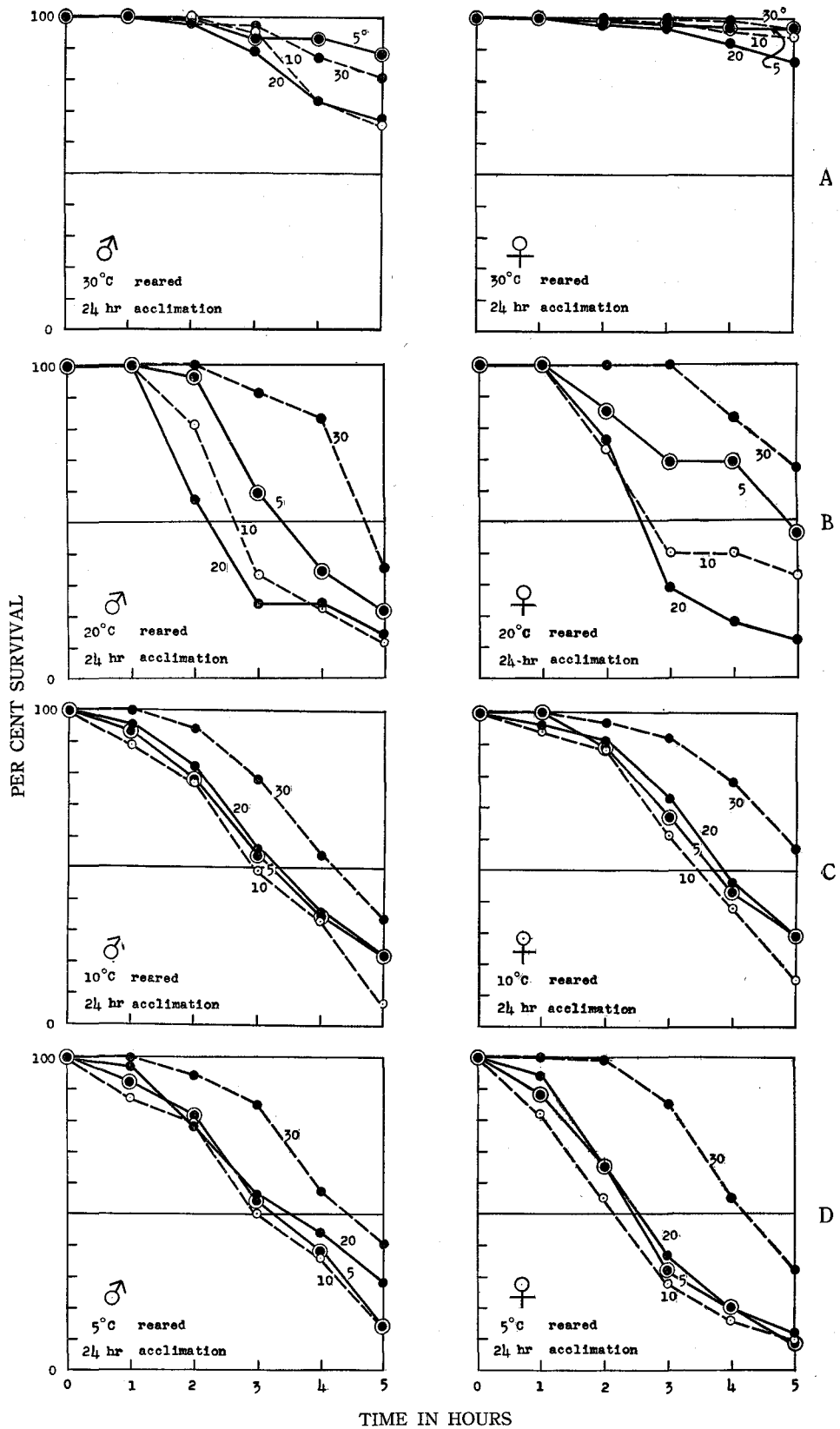
Fig. 5 (A–D). Time-survival curves at 38.6°C of adults of *Tigriopus japonicus* 24 hours after transfer from four different rearing temperatures to four different acclimation temperatures stated.
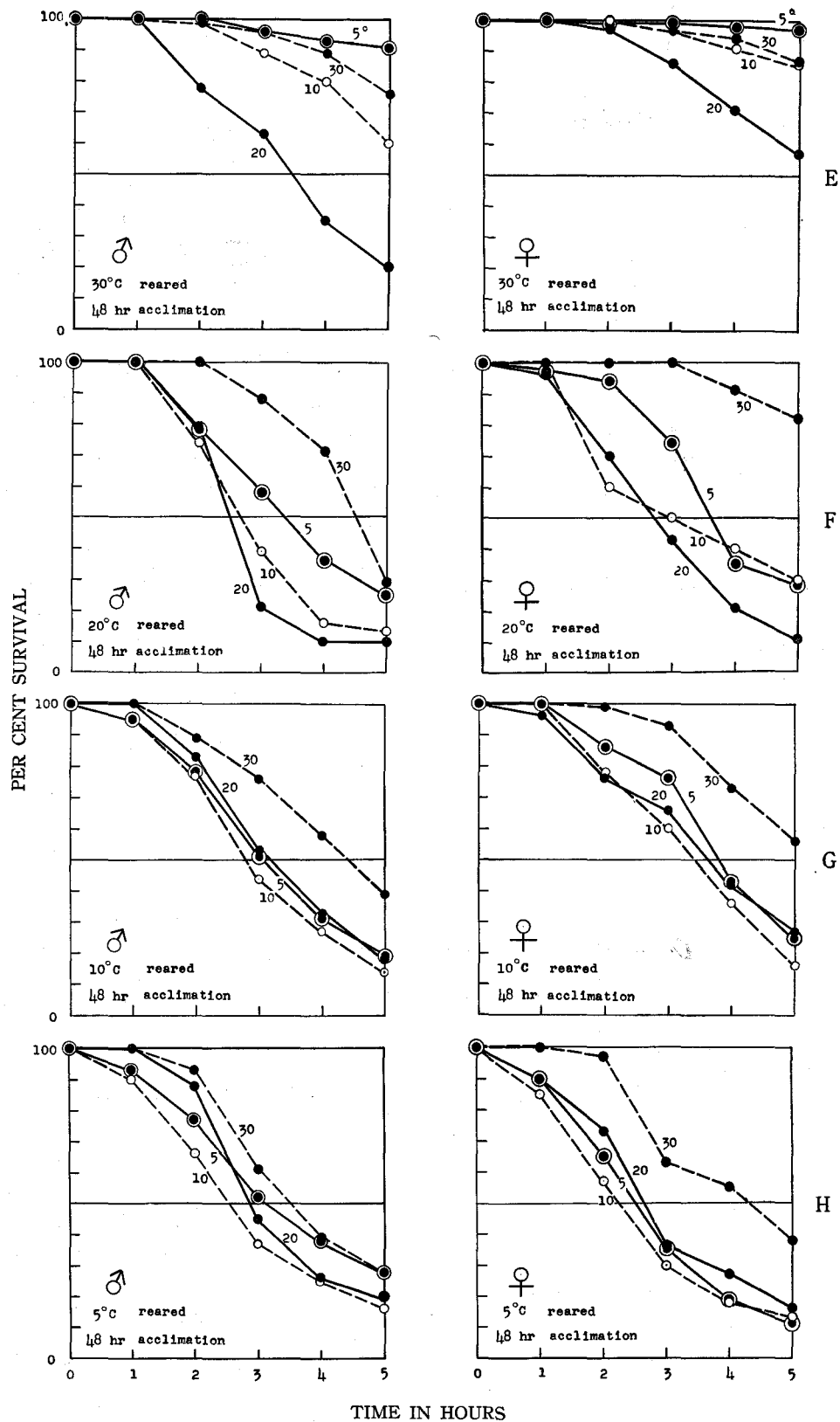
Fig. 6 (E–H). Time-survival curves at 38.6°C of adults of *Tigriopus japonicus* 48 hours after transfer from four different rearing temperatures to four different acclimation temperatures stated.

change of heat resistance due to acclimation is little significant except in 30°
acclimation.

From each of these time-survival curves, the sum of per cent survivals for
5 hours, that is the 5-hour cumulative per cent survival, was calculated as the
index of heat resistance for the convenience of ready comparison of the mode
of change of heat resistance among the acclimations. The result is shown in
Fig. 7, in which the indexes of resistance are plotted against acclimation tempera-
tures.

These acclimation temperature–heat resistance curves are all characterized by
the U-shape. In both 20°- and 30°-reared animals, the lowest resistance is shown
in 20° acclimation. In 20°-reared animals, the highest resistance is attained by 30°
acclimation group in both sexes, while in 30°-reared animals the resistance of 5°
acclimation group appears somewhat higher than that of 30° acclimation group.
In 5°- and 10°-reared animals, on the other hand, the highest resistance is shown
in 30° acclimation and the lowest resistance in 10° acclimation. It is concluded,
therefore, that 20°- and 10°-reared animals gain heat resistance whether they are
warmed or cooled, while heat resistance is reduced as a result of temperature
change when 30°-reared animals are acclimated to 20° and when 5°-reared animals
are acclimated to 10°.

When *Tigriopus* is transferred from its rearing temperature to a different
acclimation temperature, its heat resistance is considered to change gradually and
to tend to a new stationary level which is proper to that acclimation temperature.
On averages, the stationary level is higher in the order: 30° > 5° > 10° > 20° of
acclimation temperature in males; and 30° > 10° > 20° > 5° in females.

The trend of heat resistance toward the stationary level in each case is
illustrated in Fig. 8. Resistance increases rapidly in the animals warmed to 30°
from the lower temperatures and decreases also rapidly in the animals cooled
from 30° to 20°. The mode of change of resistance is, however, by no means
simple. In some cases, a phenomenon of overshooting is acknowledged (Fig. 8 B,
5° acclimation; D, 10° acclimation), and in some cases resistance appears to be
in a fairly stable state before it reaches the stationary level (Fig. 8 C, 20° and
30° acclimation; G, 30° acclimation). In other cases, resistance initially changes
in the opposite direction to the stationary level (Fig. 8 A, 5° acclimation) and
then returns back to the level (Fig. 8 D, 20° acclimation; F, 5° acclimation; G,
20° acclimation; H, 10° acclimation).

Heat resistance which, in most cases, is still changing 48 hours after transfer
is supposed to reach eventually the corresponding stationary level when tempera-
ture acclimatization is completed. The time required for complete acclimatization
will depend on the temperature of acclimation on the one hand and on the
difference between the rearing and the acclimation temperature on the other.
If, however, there has taken place some genetic variation associated with heat

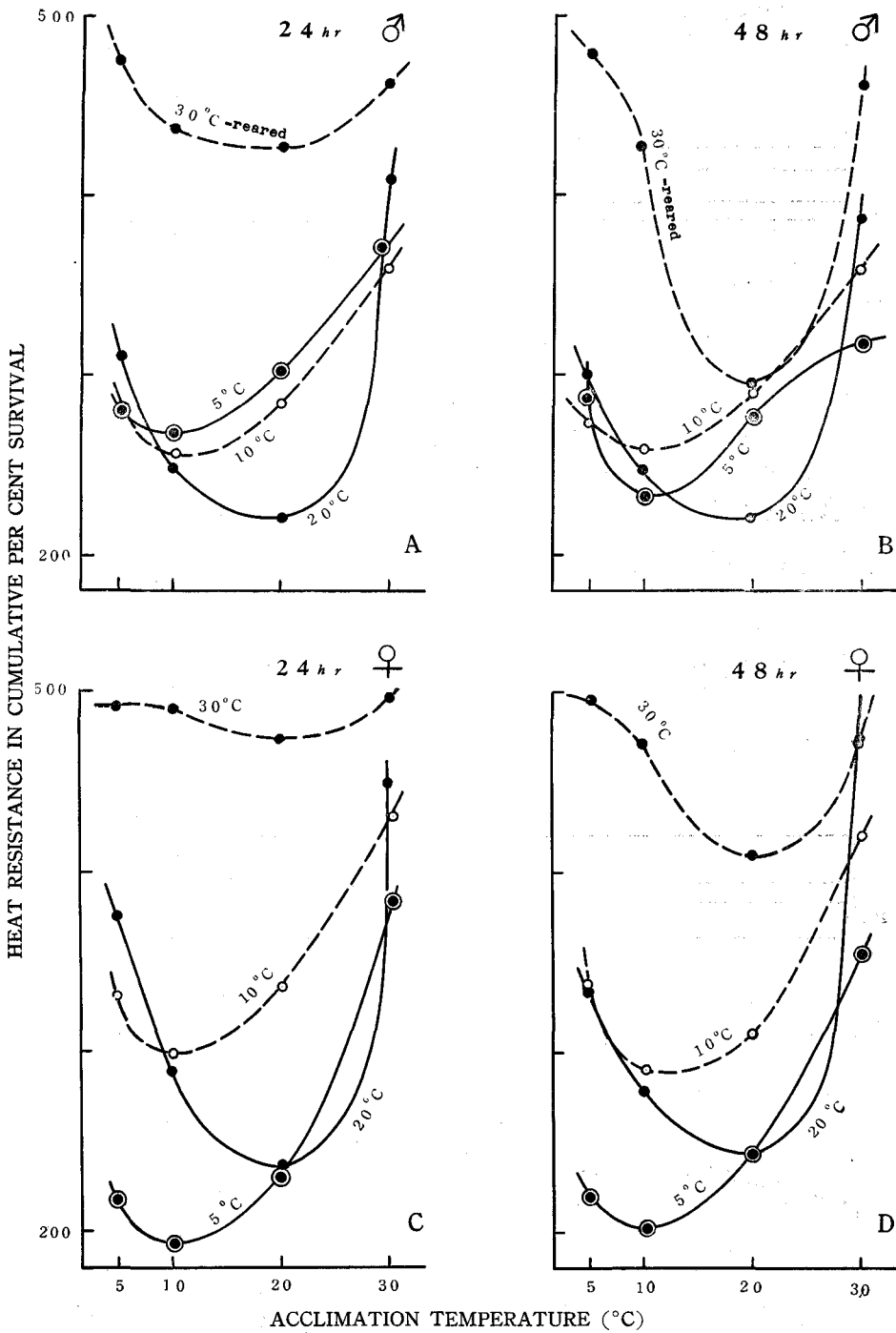Fig. 7. Heat resistance of adults of *Tigriopus japonicus*, as indicated by 5-hour
cumulative per cent survivals at 38.6°C, 24 and 48 hours after transfer
from four different rearing temperatures stated to four different acclima-
tion temperatures.

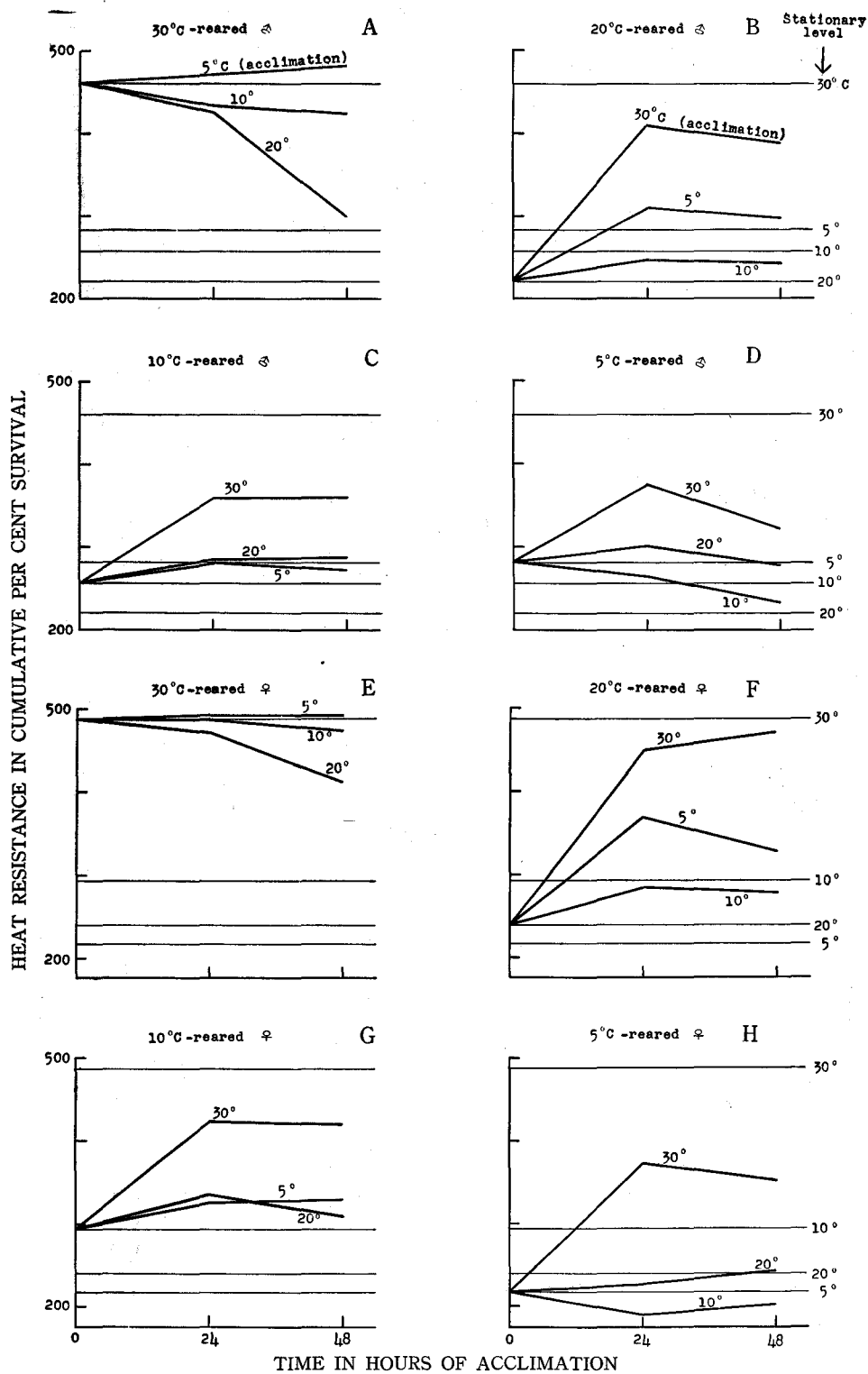Fig. 8. Change of heat resistance of adults of *Tigriopus japonicus* as indicated by
5-hour cumulative per cent survivals at 38.6°C, 24 and 48 hours after
transfer from the rearing temperatures, 5°, 10°, 20° and 30°C to four
different acclimation temperatures stated. Fine horizontal lines indicate
stationary levels of heat resistance to be reached when acclimatization is
completed at the acclimation temperatures.

resistance in a temperature group of animals during the culture extending over several months, the stationary level may be different from that which is estimated.

Fig. 8 shows the absolute rate of change of heat resistance. Since the acclimation temperature–heat resistance curves are not linear and the stationary levels are not equally spaced, it may be necessary to take into consideration the relative

Table 1. Change of heat resistance of adults of *Tigriopus japonicus*, reared at four different temperatures, 24 and 48 hours after transfer to four acclimation temperatures, in percentages of the change from the original stationary level of heat resistance for a rearing temperature to the level for an acclimation temperature, and direction of change (arrows) to reach the latter level, negative signs showing changes reverse in direction to those indicated by arrows.

♂		Acclimation temperature (°C)							
		5°		10°		20°		30°	
		after 24 hr	after 48 hr	after 24 hr	after 48 hr	after 24 hr	after 48 hr	after 24 hr	after 48 hr
Rearing temperature (°C)	5°			↓63	↓174	↓−33	↓8	↑52	↑22
	10°	↑100	↑74			↓−86	↓−86	↑51	↑51
	20°	↑138	↑120	↑72	↑66			↑79	↑71
	30°	↓−6	↓−11	↓15	↓19	↓14	↓66		

♀		Acclimation temperature (°C)							
		5°		10°		20°		30°	
		after 24 hr	after 48 hr	after 24 hr	after 48 hr	after 24 hr	after 48 hr	after 24 hr	after 47 hr
Rearing temperature (°C)	5°			↑−38	↑−19	↑57	↑119	↑57	↑50
	10°	↓−46	↓−46			↓−88	↓−34	↑68	↑66
	20°	↓−619	↓−428	↑85	↑72			↑84	↑93
	30°	↓−2	↓−2	↓0	↓7	↓6	↓31		

rate besides the absolute rate of change of heat resistance. In this connection, Table 1 was made up in which the relative rate of change is given in percentages of the whole change from an original stationary level to a new stationary level to be reached when acclimatization is completed.

Fig. 8 and Table 1 show that the initial direction and the rate of change

vary significantly according not only to acclimation temperature but also to rearing temperature. It was stated by a number of investigators (LOEB and WASTENEYS 1912, DOUDOROFF 1942, BRETT 1944, 1946) and is generally accepted that gain of heat resistance of poikilotherms in acclimation to higher temperatures is much more rapid than loss in acclimation to lower temperatures. The present data have many respects which contradict with this statement as the data obtained by TSUKUDA (1960) with the guppy, *Lebistes reticulatus*, have. The rule certainly holds good between 20° and 30° but does not over the whole range of temperature from 5° to 30°, which is conceivably due to the complicated relationship between acclimation temperatures and heat resistance such as represented by the U–shaped curves.

In the greenfish, *Girella nigricans*, DOUDOROFF (1942) observed an initial rise before gradual decline of heat resistance in fish acclimated from 26° to 14°. SPOOR (1955) also noticed a similar phenomenon in the crayfish, *Orconectes rusticus*, transferred from 22°–26° to 4°. Such initial rises are acknowledged in the present data, not only in acclimation to lower temperatures (Fig. 8 A and E, 5° acclimation; F, 5° acclimation; G, 5° acclimation) but also in the case of loss of heat resistance in acclimation to higher temperatures (Fig. 8 C, D and G, 20° acclimation).

Experiment 3.

Cyanide resistance of *Tigriopus japonicus* in relation to acclimation temperature

The resistance to cyanide of *Tigriopus* acclimated to different temperatures was tested to see whether there are or there are not physiological differences other than that in heat resistance between the individuals which have gained heat resistance through acclimation to a high temperature of 30° and those which have become resistant to heat through acclimation to low temperatures of 10° and 5°.

Materials and methods

Tigriopus was kept in a mass at 19°C for 30 days. From this culture adult animals were transferred to four different acclimation temperatures of 2°, 9°, 19° and 28°C, at which they were maintained for 4, 20 and 46 hours, in the same way as described above, before they were tested for their resistance to cyanide. The test was made by keeping them by 100 individuals in a hemispherical glass bowl, 55 mm in diameter, containing 22 ml of M/20 NaCN sea water at 19°C, for 2 hours during which mortalities were recorded every 15 minutes (Fig. 9).

Results and consideration

From each time-per cent survival curve obtained, the 2-hour cumulative per cent survival, *i.e.*, the sum of survival percentages observed every 15 minutes for 2 hours, was computed as in the case of the heat resistance test described in the foregoing chapters. Using this value as the index of cyanide resistance, the acclimation temperature–cyanide resistance curves were drawn (Fig. 10).

These curves indicate that there are pronounced differences in cyanide resistance among the four temperature acclimation groups, which vary significantly with time. After 4 hours in acclimation, the lower the acclimation temperature, the greater is cyanide resistance. Then cyanide resistance increases relatively rapidly with time in 28°-acclimated animals, while it decreases appreciably in 9°-acclimated males and 2°-acclimated females. In 20°-acclimated males and 9°-acclimated females, however, the decreasing change of cyanide resistance is little significant.

Rearing temperature   Acclimation temperature   Test
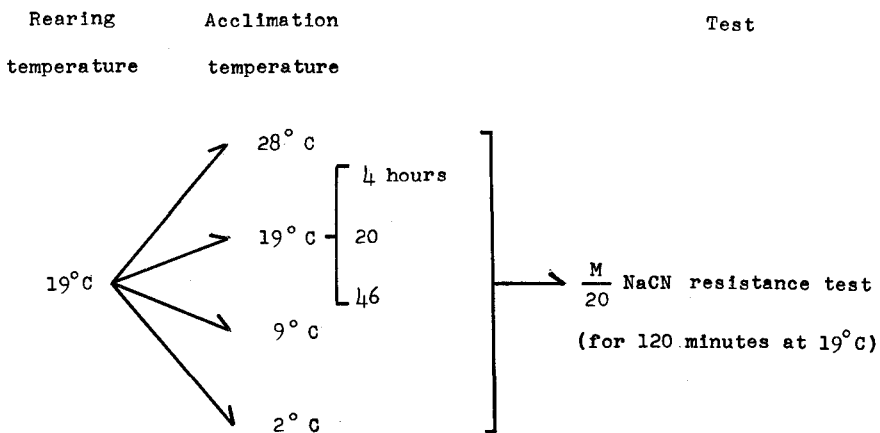

Fig. 9. Procedure of experiment 3.

Since cyanide inhibits oxidative processes, a high level of cyanide resistance is considered as indicating primarily a depressed activity of aerobic metabolism, which in turn is perhaps associated with a reduced rate of oxygen consumption. It is a well established fact that the rate of oxygen consumption of poikilotherms decreases with falling temperature within the normal range of temperature (for crustaceans, *c.f.* MARSHALL and ORR 1955; also see BULLOCK 1955, FRY 1957, PRECHT 1958), and it has also been demonstrated by many investigators (SAYLE 1928, KIRBERGER 1953, BLAZKA 1955, KANUNGO and PROSSER 1959) that temperature acclimation has usually a compensatory effect on the rate of oxygen consumption.

When animals acclimatized completely to 19° are transferred from this rearing temperature to 2°, they become considerably inactive, remaining almost immobile

at the bottom of vessel, probably, in a state of chill coma. In this state, their metabolism is supposed to be extremely depressed. Those individuals which have fallen into such a state do not restore readily their activity after they are returned back to 19°. If, however, they are kept longer at 19°, their activity recovers to some extent owing to 'capacity adaptation or acclimatization' (Precht 1958). In fact, some of 2°-acclimated animals were observed to swim slowly 2-3 days after transfer. The gradual decrease of cyanide resistance in 2°-acclimated females (Fig. 10) certainly indicates the effect of capacity acclimatization in aerobic metabolism. In males, the effect is far less explicit in 2° acclimation than in 9° acclimation.
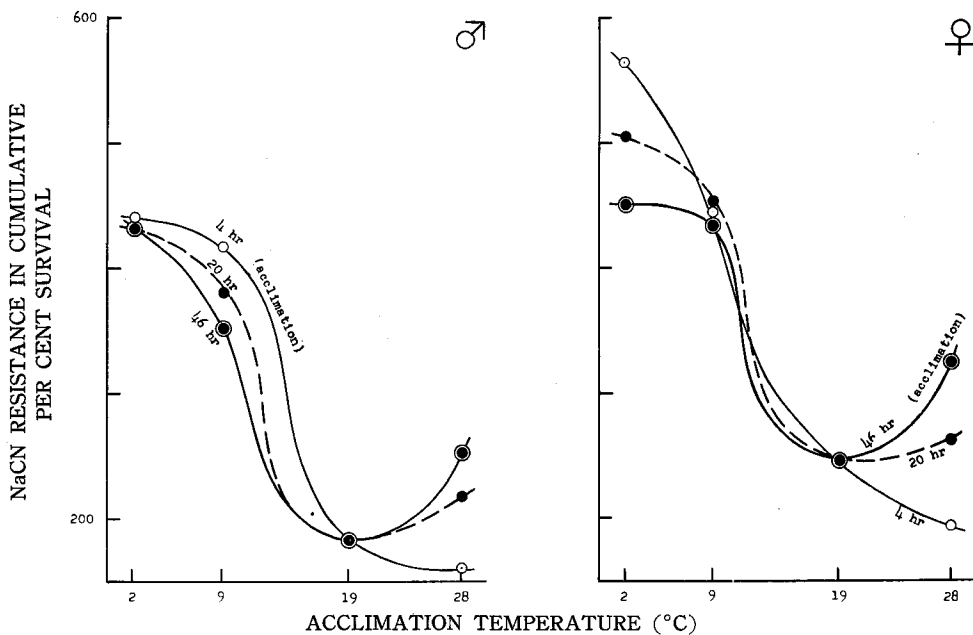

Fig. 10. Cyanide resistance of adults of *Tigriopus japonicus*, 4 (empty circles), 20 (solid circles) and 46 (double solid circles) hours after transfer from a rearing temperature of 19°C to four different acclimation temperatures. Cyanide resistance is indicated by 2-hour cumulative per cent survivals in M/20 NaCN sea water at 19°C, observed at intervals of 15 minutes.

When, on the other hand, 19°-reared animals are transferred to 28°, their activity increases. Very low values of cyanide resistance for individuals acclimated to 28° for 4 hours reflect the activated state of aerobic metabolism. Here also capacity acclimatization is evidently acknowledged in individuals acclimated to 28° for prolonged periods. The appreciable gains in cyanide resistance in 28° acclimation indicate such a compensatory effect.

Comparing the result obtained by this experiment with those obtained by

the foregoing, some parallelism is perceived between cyanide resistance and heat resistance over the range of acclimation temperature from 2° to 19°. But the relation cannot be applied to 28° acclimation in which cyanide resistance is still far lower than that in 9° acclimation even after 48 hours while the heat resistance of 30°–acclimated animals exceeds that of 5°–acclimated animals within 24 hours of acclimation (Figs. 7 and 10). SUMNER and DOUDOROFF (1938) have shown that fish acclimated to 30° are more resistant to cyanide, urethane and oxygen lack than those acclimated to 10° when compared at 20°. This relationship suggesting a reduced oxygen requirement as a result of acclimation to the high temperature agrees with that observed between 19° and 28° but does not with that observable among the temperatures lower than 19° in the present data.

The results obtained show that there are more or less distinct differences in aerobic metabolism between animals acclimated to 2° and 9° and those acclimated to 28°, and suggest that the acquirement of heat resistance at the low acclimation temperatures is different in mechanism from that at the high acclimation temperatures.

Experiment 4.

Temperature acclimatization in the heat resistance of *Tigriopus japonicus* in relation to environmental oxygen concentration

The preceding experiment has indicated a high degree of probability that the acquirement of heat resistance at the low acclimation temperatures is caused by the inactivation of aerobic metabolism due to cooling. This suggests a possibility that if the metabolic activity of animals is depressed by some agent other than coldness, they will also gain heat resistance. To examine this possibility, *Tigriopus* was acclimated to 20° and 30° in high and low concentrations of oxygen.

Materials and methods

Adult animals of *Tigriopus* sampled at random from the 20° culture were put by 100 individuals into a test tube, 30 mm in diameter by 195 mm long, containing 85 ml of sea water of a given oxygen saturation at an acclimation temperature of 20° or 30°. The test tube thus prepared was immediately closed with a rubber stopper and kept at that acclimation temperature for 35 or 59 hours before the animals were tested for their heat resistance in the same way as was described previously. The apparatus used for controlling the oxygen concentration in acclimation sea water is schematically illustrated in Fig. 11. To decrease the concentration of oxygen in sea water nitrogen gas flowed through the tubing *L* was bubbled into sea water in the equilibration tank *B* which was
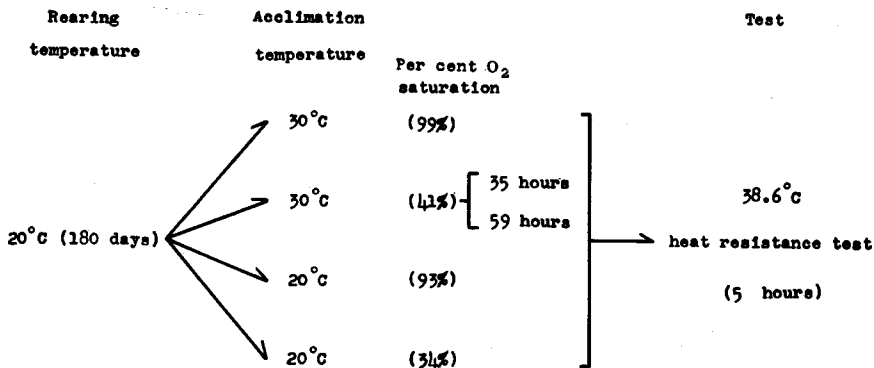
immerged into a water bath A controlled thermostatically at a given temperature. The flux of nitrogen gas was measured by a graduated cylinder F and was controlled by cocks. Sea water was sampled at times from the tank B through the tubing C and the oxygen concentration was examined by the Winkler method. When the latter was decreased to a required level, sea water was introduced into the test tube for acclimation K which had been filled with nitrogen gas at the

Fig. 11. Schematic diagram of the apparatus to obtain sea water of a
          given level of oxygen concentration.
A : thermostatic water bath.
B : sea water tank for equilibration of temperature and of nitrogen
    gas.
C : glass tubing for drawing out sea water from the tank.
D : Winkler flask.
E : reservoir for supplying sea water.
F : graduated cylinder for measuring the flux of nitrogen gas.
G : glass tubing for gas exhaust.
H : heater.
I : regulator.
J : stirrer.
K : test tube for acclimation.
L : inlet for nitrogen gas.
M : cocks.

acclimation temperature. To obtain sea water of high oxygen saturations, aerated sea water piped from the reservoir E was kept in the tank B for about 24 hours without bubbling in thermal equilibrium with the water bath A. Thus, high and low levels of oxygen concentration were prepared for either acclimation temperature (Fig. 12).

Rearing temperature	Acclimation temperature	Per cent O$_2$ saturation		Test

Fig. 12. Procedure of experiment 4.

Results and consideration

The results obtained are shown in Fig. 13 A and B in the time–survival relations, from which it is obvious that 30°–acclimated animals are by far more resistant to heat than 20°–acclimated ones and that animals acclimated at low levels of oxygen saturation are always significantly more heat resistant than those acclimated at high levels.

BRETT (1944, 1946) found in the bullhead, *Ameiurus nebulosus*, that a continuous low oxygen saturation of the water in the acclimation tank inhibited acclimatization to heat up to 23 hours. Namely, fish acclimated from 20° to 28° in adequate oxygen saturation which ranged from 64 to 74% appeared to have completed virtually temperature acclimatization within 23 hours, while those acclimated from 17° to 27° in very low oxygen saturation which ranged from 2 to 26% showed a distinct lack of acclimatization. He considered that oxygen played some positive role in the mechanism of heat acclimatization. His findings apparently conflict with the present results. It must be noticed, however, that the level of insufficient oxygen adopted by Brett is extremely low in comparison with that used in the present experiment and that the level of sufficient oxygen adopted by him is also markedly low. Exact comparison is, therefore, impossible between the two data. In *Tigriopus*, the acclimation in 10.7% O$_2$ saturation was found impracticable causing 22 to 35% mortalities, though the acclimation in 34% or 41% O$_2$ brought about no ill effect. Superposing the present data on the Brett's, it would be suggested that there might be an optimal O$_2$ concentration for temperature acclimatization to take place. But, this conception should be evaluated by a further investigation.

At all events, *Tigriopus* appears to be relatively sensitive to oxygen deficiency, and its aerobic metabolism is supposed to be suppressed not a little in such low levels of oxygen as 34% and 41%. This inactivated state must have some
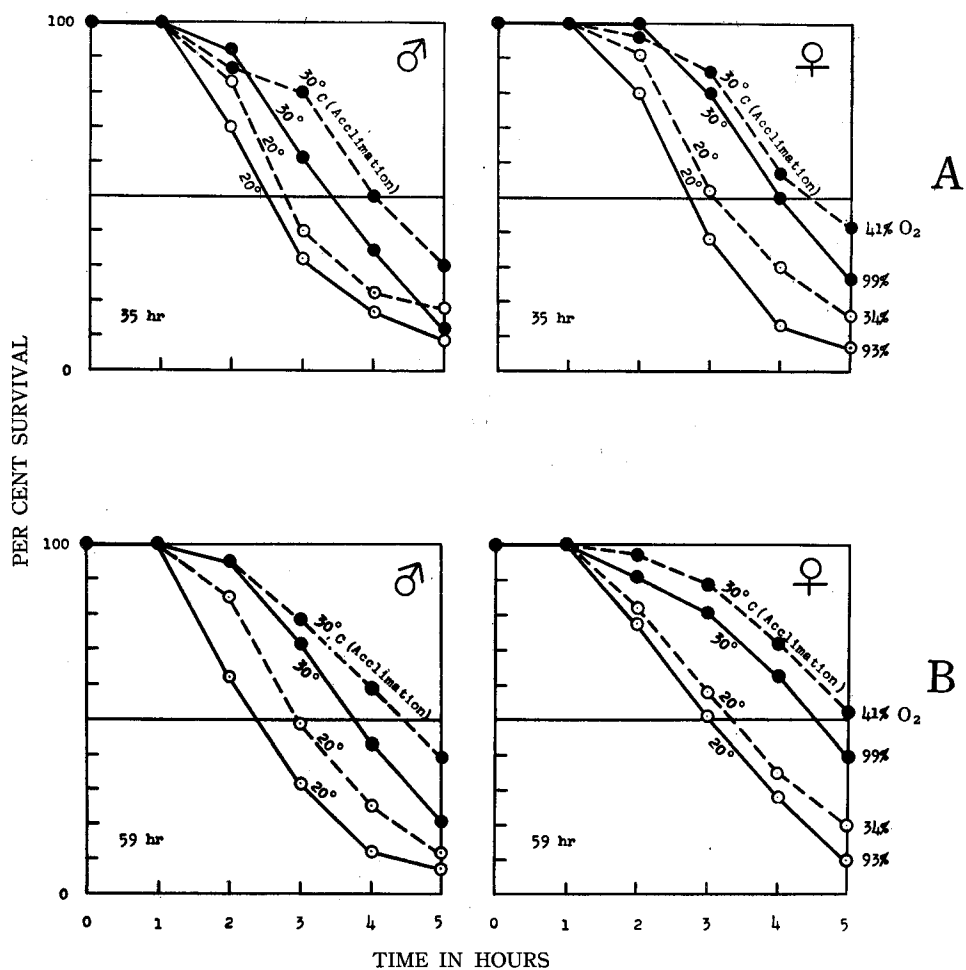
Fig. 13 (A–B). Time-survival curves at 38.6°C of adults of *Tigriopus japonicus* acclimated to 20° (empty circles) and 30°C (solid circles) for 35 and 59 hours at high (solid lines) and low (broken lines) levels of O_2 saturation.

favorable effect on the heat resistance of animals in the subsequent exposure to a high temperature.

Experiment 5.

Heat resistance in relation to salinity, of *Tigriopus japonicus* acclimated to dilute and concentrated sea waters

The foregoing series of experiments has been focused on the mechanism through which *Tigriopus* gains heat resistance when it is warmed or cooled

from its optimal temperature. Viewed from such a standpoint, the present ex-
periment is rather secondary in nature. As already mentioned, salinity is without
doubt an environmental factor for *Tigriopus* no less important than temperature.
It is expected that the former is closely associated with the latter in their effects
on the physiology of *Tigriopus* as it is in their fluctuations in the natural environ-
ment of this species. So it is highly interesting not only from the physiological
viewpoint but also from the ecological viewpoint to see how the heat resistance
of this species is related with ambient salinity.

Materials and methods

Adults of *Tigriopus japonicus* from the culture in 100% sea water (18.46‰
Cl) at 20°C were acclimated to 25% and 150% of sea water for definite periods
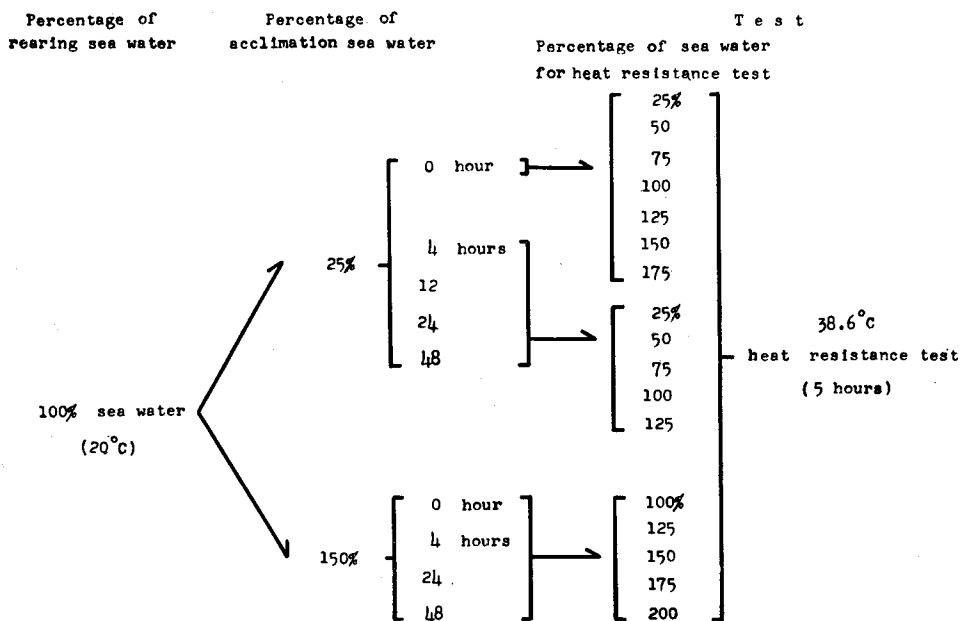

Fig. 14. Procedure of experiment 5.

of time as shown in Fig. 14. Then they were tested for their heat resistance, as
described above, in various per cent concentrations of sea water (Fig. 14). The
concentrations used for either salinity acclimation group were within the range
of concentration to which *Tigriopus* could be transferred without deleterious
effects at 20°C (MATUTANI, unpublished data).

Various concentrations of sea water were obtained by diluting 200% sea
water, which was prepared by evaporation at a temperature below 40°C, with
fresh water purified by ion exchange.

Results and consideration

For the convenience of comparison, the values of heat resistance, expressed in 5-hour cumulative per cent survivals, are plotted against the per cent concentration of test sea water in Figs. 15 and 16.

The 0 hour curves *i.e.* curves for the control 100%-acclimated animals have their apexes at 150% without exception and are almost symmetric in shape falling down gradually on either side of this concentration. When animals are acclimated to 25% sea water, their heat resistance markedly decreases on the whole with the duration of acclimation time and at the same time the concentration in which they show the highest resistance to heat becomes lower (Fig. 15). After 48 hours of acclimation, the maximum resistance is given at 50% in males and at 75% in females. In 150%-acclimated animals, on the other hand, heat resistance increases pronouncedly within 24 hours of acclimation and then it decreases to some extent (Fig. 16). After 4 hours, the increase in resistance is much larger on the higher concentration side of 150% than on the lower side. After 24 hours, curves become much flattened, especially in females, and the concentration in which resistance is highest is found shifted to 175% from 150%. After 48 hours, however, resistance falls appreciably on the higher side in males and on both lower and higher sides in females, though it is well preserved in 150% in both sexes.

There are not a few cases reported with various animal forms and with different levels of organization in which heat resistance is higher in hypertonic media than in isotonic or hypotonic media. This is the case, for example, with the frog muscle (VERNON 1899), *Paramecium* (HUTCHINSON 1913, 1915) and the periwinkle, *Nodilittorina granularis* (OHSAWA and TSUKUDA 1956). There is however an optimum salinity, for 100% sea water-acclimated animals to withstand heat, at about 150% sea water, above which resistance decreases. TOKIOKA and SUZUKI (1939) reported the similar tendency.

The phenomenon that the heat resistance of marine invertebrates increases with the salinity to which they are acclimated has also been observed by some authors. Thus, KINNE (1958) has shown that the heat resistance of the polychaete, *Nereis diversicolor*, the amphipod, *Gammarus duebeni*, and the isopod, *Sphaeroma hookeri*, is lowered in low salinity-adapted animals and raised in high salinity-adapted animals. According to his view, one cause for the shift of heat resistance is obviously the alteration of water and ionic balance in animals under stress. It is likely that dehydration is involved in this phenomenon, which can increase general protoplasmic resistance to extreme temperatures (PRECHT 1958).

There is at present no information available on the osmotic relation of *Tigriopus* to its medium, though there are detailed studies on the osmotic regulation of *Gammarus* (BEADLE and CRAGG 1940) and *Artemia* (CROGHAN 1958 a, b).
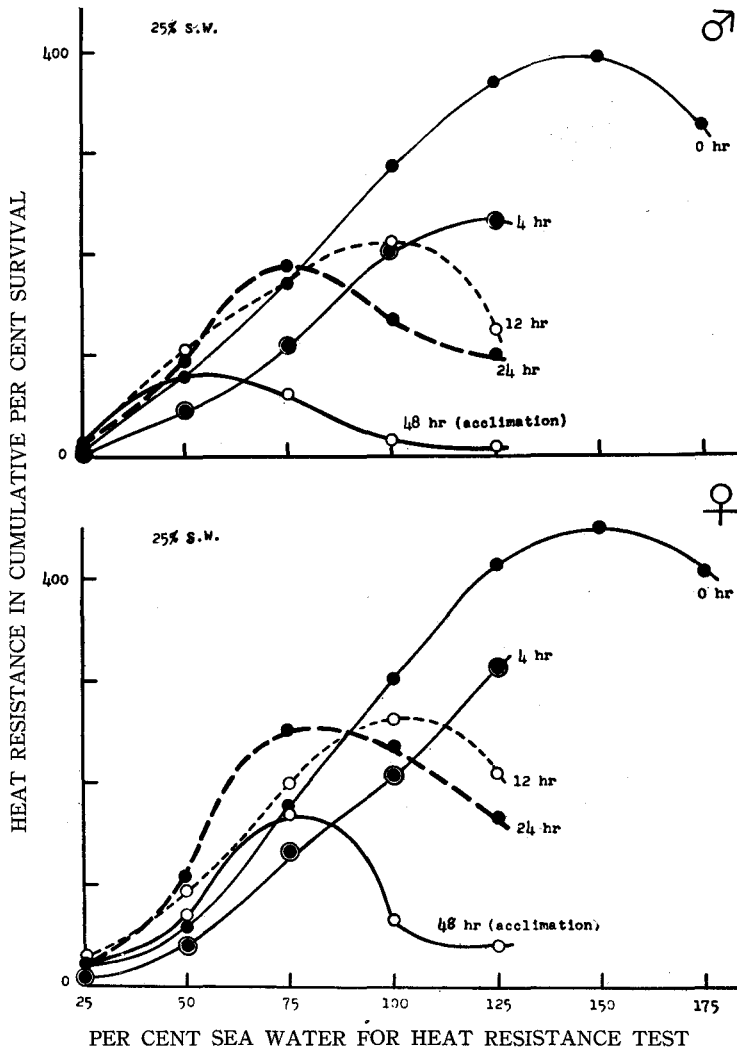
Fig. 15. Heat resistance in various per cent concentrations of sea water of
adults of *Tigriopus japonicus* acclimated from a rearing concentra-
tion of 100% to 25% sea water at 20°C for definite lengths of time
from 4 to 48 hours. Heat resistance is indicated in 5-hour cumula-
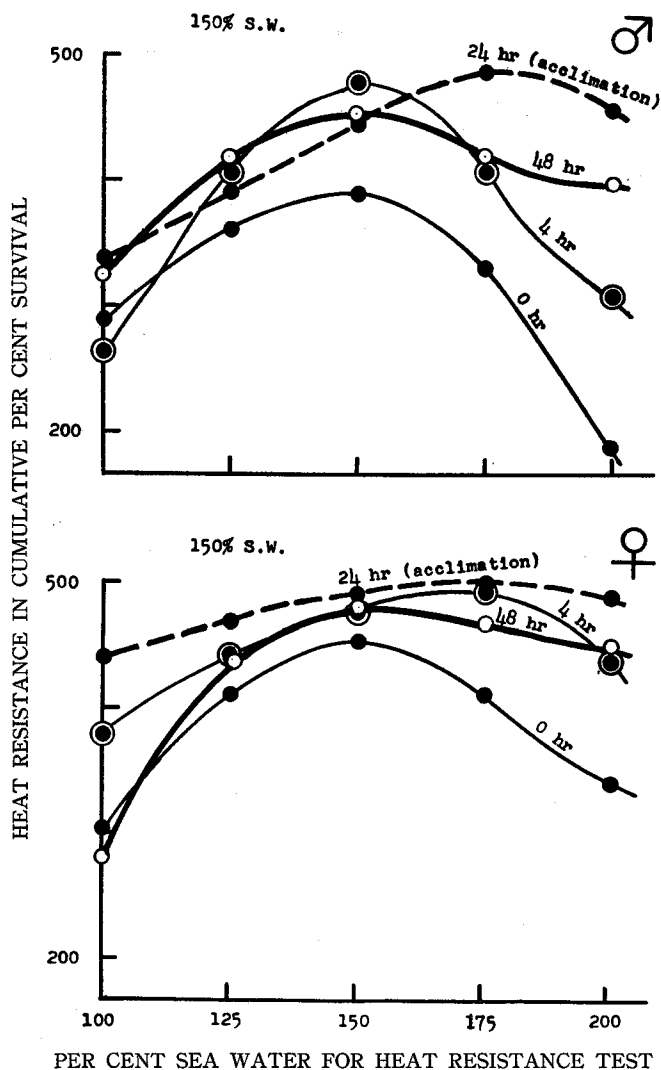tive per cent survivals at 38.6°C.

Fig. 16. Heat resistance in various per cent concentrations of sea water of adults of *Tigriopus japonicus* acclimated from a rearing concentration of 100% to 150% sea water at 20°C for definite lengths of time from 4 to 48 hours. Heat resistance is indicated in 5–hour cumulative per cent survivals at 38.6°C.

The relatively rapid decrease of the optimum salinity for heat resistance in 25% sea water–acclimated animals and the little increase of the optimum salinity in 150% sea water–acclimated animals appear to indicate that the ability of osmotic regulation of this species is comparatively low in the hypotonic medium and high in the hypertonic medium. But there is no reason to regard the shift of the optimum salinity as reflecting the osmotic property of animals. It is, nevertheless, indubitable that *Tigriopus* is euryhaline, a fact which suggests a high degree of probability that it possesses a somewhat evolved osmoregulatory function in comparison with its pelagic relatives. At any rate, the explanation of the phenomena observed must wait for further experiments.

Apart from the consideration on the underlying physiological processes, the relations obtained by the present experiment are very suggestive from the ecological point of view. Tide pools inhabited by *Tigriopus* are situated well above the high tide mark and rarely overflowed by waves unless the sea is raging. Sea water in such tide pools are readily concentrated by evaporation or diluted with rain water. Ordinarily, an extreme elevation of water temperature under the direct sun in summer months is accompanied with accerelated concentration by evaporation. The increase of heat resistance in a hypertonic medium is certainly advantageous for the life of *Tigriopus*. On the other hand, a rapid elevation of water temperature after a heavy rain, if it occurs, will constitute a crisis.

Discussion

The present study was undertaken for the purpose of elucidating the process of temperature acclimatization in the heat resistance of the eurythermal copepod *Tigriopus japonicus*. Exp. 1 revealed an unexpected phenomenon that heat resistance was gained not only by animals acclimated from 20° to 30° but also by those acclimated from 20° to lower temperatures. This finding has orientated the subsequent study in the direction of investigating the mechanism of acquisition of heat resistance in low temperature acclimation in comparison with that in high temperature acclimation.

The possibility that a change in the ambient temperature, whether upward or downward, might alone induce an increase in heat resistance has been denied by Exp. 2, which showed that heat resistance was markedly reduced in many cases, when the ambient temperature was changed, *e.g.*, from 30°to 20° and from 5° to 10°.

Then it has remained as the most important problem whether the mechanism underlying the acquisition of heat resistance in low temperatures is or is not the same in nature with that in high temperatures. Animals acclimated to 5° or 10° appear to be so different in their general physiological conditions from those

acclimated to 30° that it is quite unlikely that the mechanisms should be the same. The former are in a state like chill coma for some time after transfer, while the latter show much increased activity. Phenomenally it can be said that inactivation at low temperatures and activation at high temperatures bring about the same result which is the gain of heat resistance. Those relations seem to diminish very much the probability that heat resistance is gained through the same process in low temperature-acclimated animals and in high temperature-acclimated animals.

The working hypothesis preferred is that the acquisition of heat resistance at low temperatures is effected secondarily by the inactivation of general metabolism of animals and is accordingly altogether different in mechanism from that at high temperatures which is temperature acclimatization in the strict sense of the term. Temperature acclimatization in this sense is observable in many poikilotherms and may involves some fundamental alterations in their physiological properties. On the other hand, it is a problem difficult to interprete how the inactivation of metabolism causes the gain of heat resistance. A solution of the problem has perhaps to be pursued in the mechanism of heat death.

Concerning the mechanism of heat death, there are a few theories, none of which is decisive. The mechanisms may be different with different high temperatures at which resistance is tested and with different conditions of the resistance test. An important theory is that death at high temperatures is asphyxial (cf. FISHER 1958). Since the test temperature of 38.6°C is relatively low for a high lethal temperature, it seems more probable that on transfer to this temperature the metabolic rate of Tigriopus is elevated immediately up to the limit beyond which oxygen deficiency in tissues has a fatal effect than that the thermal alteration of body substances takes place in tissues. If the heat death of Tigriopus is asphyxial in the present case, the heat susceptibility of animals is directly related to their oxygen requirement at the test high temperature.

According to the experience of the writer, the optimum temperature for Tigriopus is 20° or thereabout. Hence animals acclimated to 20° are considered to have their physiological activities in most harmonized and efficient conditions. If such animals are put at the test temperature, their metabolic rate is accerelated extremely till at last heat death occurs. In animals acclimated to 30° the metabolic activity becomes decreased through temperature acclimatization and, when exposed to the test temperature, it does not rise so high as in 20°-acclimated animals. This contributes to heat resistance. On the other hand, when animals are acclimated to low temperatures such as 5° and 10°, their metabolic activity becomes considerably suppressed, and, on transfer to the test temperature, metabolic rate is elevated far less readily in them than in 20°-acclimated animals. This results in an increased level of heat resistance.

Thus, it is inferred that so far as the range of temperature is concerned

within which the acclimation experiments have been carried out, the higher the acclimation temperature, the greater is the effect of temperature acclimatization and the smaller is the effect of metabolic inactivation. Both effects would result jointly in the U–shaped acclimation temperature–heat resistance curves.

This theory finds strong support in the results obtained by Exp. 3 and also in the results by Exp. 4. The increased cyanide resistance in animals transferred to 20° from the lower acclimation temperatures at the early period of acclimation indicates the inactivated state of aerobic metabolism in these animals, and the decreased cyanide resistance in animals transferred from the higher temperature the activated aerobic metabolism in them. Temperature acclimatization presents itself as an effect to reduce the differences in cyanide resistance.

In this case, however, cyanide–insensitive metabolism cannot be neglected. There is evidence suggesting the alteration of metabolic pattern as a result of acclimatization to temperature (EKBERG 1958) as in the case of acclimatization to oxygen (PROSSER *et al.* 1957). After EKBERG, cold–acclimated goldfish depend relatively more on an active cyanide–sensitive path and warm–acclimated ones on an iodoacetate–sensitive path. There is, however, no information available at present on the cyanide–insensitive metabolism of *Tigriopus*. The problem is open for further study. The acquirement of heat resistance by animals acclimated to low oxygen can also be interpreted as resulting from the inactivation of aerobic metabolism under the condition of oxygen deficiency.

A dualistic conception about the mechanism of acquirement of heat resistance in *Tigriopus* has been concluded from the results of the present series of experiments. It is not clear whether this conception is restricted to *Tigriopus* or applicable to other poikilotherms. There is a possibility that such phenomena as have been observed in *Tigriopus* may be found in other forms if acclimation experiments are performed over relatively wide ranges of temperature, since, in *Tigriopus*, only simple relations showing the ordinary effect of temperature acclimatization would have been obtained if acclimation had been confined within a narrow range of temperature, say, from 20° to 30°.

Summary

Change of heat resistance was investigated in relation to the environmental temperature and salinity with the euryplastic microcopepod, *Tigriopus japonicus*, which inhabits tide pools situated well above the high tide mark. Heat resistance was tested by exposing animals to 38.6°C for 5 hours and determining the mortality every hour in definite lengths of time after acclimation to various conditions.

1. When animals from 20°–reared culture were acclimated to four different temperatures of 5°, 10°, 20° and 30°C, the highest heat resistance is shown by 30°–acclimated individuals and the lowest resistance by 20°–acclimated ones, those

acclimated to 5° being next to the former in resistance. Thus U–shaped accli-
mation temperature–heat resistance curves result in either sex over the range from
5° to 30°C.

2. When animals which have been reared at four temperatures of 5°, 10°,
20° and 30° are respectively transferred to the four acclimation temperatures,
also U–shaped acclimation curves are obtained in each case. Heat resistance is
lowest in 10° acclimation for 5°– and 10°–reared animals and in 20° acclimation
for 20°– and 30°–reared animals. In 30°–reared animals, 5° acclimation results in
a slightly greater resistance than 30° acclimation. The results disproves the
possibility that a change in the environmental temperature alone may induce a
gain of heat resistance. Moreover, they show the relations which contradict
with the well–established principle of temperature acclimatization that heat
resistance is gained much more rapidly on warming than it is lost on cooling.

3. In 20°–reared animals, the lower the acclimation temperature, the higher
is the resistance to cyanide when tested at 20°. This suggests that aerobic
metabolism is inactivated in acclimation to lower temperatures and activated in
acclimation to higher temperatures so that it does not restore readily its original
level on returning to 20°. Cyanide resistance somewhat decreases gradually with
time in 5° and 10° acclimation and increases in 30° acclimation, conceivably owing
to the effect of true temperature acclimatization.

4. Those animals whose respiration has been suppressed for 35 and 59 hours
in reduced oxygen concentrations, i.e. 34% oxygen in 20° acclimation and 41%
oxygen in 30° acclimation, prove appreciably increased heat resistance in com-
parison to those which have been kept in sufficient concentrations of oxygen,
i.e. in 93% oxygen in 20° acclimation and 99% oxygen in 30° acclimation.

5. Heat resistance changes markedly with the salinity of acclimation sea
water as well as with the salinity of test sea water. The heat resistance of the
control, i.e., 100% sea water–acclimated animals is highest in 150% sea water and
declines gradually whether the concentration of test sea water is decreased or
increased. In 25% sea water–acclimated animals, heat resistance falls rather rapidly
with time and the concentration of test sea water in which they are most resistant
to heat moves toward lower concentrations till it reaches 50% after 48 hours of
acclimation. In 150%–acclimated animals, on the other hand, heat resistance
increases significantly till after some 24 hours and the peak appears to be shifted,
though slightly, toward higher concentrations.

These relations are considered to be advantageous for *Tigriopus* to survive
elevations of water temperature in summer months which are liable to be accom-
panied by concentration of sea water due to evaporation.

6. It is inferred from the above results 1–4 that the acquisition of heat
resistance in acclimation to temperatures below 20°, i.e. the optimum temperature
for the life of *Tigriopus*, is different in mechanism from that in acclimation to

higher temperatures. The latter is certainly caused by temperature acclimatization in the strict sense of the term, while the former should be ascribed to the in-activation of aerobic metabolism by cold, which leaves its effect fairly long after temperature is raised.

Acknowledgements

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EXPLANATION OF PLATES XVI–XVII

PLATE XVI

Fig. 1. View of the rocky shore of Sakinoyu, near the Seto Marine
Biological Laboratory, where *Tigriopus japonicus* MORI occurs
abundantly.

Fig. 2. Near view of the natural habitat of *Tigriopus japonicus* MORI,
showing two tide pools on the upper rocky shore, where ex-
perimental materials were collected.

PLATE XVII

Fig. 3. Adult male of *Tigriopus japonicus* MORI.

Fig. 4. Adult female carrying an egg sac.

Fig. 5. Adult male killed by heat.

Fig. 6. Adult female killed by heat.