Effect of temperature on waterflea *Daphnia magna* (Crustacea:Cladocera)

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Acclimation of the waterflea *Daphnia magna* Straus (Crustacea: Cladocera) at 2 ^{o} to 12 ^{o} C above their habitat temperature (16 ^{o} C) for 6 months increased the rate of their metabolic activity (respiration and heartbeat rates). Temperature-enhanced activity appeared to be supported by cellular ATP synthesis (increased cytochrome c oxidase and succinic dehydrogenase activities) and hydrolysis (increased ATPases activities), indicating high rate of intermediary metabolism, the substrates for which may come from the stored glycogen and fat in addition to ingested food. Temperature-enhanced activity was associated with loss of body mass and decrease in body size, both of which may result from hyperactivity (causing lack of replenishment of consumed stored glycogen and fat) and loss of body water (due to hyperosmolarity of water because of evaporation). Hyperthermia-caused hypoxia was associated with increase in hemoglobin (Hb) synthesis (a typical daphnid response to hypoxia). These responses to hyperthermic stress started showing up after one month of acclimation at 27 ^{o}-29 ^{o} C and after 6 months at lower temperature ranges (18 ^{o}-20 ^{o} C) indicating the possibility of initiation of similar responses in field populations at current expected rise in global temperature due to climate change. Since the thermal stress will continue for generations, the effects observed with *D. magna* may start showing up in other small aquatic poikilotherms; indicating the speeding up of their life processes and of their gametes, embryos, larvae, and young adults. Such responses can affect aquatic ecosystem dynamics.

Recent public awareness of global climate change[1-3] has created concerns about its impact on the Earth's ecosystems extending all the way to human societies. This has prompted investigations on various aspects of the effects of global warming on world's ecosystems. The expected increase in surface temperature, which can range from > 1^{o} to 2^{o} C [1-3], can affect the life processes of those organisms whose metabolism is increased by temperature (thermoconformers / poikilotherms)[4]. This thermal input can be more stressful to aquatic poikilotherms at lower levels of organization and to their gametes, embryos, larvae, and young adults as well as to those of other aquatic animals. This is assumed; because water, which is ~1000-time denser than air and contains 0.008% oxygen (v/v) compared to >20% in air [5,6], looses its dissolved oxygen with increase in temperature and salinity. Thus, an increase in water temperature can (i) increase the metabolism of its organisms; (ii) decrease its oxygen concentration at a time when organisms need more oxygen to support their temperature-enhanced metabolic activity, (iii) increase salinity due to evaporation. These secondary factors can put poikilotherms under osmotic and metabolic stresses, which can make them more susceptible to other environmental challenges, such as chemical pollutants[7] (whose concentration in water may increase due to evaporation and desorption), pathogens, etc. Such compounding effects of thermal inputs can be damaging to entire ecosystem, the life processes of whose organisms may be speeded up.

In nature, populations of an allopatric species (eurythermic) with wide geographical distribution have their metabolic rates adjusted to ambient temperature and its diurnal and seasonal fluctuations [4,6], mostly by avoidance behavior. Also, the metabolism within the same population can vary among its individuals (embryos, larvae, young adults, mature and old male and females, etc). Thus, the ability of the individuals in a population or of the populations to tolerate increase and fluctuations in temperature, can determine their survival under current thermal stresses.

This laboratory has been studying the effects of temperature on poikilotherms including eurythermic aquatic (benthic zebra mussels, shallow water crayfish, and subsurface daphnids) [8-10] and stenothermic semiterrestrial (earthworm *Lumbricus terrestris*) [11, 12] invertebrates. The metabolism of all these species increases with temperature, which also makes them more sensitive to chemical pollutants, such as heavy metals (metabolic inhibitors) [8-12].

RESULTS

This report provides preliminary data about effects of increasing temperature on aquatic organisms using waterflea *Daphnia magna* as a model. These findings indicate that as the temperature is increased up to 6° C and higher above the ambient (16° C), the daphnids become more active, increase their rates of breathing (Fig. 1) and heartbeat (Fig. 2) and adjust to a lower body mass and smaller size (Fig.1). The demand for more oxygen by the increased rate of metabolism is met by increased rates of hemoglobin (Hb) synthesis (Fig. 3), respiration, heartbeat, etc., which are compensatory responses to hypoxia [13-15]. As the normoxia (10-11 mg oxygen.L-^{1}) lowers to moderate (7 mg.L-^{1} or less) and nearly severe (5 mg.L^{1} or less) hypoxia (Table 1) the more active daphnids need more fuel to produce energy needed to support the increased rate of metabolism. This fuel may come from increased food intake and its consumption and the mobilization of fat stored in fat cells. The increased utilization of food fuel to provide energy for increased activity by daphnids reduced their body weight and size (Taable 1) at and above 18° C (Fig. 1). The reduction in body size and mass, as indicated by the wet weight: dry body weight ratios, which decreased with temperature (Table 1), seems to be caused mostly by loss of body water related to an increase in osmolarity of habitat water (due to evaporation) [10].

The increase in activity and need for transporting and utilizing oxygen to support high level of activity involves various biochemical and physiological adjustments. The hypoxia, as has been reported [13-15], more than doubles the synthesis of Hb2 isoform (Fig. 3) with higher affinity for oxygen [16, 17]. This should facilitate the transport of oxygen to cells where glucose and fatty acids are broken down to provide energy to support the increased rate of metabolism. The activity of cytochrome c oxidase increased by 100% at 29° C as compared with that at 16° C (Fig. 3). Additionally, the activities of mitochondrial succinic dehydrogenase (umole $K_3Fe(CN)_6$ reduced.min.mg protein-1: 16-18° C= 8.17±0.782 versus 15.57±1.12 at 27° C) and ATPases also increased by about 100% (Fig. 4), the latter indicating that increased activity is supported by the hydrolysis of ATP[11]. A slight lowering of the activities of these enzymes at 29° C may indicate this temperature to be the upper limit of these mechanisms to support the sustained hyperactivity.

The foregoing changes observed at above ambient temperatures, 2° C and higher, may indicate the possibility of these adjustments at lower range of temperature increase (1° to 2° C) in field populations of this and other aquatic poikilotherms. The impact of such changes at evolutionary and ecological level needs to be investigated.

DISCUSSION

D. magna has been shown to have genetic ability to tolerate high temperatures (25^0 C) [18-20] and low oxygen concentration better than *D. galeata mendotai* [21], *D. pulex* [22], and *D. obtuse* [13]. The hypoxic challenge induces compensatory mechanisms, which, in *D. magna,* include increases in respiratory activity [27], heme synthesis [13], and cytochrome c oxidase activity. The high rate of metabolic activity may demand increased utilization of glucose and stored fat. The depletion of these stores and lack of replacement of fat under sustained hyperactivity, may contribute partly to a decrease in body weight, most of which results from the hyperosmotic stress. Both of these stresses may contribute to the reduction in size and mass of *Daphnia*.

Induction of Hb synthesis by hypoxia has been long known in *Dphnia* [13-15]. The di-domain *D. magna* Hb (580 kDa), composed of at least 16 subunits, 36k Da each [23] , is a product of Hb gene *hb,* expressed in the epithelium of epipodites (bathed in oxygen-rich water) and fat cells (bathed in oxygen-poor hemolymph [24]). The isoforms of Hb synthesized under various oxygen concentrations are: Hb1: 11 mg oxygen.L $water^{-1}$ (normoxia) Hb2: ~6 mg oxygen.L^{-1} (moderate hypoxia): Hb2 , and Hb3: -3 mg.L-1 (severe hypoxia) [25, 26], The synthesis of new Hb2 and Hb3 isofroms, with 3- to 8-time higher affinity for oxygen, is increased by at least 4- to 8-fold [27]. Hb3 is synthesized in fat cells [24]; and if *Daphnia* body fat is depleted to support the high rate of metabolism at 27-29^0 C then fat cells may become dedicated to HB synthesis. Thus, fat- and water-depleted lighter and smaller daphnids (Fig. 1) may be better adapted to carry on increased synthesis of Hb3 to support the sustained high rate of metabolism. Induction of Hb synthesis via hypoxia-induced transcriptional activation of *hb* genes [24-27] also seems to increase the synthesis of other hemoproteins, such as cytochrome c oxidase in mitochondria. This may indicate the increased activity of electron transport system components to produce more ATPs and of ATPases to hydrolyze them to provide energy for increased locomotory, respiratory, and circulatory activities.

The compensatory mechanisms at elevated temperatures may be working at full/ peak capacity and may become sensitive to any interruption by other environmental stresses, such as chemical pollutants, pathogenic agents, etc. Thus, in addition to the observed direct effects on poikilotherms, the hyperthermic stress can exagerate the negative effects of otherwise non-expressive environmental factors. For example, an increase in rate of respiration should increase the rate of inflow of water and, thus, of dissolved pollutants into the bodies of daphnids. The hyperthermia can also increase the concentration of chemical pollutants in water due to (i) their desorption from surfaces [28], (ii) evaporation of water, and (iii) increase in solubility of chemicals in water [29]. Daphnids and other small organisms, their gametes, eggs, embryos, larvae, young adults, etc., because of their high rate of metabolism and capacity to reach rapid thermal equilibrium [30] , can become very sensitive to chemical pollutants during hyperthermia (due to increased chemical pollutant intake). The ability to tolerate the resulting compounded environmental stresses, which can become additive if not synergistic, can be seriously compromised [31-33]. Such effects have been observed in several invertebrates in the vicinity of power plants [29,31,34, 35], and in laboratory testing of heavy metals [36, 37] with zebra mussels *Dreissena polymorpha* [8], fiddler crab *Uca pugolator* [38], crayfish *Orconectis immunis* [9], shrimps

Crangon crangon [35] and *cumeta* [39] , waterflea *D. magna* [10], and earthworm (*Lumbricus terrestris*) [11].
In bodies of water temperature fluctuations can affect the availability of phytoplankton and their consumers, such as daphnids [40, 41] and other predators. The negative effect of temperature and other factors on the physiology of aquatic foodchain organisms can affect the entire ecosystem. For example, a decrease in body size over a period of several generations can change the trophic dynamics of the freshwater foodchain. If the responses observed in *Daphnia* and expected in other small aquatic organisms have been initiated by current rise in global temperature then these slow changes may soon start becoming apparent in aquatic ecosystems. Further laboratory and field investigations are, therefore, needed to understand and predict the effects of global climate change on aquatic ecosystems.

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Materials and Methods

Animals. Waterflea *D. magna* were purchased from NASCO (Fort Atkinson, WI) as well as kindly provided, as a start-up culture, by USEPA (Duluth, MN). Both were mixed and raised in a microcosm (20-gallon clear plastic aquaria with running water (1 ml/ min). The microcosm contained 10 gallons of water, freshwater mussels, and *Elodea* and was aerated gently. The rate of inflow of water balanced the evaporation of water at 16-25^0 C but at higher temperature evaporation exceeded the inflow to simulate the natural conditions. This balanced the evaporation up to 25^0 C but not above. The evaporative loss of water simulated natural environmental conditions. Daphnids were fed a mixture of YCT[42] plus Frog Brittle Food (NASCO) and Daphnia Food (Kiyuria product, Hikari Sales, USA) mixed in 1:1:1 (w/w) ratio in a form of a 5% slurry. Reconstituted water[32] , at 22-24^0 C (pH 7.90), contained about 250 nmole (8 mg) oxygen. L^{-1}. Batches of daphnids were acclimated at 16-18^0 C (habitat temperature), 16-18, ^{o} 18-20 ^{o}, 20-22^0, 24-26^0, 27^0, and 29^0 C for more than 6 months.

Body weight. Fifty adult non-gravid daphnids were removed; and after soaking their water on paper towel, weighed on a microbalance ((Sartorius Handy microbalance). Then, after drying them in an oven at 65^0 C for 24 hr, they were weighed again to get the dry weight. The wet weight: dry weight ratios were calculated for each acclimation temperature.

Body length. Five daphnids were removed and anesthesized by placing them in carbonated water. Each daphnid was placed on a glass slide with a transparent ruler underneath the slide. The body length was measured from the base of the spine to the top

of the helmet. The average length of daphnids in each of the groups from various acclimation temperatures was calculated.

Osmolarity of water at various temperatures was determined using 50 µ l of water on Microosmometer (µ-Osmette™, Precision Systems) [11]. The osmolar solution standards were purchased from Precision Systems. Initial osmolarity of water was adjusted to 130 mosmolar. The pH was determined using an Orion electrode and Acumet pH meter (Model 10, Fisher Scientific).

Chemicals. The chemicals were purchased from Fischer Scientific and biochemicals from Sigma-Aldrich (Milwaukee, WI).

Oxygen concentration and consumption. Batches of 50 non-gravid adult daphnids were added to 300 ml of the habitat water in BOD bottles (Fischer scientifics). Oxygen consumption was determined at the acclimation temperature of daphnids. At each temperature the oxygen concentration in water at zero-time was recorded. During experimentation oxygen concentration in water with and without daphnids was monitored using an oxygen electrode (Probe #970899) and a Meter (# 0720A9, Thermoelectron Corp., Beverly, CA) [9]. Oxygen concentration in water was measured continuously with time for 3 hr. The decrease in oxygen concentration in water was taken as a measure of the amount of oxygen consumed to determine the rate of oxygen consumption (nmole or mg oxygen.hr.g^{-1}).

Rate of heartbeat. Heart beat rate was measured using *Daphnia* Heart Rate Kit (36W1258, Wards Natural sciences, Rochester, NY). Each daphnid was placed in 1 ml of habitat water, in a transparent plastic curette, under nylon net (which arrested their movement). The heart rate was observed under a dissecting microscope (x 10 magnification) at the acclimation temperature. The rate was measured for 3 minutes using an electric timer. At least 5 daphnids were used for each determination and each experiment repeated at least five times. The mean value with standard deviation was used for making comparisons.

Hb concentration. Hb concentration in daphnids was determined by a modification of published methods [42, 43]. Fifty adult daphnids were rinsed with 0.1M phosphate buffer (pH 7.4) and then homogenized at 4^0 C in 3.0 ml of the same buffer using a Potter-Elvejhm homogenizer [44]. The homogenate was centrifuged at 3,000g for 10 min in a Sorvall RC-2B refrigerated centrifuge. The pellet contained more than 95% of the cytochrome c oxidase activity. The pellet was resuspended in 0.7 ml of the buffer. The suspension was divide equally in to two 1-ml cuvetts and equal absorbency was established at 420 nm using Spectronic spectrophotometer (Model genesis 20). The sample cuvette was then bubbled with carbon monoxide for 20 minutes and the difference spectra recorded. Hb concentration was estimated using an extinction coefficient of 57.5 [24] and expressed as µmole Hb. mg $protein^{-1}$.

Mitochondrial electron transport system (ETS) activity.

Cytochrome c oxidase. The subcell suspension, prepared as above, was used to assay ETS activity [45]. To the 2.4 ml buffer (0.1 M phosphate, pH 7.4) was added 0.4 ml of 10 mM cytochrome c (reduced with dithionite). After stabilization of the reading, the reaction was started by adding 0.2 ml of the subcell suspension and reading the absorbance at 550 nm for 2 min (at 15 second intervals) at room temperature. The protein concentration was determined using the BioRad reagent and the activity expressed, using extinction coefficient (19.1 x 10^3 $M.cm^{-1}$) as µmole cyt c oxidized.min.mg $protein^{-1}$.

Succinic Dehydrogenase. The 3.0 ml reaction mixture[46] contained 0.3 ml of 0.1 M sodiem azide, 0.6 ml of 0.01 M potassium ferricyanide, 1.1 ml of 0.2 M phosphate buffer (pH 7.8). The mixture was incubated with 0.7 ml of the subcell suspension at 30o C for 2-3 min. Following this the reaction was started by adding 0.2 ml of 0.2 M succinic acid as a substrate. After 30 minutes of incubation the reaction was terminated by adding 2.0 ml of 10% TCA. Optical density was recorded at 420 nm.

ATPases. To 1.0 ml of the reaction mixture containing 5 mM ATP, 2 mM $MgCl_2$, 20 mM Tris-HCl buffer (pH 8.5) was added 0.2 ml of subcell suspension and the mixture incubated at 30^o C for 10 min [47]. To a similar incubation mixtures was added 25 ug Oligomycin to determine the sensitivity/inhibition of the ATPase.The reaction was terminated with 10% TCA followed by centrifugation. The supernatant was analyzed for inorganic phosphate concentration[48]. The activity was expressed as nmole phosphorus released. min.mg $protein^{-1.}$

Statistical analysis. Each experiment was repeated at least 5 times and the mean of the five experiments with standard deviation used to make comparisons with other experiments. Statistical analysis of data used means with standard deviations using ANOVA [49]. The values of p= 0.05 were taken as significant.

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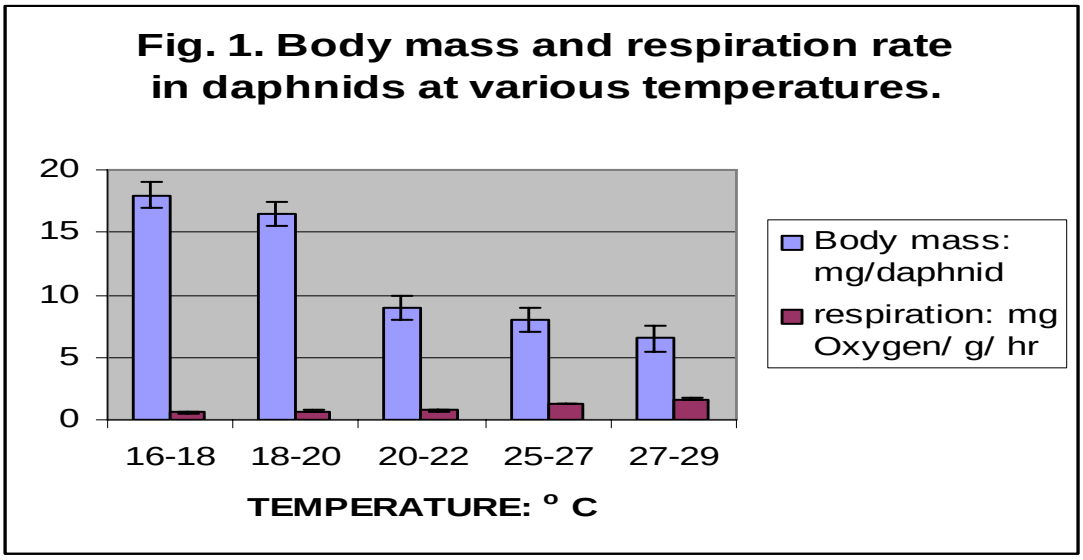

Fig. 1. Body mass and rate of respiration in daphnids acclimated at various temperatures.

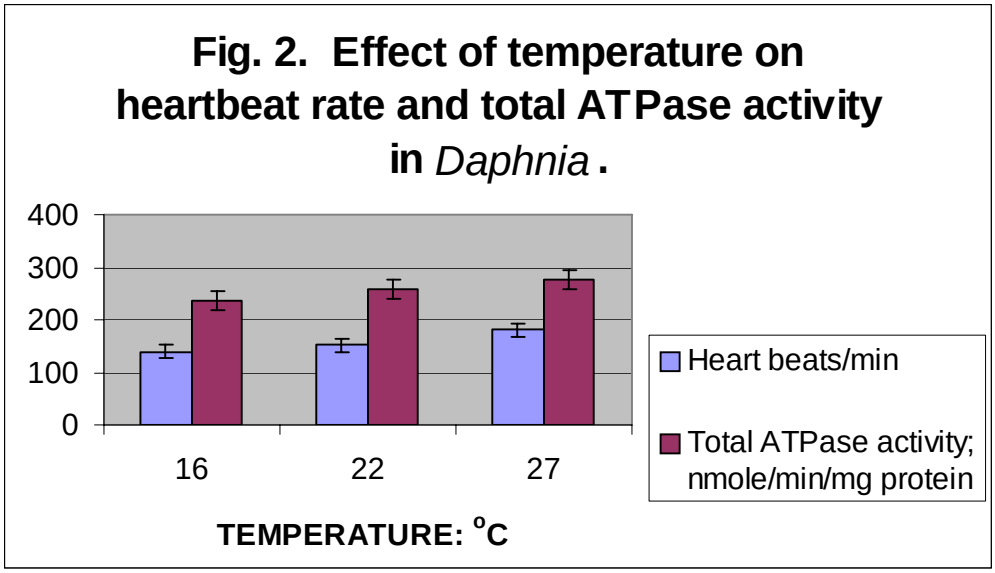

Fig. 2. Heartbeat rate and total ATPase activity in Daphnia acclimated at various temperatures.

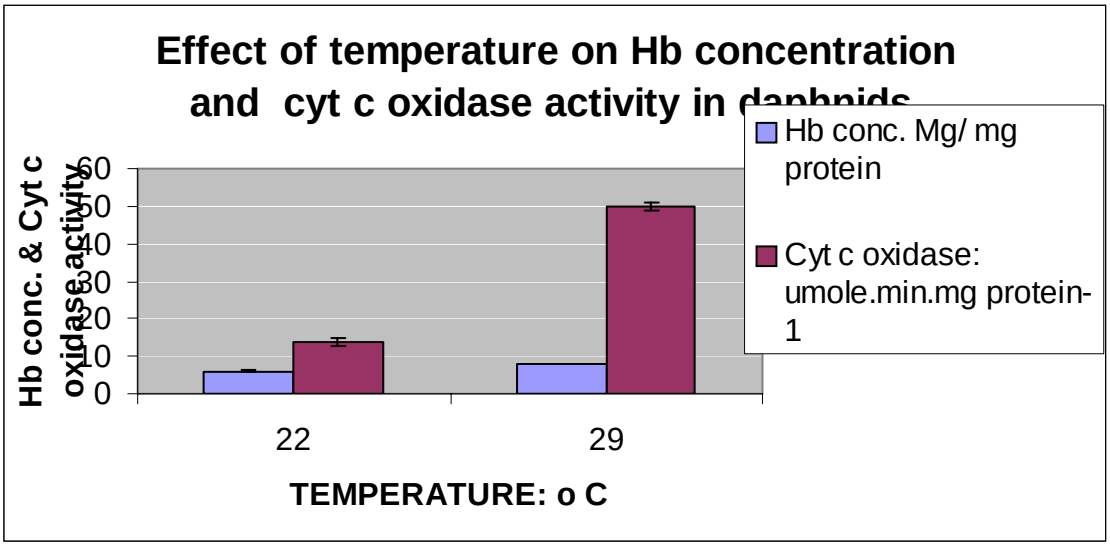

Fig. 3. Hb concentration and cytochrome c oxidase activity in *Daphnia* acclimated at various temperatures.

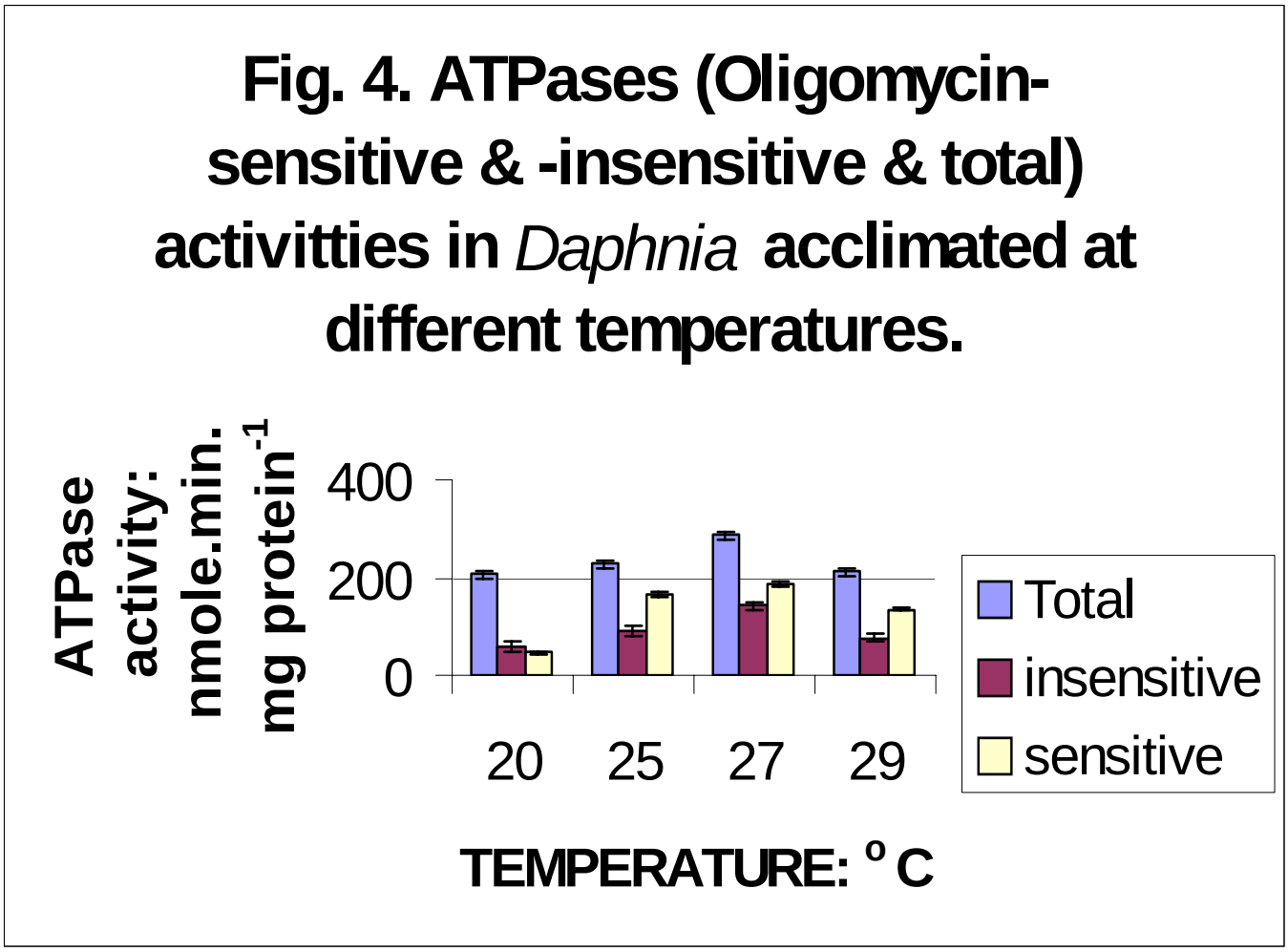

Fig. 4. Various ATPases activities in Daphnia acclimated at various temperatures.

Table 1. Effect of temperature acclimation for 6 months on respiration, body weight, body length, and osmolarity in *D. magna*.

Temperature	Water		Body wt [a]		Body Size
^{0} C	O_2 conc. nmolar	osmolarity [b] mosmolar	Dry wt. mg/ daphnid	Ratio wet wt:dry wt	length: mm[c]
16	329	130	17.50±2.01	14.52±1.24	4.25
18-20	277	130	16.90±1.48	14.06±1.37	3.84
22-23	251	128	15.50±1.56	13.95±1.08	3.57
24-25	238	120	8.95±1.03	13.80±1.24	3.38
26-27	213	113	8.10±1.13	12.07±1.07	3.15
29	173	105	6.51±1.01	11.64±1.36	2.95

a: Two batches of 50 daphnids weighed in 3 different experiments. Values are mean ± standard deviation.
b: Osmolarity determined one month after starting the acclimation. The osmolarity at the start was adjusted to 130 mosmolar. Each value is an average of 5 determinations.
c: Average of at least 5 daphnids.