RESISTANCE TO FREEZING IN POIKILOTERMS.

Especially important in marine teleosts and insects.

- BEHAVIORAL (avoidance; many animals, especially insects, die well before freezing)
- TOLERANCE TO FREEZING (tolerate internal ice formation; with colligative or noncolligative cryoprotectants)
- COLLIGATIVE ANTIFREEZE COMPOUNDS: glycerol, sugars (insects, other arthropods, reptiles)
- NON-COLLIGATIVE ANTIFREEZE COMPOUNDS: much more efficacious, proteins, glycoproteins (fishes, insects)
- SUPERCOOLING (superfusion): decrease of ice nucleating agents.

FREEZING TEMPERATURE:

Marine animals (Invertebrates): -1.86 °C (like sea water)

Vertebrates: -0.6/-0.7 °C

HIGHER FREEZING RISK:

Marine vertebrates
Small terrestrial animals
Intertidal invertebrates

TOLERANCE TO FREEZING.

a) SLOW and CONTROLLED ICE FORMATION:

Extracellular ice-nucleating agents:

Ice Nucleating Proteins (INPs) in invertebrates.

The cells and the extracellular unfrozen parts increase their osmolarity (ice excludes solutes): this leads to distorsion, but protects from freezing (decreases freezing point).

b) CRYOPROTECTANTS:

COLLIGATIVE (high concentrations):

typically polyols, sugars,
glycerol is common (about 30% of weight
in certain insects; 200 mM in water
is about 1/275)

NON-COLLIGATIVE (< 200 mM):

e.g., threalose or proline in insects
protect cell structures by substituting water

Synthesized from glycogen.

CELLULAR HOMEOSTASIS IN FREEZING CONDITIONS

- CELLS SUSTAIN HIGH IONIC STRENGTH

- LOW T WITH HIGHLY REDUCED RESPIRATION, CIRCULATION and METABOLISM.

Energy production: often **phosphagens (P-creatine, P-arginine) to produce ATP**

↓

anaerobic glycolysis

↓

(Accumulation of LACTATE and ALANINE)

These are common strategies in invertebrates (Protostomia: insects, annelids, molluscs, nematodes).

They can resist for weeks at temperatures down to -35 °C (down to -70 °C in arctic species)

It is rare in vertebrates (found in some Amphibia and Reptilia).

ORGANISMS WHICH DO NOT TOLERATE FREEZING

SUPERCOOLING plus ANTI-FREEZING AGENTS

a) ELIMINATION OF ICE-NUCLEATING AGENTS

b) ↑ SOLUTES LIKE GLYCEROL, etc. (e.g., arthropods)

Invertebrates can resist down to -60 °C without freezing.
Some species of Antarctic acarids remain active at -10/-20 °C.

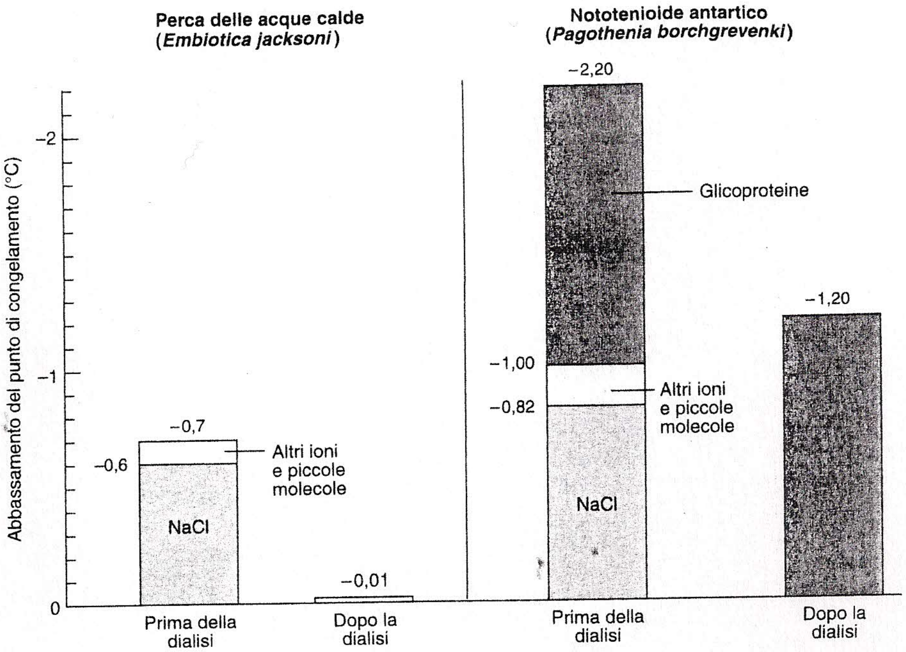
c) TELEOSTS OF POLAR WATER ARE AT HIGH RISK:

ANTI-FREEZING PROTEINS and GLYCOPROTEINS
(200 times as effective as NaCl)

EXTRACELLULAR [NaCl] higher than usual

Proteins confer THERMAL HYSTERESIS to aqueous solutions ($T_{freeze} < T_{melt}$ by 2-3 °C)

Figura 9.40. I livelli di NaCl nel sangue di teleostei di zone temperate e polari. Nella perca delle acque calde il punto di congelamento del sangue prima della dialisi era −0,7 °C e saliva a −0,01 °C dopo la dialisi (rimozione dei piccoli soluti). Ciò significa che l'NaCl e gli altri piccoli soluti sono la causa principale dell'abbassamento del punto di congelamento (FPD, *Freezing Point Depression*). Nel nototenioide antartico il livello di NaCl è alto e la sua rimozione innalza il punto di congelamento; tuttavia, le glicoproteine non dializzabili sono responsabili per più della metà dell'FPD totale. (Da EASTMAN, 1993).



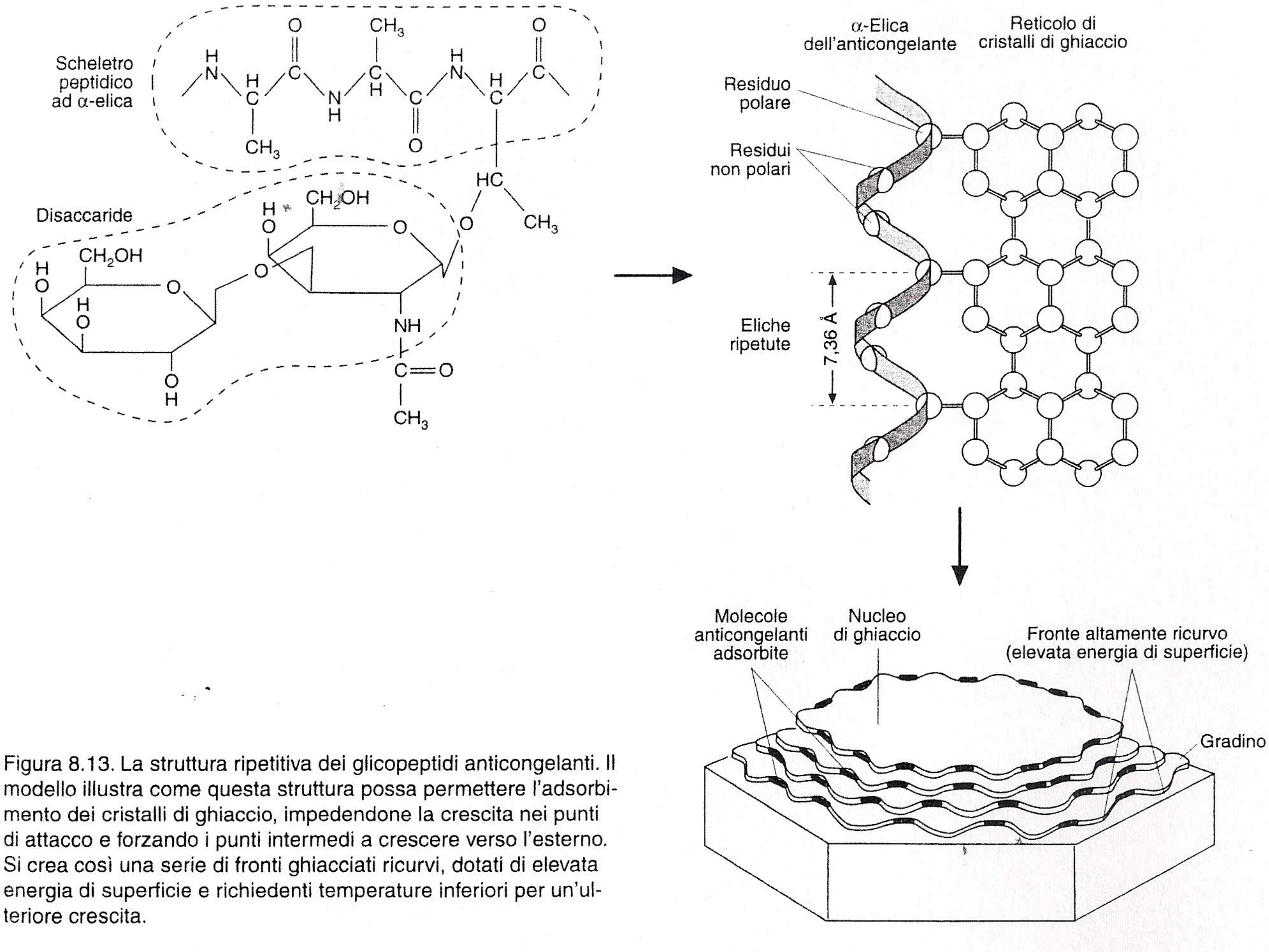


Figura 8.13. La struttura ripetitiva dei glicopeptidi anticongelanti. Il modello illustra come questa struttura possa permettere l'adsorbimento dei cristalli di ghiaccio, impedendone la crescita nei punti di attacco e forzando i punti intermedi a crescere verso l'esterno. Si crea così una serie di fronti ghiacciati ricurvi, dotati di elevata energia di superficie e richiedenti temperature inferiori per un'ulteriore crescita.

Table 11.12 Freezing points (FPs) and melting points (MPs) due to antifreeze glycoproteins in polar marine fish.

Genus	Environment (temperature, °C)	FP, organism (°C)	FP, blood (°C)	MP, blood (°C)	Antifreeze molecules
Gadus	Deep, ice free	−1.0	−1.1	−0.7	AFGPs
Chaenocephalus	Shallow, ice free (−1.0)	−1.5	−1.5	−0.9	
Rhigophalia	Deep, ice free (−1.9)	−1.9	−2.0	−0.9	AFP 1–3
Notothenia	Shallow, icy (−1.9)	−2.0	−2.1	−1.1	AFGPs
Pagothenia	Shallow, icy (−1.9)	−2.4	−2.7	−1.1	AFGP 1–8

AFGP, antifreeze glycoprotein; AFP, antifreeze protein.

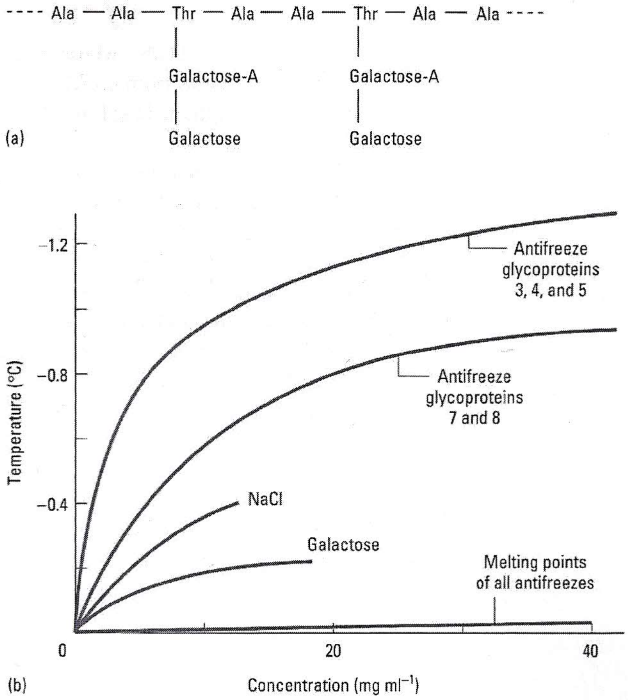


Fig. 11.40 The antifreezes of Antarctic fish. (a) The structure of the antifreeze glycoprotein (AFGP) from an Antarctic fish, with the typical Ala–Ala–Thr repeat and attached disaccharides. (b) The freezing point depressions produced by antifreeze proteins (AFPs) and AFGPs (NB 3, 4, and 5 are larger than 7 and 8), in comparison with the much lesser effects of NaCl and of galactose; note also that the AFPs of all kinds have little or no effect on melting points.

AFGPs: LIVER → BLOOD

REMAIN IN EXTRAC. FLUIDS

TENS OF GENE COPIES

EVOL. CONVERGENCE between arctic and antarctic species

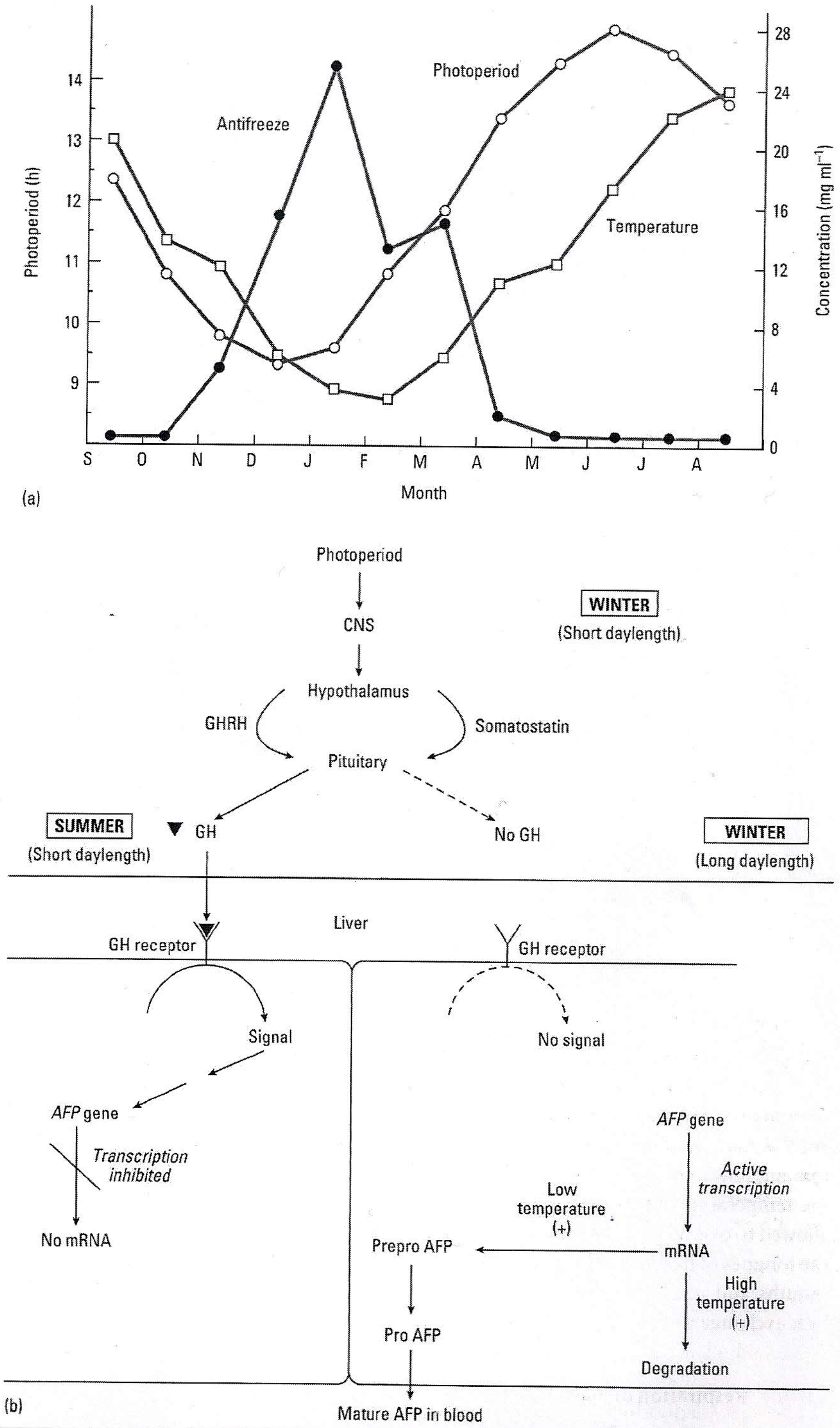
AFPs: MUCH MORE VARIABLE FROM A STRUCTURAL POINT OF VIEW.

Several classes, rich in Ala and Cys

CIRCADIAN CLOCKS:

often $Q_{10} \simeq 1$

From: Willmer et al. 2005



g. 11.41 (a) Seasonal production of antifreeze roteins (AFPs) in the winter flounder. (b) egulation of this AFP production by photoperiod nd the intermediary effects of growth hormone GH) initiating transcription. Note that post-anscriptional control by temperature also perates, to insure that AFP is only present if mperatures are low. CNS, central nervous system; HRH, growth hormone-releasing hormone. , Adapted from Cossins & Sheterline 1983; adapted from Chan *et al.* 1993.)

CIRCADIAN CLOCKS $Q_{10} \simeq 1$ (OFTEN)

From: Willmer et al. 2005

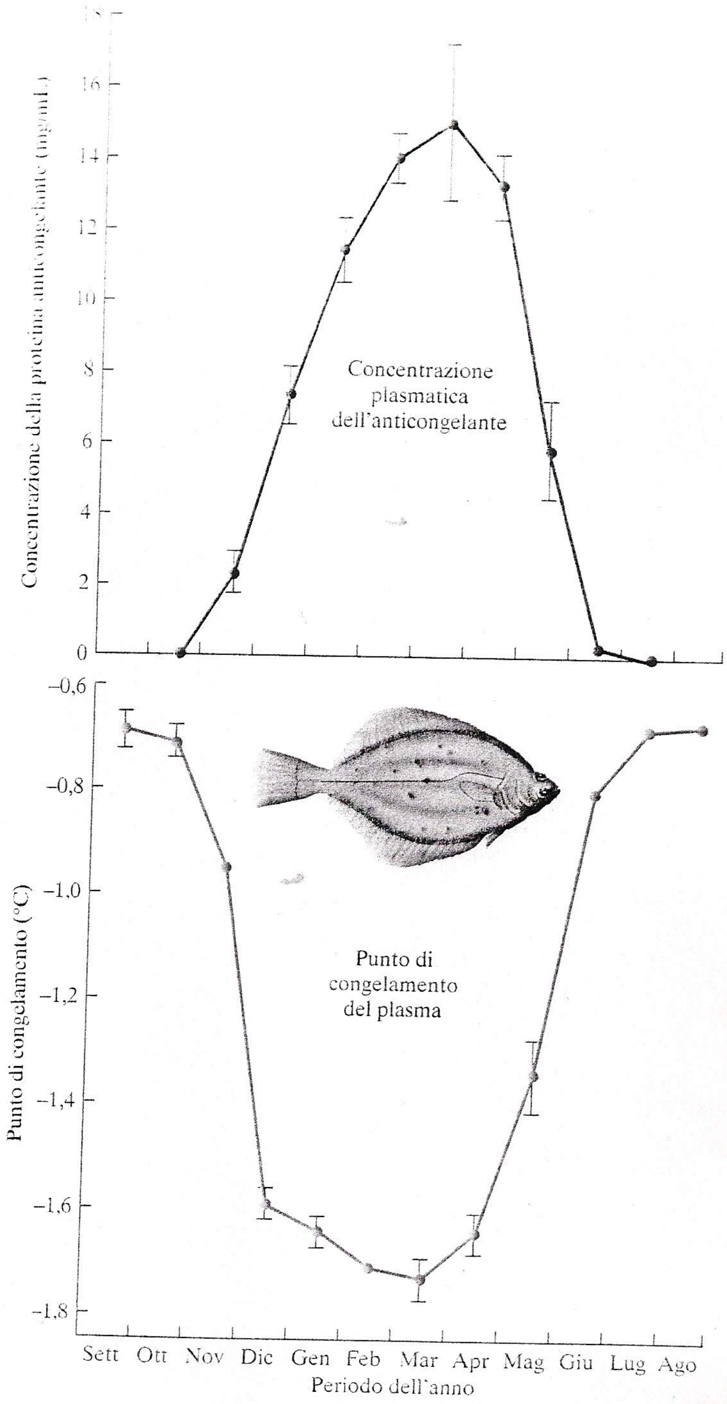


Figura 8.20 Variazioni stagionali della protezione invernale anticongelante nella passera di mare (*Pleuronectes americanus*). La concentrazione di proteina anticongelante nel plasma sanguigno sale con l'avvicinarsi dell'inverno, perché aumenta l'espressione dei geni codificanti per la proteina anticongelante. Il punto di congelamento del plasma si abbassa in sincronia e d'inverno risulta inferiore alla temperatura minima invernale; l'animale si protegge cosi dal congelamento. La passera di mare è una specie importante dal punto di vista commerciale, che ha la particolarità di riprodursi nell'acqua gelida alla fine dell'inverno o all'inizio della primavera. [*Fonte*: Fletcher et al., 1998.]

OTHER BIOCHEMICAL ADAPTATIONS TO TEMPERATURE.

1) HOMEOVISCOUS ADAPTATION (HVA; in cellular membranes)

a) Ratio between saturated and unsaturated fatty acids.
b) Cholesterol %.

Short and unsaturated fatty acids increase membrane fluidity.

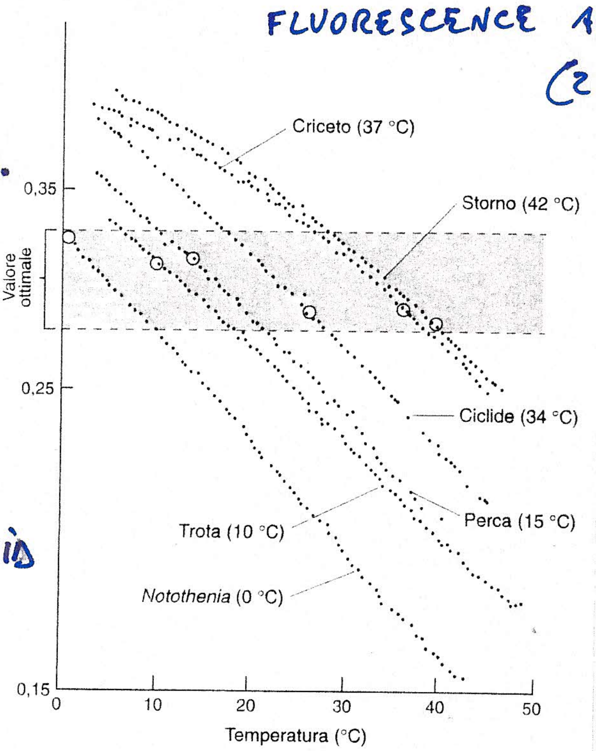
Cholesterol changes membrane properties in different ways.

Membrane composition also depends on diet, and this may have seasonal meaning.

Table: Ratio between saturated and unsaturated fatty acids in several phospholipids of animals adapted to different temperatures (from Willmer et al., Environmental Physiology of Animals, 2005). Cerebral tissue.

Species	Body T (°C)	Choline	Ethanolamine	Serine inositol
Arctic sculpin	0	0.59	0.95	0.81
Goldfish	5	0.66	0.34	0.46
	25	0.82	0.51	0.63
Desert pupfish	34	0.99	0.57	0.62
Rat	37	1.22	0.65	0.66

FLUORESCENCE ANISOTROPY (2 probes)



<FLUID.

>FLUID

Figura 8.6. Adattamento omeoviscoso (HVA) nelle membrane cerebrali di alcuni vertebrati, valutato con la polarizzazione di fluorescenza (vedere il testo per i particolari; la diminuzione della polarizzazione indica un aumento della fluidità e una diminuzione dell' ordine). I valori che si riferiscono alla normale temperatura corporea di ciascuna specie (in parentesi) corrispondono ai cerchi vuoti. (Da BEHAN-MARTIN *et al.*, 1993).

2) PROTEIN ADAPTATIONS

A) SHORT-TERM REGULATION (HOURS).

a) Direct: change of concentration or activity, by increased/decreased expression, or increased/decreased activity (e.g., by phosphorylation), or compartment changes.

b) Indirect: changes in ionic strength, or higher pH because of lower T (which alters protein stability), etc.

B) MEDIUM-TERM (ACCLIMATATION): DAYS TO WEEKS.

a) Synthesis or degradation of limiting enzymes

(e.g., ↓ T → ↑ mitochondrial proteins)

b) Regulatory enzymes (e.g., control of membrane fatty acids)

c) Isozymes specific for certain T ranges (e.g., myosin ATPase in some fishes)

d) HSP proteins.

C) LONG-TERM (EVOLUTIONARY ADAPTATION)

a) Structural proteins, e.g., arctic tubulins.

b) Enzymes (thermal stability, kinetics).

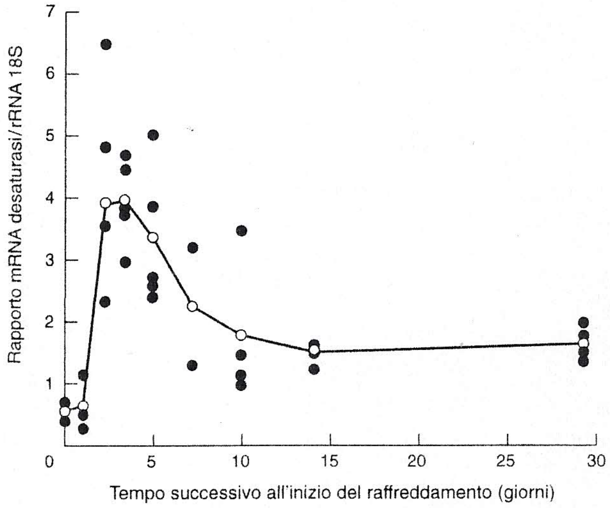


Figura 8.7. Effetto dell'acclimatazione al freddo sui livelli dell'enzima desaturasi della carpa, valutato come rapporto tra il trascritto di mRNA per la Δ^9 desaturasi nel fegato e di un rRNA 18S standard. I cerchi pieni si riferiscono ai singoli animali, i cerchi vuoti ai valori medi. Risulta chiaramente l'aumento durante i primi giorni del raffreddamento. (Da GRACEY *et al.*, 1996; cortesia della Cambridge University Press).

t_0 : ACCLIMATED AT 30°C

↓

PROGRESSIVE COOLING TO 10°C

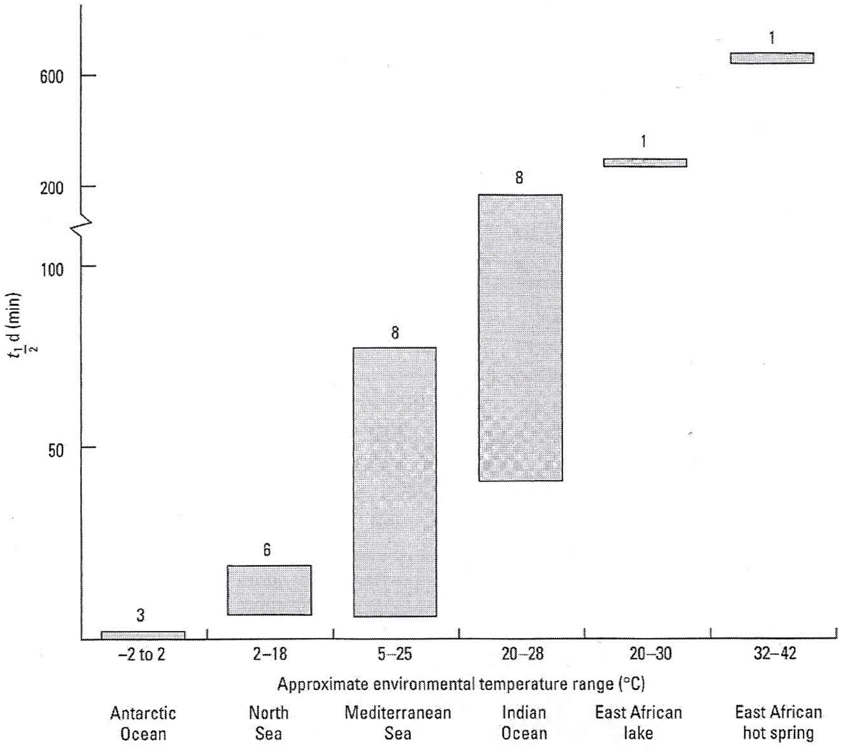


Fig. 8.3 Thermal stability of enzymes in marine animals is related to their thermal habitat. The thermal denaturation half-times at 37°C for myofibrillar ATPase from fish species taken from six habitats are compared. Those from cold seas denature within a few minutes, while those from the Indian Ocean (normally 20–28°C) have half-times of up to 3 h. Note that enzymes in fish from warm freshwater lakes and springs are even more thermally stable. Numbers above bars indicate numbers of species tested. (Adapted from Johnston & Walesby 1977.)

Table 8.2 Substrate turnover rates (K_{cat}) for lactate dehydrogenase (LDH) from barracuda (see also Fig. 11.9), showing the similar (conserved) values for different species when measured at normal operating temperatures.

Species (distribution)	Normal mean temperature of habitat (T_m) (°C)	K_{cat} at 25°C (s^{-1})	K_{cat} at T_m (s^{-1})
Sphyraena argentea (northern)	18	893	667
Sphyraena lucasana (intermediate)	23	730	682
Sphyraena ensis (southern)	26	658	700

- STABILITY

- KINETICS : HIGHER K_{CAT} IN SPECIES WITH COLDER HABITATS

- SENSITIVITY TO LIGANDS :

OFTEN SMALLER K_M IN COLD HABITATS

HSP: HEAT SHOCK PROTEINS (or STRESS PROTEINS).

Different types of HSPs can cooperate. They have conserved structure.

Big: HSP 60, 70, 90, 100 → Animals

Small: HSP 27, 10, ubiquitin → More common in plants

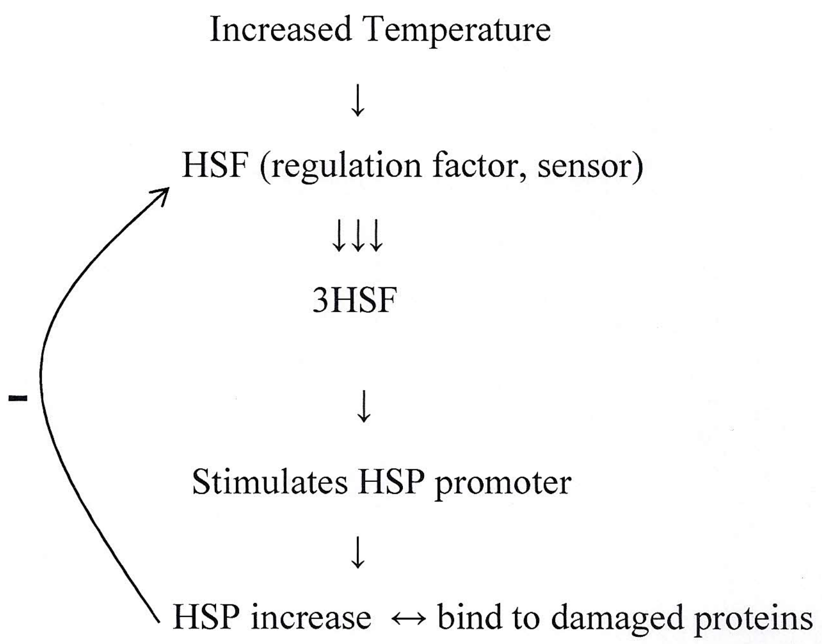
Δ Temperature →
Hypoxia-hyperoxia →
Osmotic shock →
Δ pH → ↑ HSP (min/hours)
Alcohols →
Ionizing radiations →
Heavy metals, free radicals, etc. →

Activation thresholds and final concentrations depend on species and environment.

HSP FUNCTION: “**CHAPERONS**”

(induce degradation, or impede structural alteration and then release the protein).

REGULATION:



The larger is the number of damaged proteins,
the less effective is the negative feedback on HSF,
as these proteins sequester HSP.