Too hot or too cold:
the biochemical basis of temperature-size rules for fish and other ectotherms

by

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Abstract

The well-established temperature-dependence of growth parameters and maximum sizes of fish and other water-breathing ectotherms (WBEs) form the basis for various 'temperature-size rules' for fish and WBEs. Numerous adaptationist interpretations of these rules exist, but their biochemical basis is largely ignored. One fundamental, but frequently overlooked component of the mechanism that leads to temperature-size rules is that proteins only 'work' if their native quaternary structure (or folding) is maintained. However, proteins have half-lives that are U-shaped functions of temperature, which means that higher or lower than optimal temperatures increase their rates of spontaneous denaturation in aqueous solutions, i.e., within body cells. Proteins that lose their quaternary structures, either because the surrounding water is too hot or too cold, cease to function and in most cases, need to be resynthesized. Thus, protein denaturation may explain why the metabolic rates of fish and other ectotherms increase with temperatures both above 4 $^{\circ}$C, the temperature at which hydrogen bonding in water is the strongest and hydration of protein nonpolar groups the weakest, and below 4 $^{\circ}$C, the regime of 'cold denaturation.' Considering this biochemical basis of temperature-size rules for fish and other WBEs would enable biologists to better understand, and possibly mitigate adverse consequences of climate warming for marine and freshwater biodiversity.

Introduction

When marine or freshwater biologists or fisheries scientists study and document fish growth and related processes, they usually pay scant attention to the underlying biochemical processes. This is evident in recent publications where the choice of fish growth models (von Bertalanffy, Gompertz, logistic, polynomials, etc.) is a purely statistical exercise, often aided by the criterion proposed by Akaike (1974), resulting in growth curves being selected for the description of length-at-age data with no basis in biology. Such approaches obfuscate, rather than elucidate the relationships between growth and temperature.

The aim of this contribution is thus to clarify key concepts that can advance a definition of growth in water-breathing ectotherms (WBEs), such as most fishes, crustaceans and mollusks, and a variety of other phyla, emphasizing the underlying biochemical mechanism giving rise to macroscopic phenomena described by various temperature-size rules (TSR) purporting to describe and/or explain temperature-related size variation in WBEs (e.g., Atkinson 1984).

Growth and Pütter's Equation

Pütter (1920) conceptualized growth as the net result of two processes with opposite signs affecting the body weight (W; actually mass) of WBEs, one adding to, and the other subtracting from it, i.e.,

$$dW / dt = HW^d - kW^m \qquad (1)$$

where dW / dt is the growth rate, and HW^d and kW^m are conventionally referred to as anabolic and catabolic terms, respectively.

The integration of Equation (1) with $d = 2/3$ and $m = 1$ yields the ubiquitous von Bertalanffy Growth Function (von Bertalanffy, 1938), with growth ceasing when $HW^d = kW$, which implies that asymptotic (= final) length and weight will be reduced as temperature increases affect k more strongly than H. There is a huge number of studies of this specific temperature effect (see, e.g., Dimarchopoulou and Tsikliras 2022, Palomares et al. 2021 and references therein), including new evidence presented below. However, before we delve further into the implications of Equation (1), we first elaborate upon its exponents, d and m.

In animals, adenosine triphosphate (ATP) is required to synthesize the proteins from which body cells and tissues are made. ATP is derived from oxidative phosphorylation in the inner mitochondrial membrane, with the Krebs Cycle providing substrates, and where oxygen is required as the final electron acceptor (Cox and Nelson 2008). This requires an oxygen supply that must enter the body through a respiratory surface.

In biological organisms, respiratory surfaces grow in proportion with the square of body length, i.e., $S \propto L^2$ in the case of isometry (i.e., the same, or 'proper' dimension). However, the exponent can be < 2 (negative allometry) or > 2 (positive allometry). The body weight (W, strictly 'mass') of WBEs can increase isometrically with L ($W \propto L^3$), or with negative (< 3), or positive allometry (> 3). Length-weight relationships (LWR) of the form $W = a \cdot L^b$, with $b = 3$ or $b \approx 3$, are most frequent in fishes (Froese 2006), crustaceans (Pauly et al. 2022), and other WBEs (see www.sealifebase.org).

Next, we must deal with the manner that oxygen enters a WBE's body. Fick's Law states that the rate of diffusion of oxygen through a respiratory surface can be quantified by

$$R = U \cdot S \cdot dP / WBD \qquad (2)$$

where R is the oxygen uptake (i.e., supply to the body in mL/hour), U is Krogh's diffusion constant (i.e., the amount of oxygen (in mL) that can diffuse through membranes with an area of 1 mm^2 in one minute through a given type of material or tissue), S is the respiratory area (e.g., the sum of the lamellar area of gills), dP the difference between the oxygen pressure on either side of the membrane in atm, and WBD is the water-blood distance, i.e., the thickness of the membrane in question (De Jager and Dekker 1975; Pauly 2021).

One can reasonably assume that, of the parameters in Equation (2), only S changes with the size of WBEs, so oxygen uptake (R) can be considered directly proportional to S, i.e., $R \propto S$ (De Jager and Dekker, 1974). Combining Fick's Equation with the preceding considerations leads to the exponent of the anabolic term of Equation (1), HW^d, having a d-value of 2/3 when the respiration of a WBE is proportional to its length squared ($R \propto S \propto L^2$) and its body weight to its length cubed ($W \propto L^3$).

Examples of WBEs with isometric respiration are the guppies (*Poecilia reticulata*) that von Bertalanffy used to test his growth theory (Bertalanffy 1951) and subsequently, the brine shrimp

Artemia salina (von Bertalanffy and Krywienczyk 1953). However, while such small WBEs often have gills that grow near isometrically, or even lack gills and respire through their integument, where $S \propto W^{2/3}$ applies (as in chaetognaths; see Pauly et al. 2021), the gills of larger WBEs usually grow with a positive allometry, i.e., with *d*-values commonly ranging between 0.7 and 0.9 (Muir and Morgan 1969; De Jager and Dekker 1975; Pauly and Cheung 2017).

Important here is that the estimates of *d* be those pertaining to juvenile and adult WBEs, which are always < 1, and not the *d*-values observed in fish larvae, which commonly exceed 1; see De Sylva (1974) for larvae of Atlantic herring (*Clupea harengus*) and European plaice (*Pleuronectes platessa*). The growth of fish larvae, consequently, does not become limited by their oxygen supply as they gain weight (Bochdansky and Leggett. 2001; Pauly 2019, 2021).

Also, given Fick's Law, *d* can be estimated from respiratory or gill area studies (see De Jager and Dekker, 1975). However, only studying a small range of body sizes within a species of WBEs, subjecting the studied WBEs to various stresses, or not being aware of pitfalls in the study of gill areas (Hughes 1984), can easily produce erroneous estimates of $d \geq 1$.

In adult WBEs, $d \geq 1$ would imply that the membrane through which oxygen is supposed to diffuse would fill up a 3-dimensional space, which would prevent water from flowing *through* the gills, with oxygen-rich water entering through one side, and oxygen-poor water leaving the gills (Pauly 2021). For fish larvae, the gills can expand rapidly within an initially 'empty' head, and thus $d \geq 1$ (see De Sylva 1974), but such values are an exception in adult WBEs, and apply to some fish, crabs, and other ectotherms that rely mainly on *air*-breathing, and which drown in well-oxygenated water despite having (small) gills. Here, we consider only *water*-breathing ectotherms, constituting most species of interest in aquatic biology and fisheries.

The catabolic term of Pütter's equation, kW^m, is where the real problems begin. This term has a multiplicity of names in German ("Abbau, Abnützung, Auflösung, Zerfall, Zerstörung") in the writings of Pütter (1921) and von Bertalanffy (1951). In English, the terms "degradation" or "breakdown" is often used (Jobling 1993), but without elaboration, or with elaborations that are seriously misleading.

For example, Ursin (1967, 1979) argued that perceiving catabolic processes as proportional to weight (i.e., $m = 1$) is *"too simple because it overlooks the fact that oxygen required by catabolic*

processes must enter the body through a surface" (Ursin 1979, p. 69). Thus, he reformulated Equation (1) with *d* as exponent of the anabolic term, and an invented new exponent (*n*) for the catabolic term which did generate asymptotic growth curves in a variety of fishes (only because $n < d$). This obviously led nowhere, because conceiving catabolism as a process requiring oxygen resulted in numerous contradictions, notably that dead flesh would not decay.

Another definition of catabolism may be generally correct, but is not suitable in the context of Pütter's equation is implied by Hochachka (1969), who wrote that *"[d]uring initial phases of catabolism, large molecules are broken down to yield, apart from CO_2, and H_2O, a quite restricted group of small organic molecules, liberating about one-third of the available free energy in the process."* Here, the problem is that this definition of catabolism largely overlaps with the processes wherein the components of the food of a WBE (along with its denatured proteins) are used as substrate for the Krebs Cycle at the end of which the ATP is generated that is used for the synthesis of new proteins, i.e., anabolism.

To avoid contradictory and overlapping definitions and the confusions they create, catabolism, at least in the context of Equation (1), must be conceived as 'removing' proteins from the stock of 'live' proteins through a process that does not require oxygen, or more precisely, that does not *consume* oxygen. Such a process exists and is ubiquitous in living organisms: spontaneous denaturation.

Protein denaturation

Disruption of the highly ordered quaternary structure or conformation of globular proteins, such as enzymes, from their natural or 'native' states is called ´denaturation´. Denaturation occurs spontaneously in biological organisms either from disruption of the weak chemical linkages stabilizing the conformation of the native state or from preferential hydration of the denatured state. Denaturation is easily achieved upon heating (heat denaturation) with the kinetic energy of random Brownian motion of proteins in aqueous solutions. It also occurs at low temperatures (cold denaturation) due to hydrophobic interactions among protein nonpolar groups favouring, somewhat surprisingly, their hydration in the denatured state (Privalov 1990).

Consequently, proteins in aqueous solutions, that is, in the bodies of WBEs, have half-lives that are U-shaped functions of temperature, such that higher or lower than optimal temperatures increase their rates of spontaneous denaturation. Proteins that lose their quaternary structures, either because the surrounding water is too hot or too cold, cease to function and in most cases, need to be resynthesized. Thus, spontaneous protein denaturation explains why the metabolic rates of WBEs increase with temperatures, both above and below 4 $^{\circ}$C, the temperature at which hydrogen bonding in pure water is the strongest and hydration of protein nonpolar groups the weakest. In the aqueous solution that form the bulk of contents of living cells, different proteins will have their stability optima at different temperatures, but will retain their U-shape.

Once a protein has lost its relatively fragile quaternary structure, it becomes a "random coil" (Smith et al. 1996), i.e., an essentially useless jumble of amino acids. As such, denatured proteins become part of a WBE's amino-acid pool, where they join the amino acids originating from the food of that animal. Thus, none of the oxygen supplied by a respiratory surface is required to remove a substantial part of the stock of 'working' proteins from the body of a WBE. These denatured proteins, however, must be immediately replaced if that WBE is not to succumb to entropy.

The biochemical basis of the mechanism of protein denaturation is detailed in the following:

1) The quaternary structure or conformation of proteins is typically stabilized by weak chemical linkages, such as hydrogen and disulfide bonds, and hydrophobic interactions among nonpolar groups within the polypeptide chain (Privalov 1990). The weak chemical interactions are easily disrupted in aqueous solution (for example, by shaking), by random Brownian motion of the protein molecules, the addition of denaturants, or the input of thermal energy that can overcome the strength of these bonds (~ 0.4 - 4 kJ / mol). The breaking of many of the weak intramolecular linkages responsible for the highly ordered quaternary structure of a protein in its native state results in protein denaturation.

2) Protein denaturation typically proceeds to irreversible degradation and hence to biological inactivity. In some proteins, such as with electrostatic hinges, the reversible process of renaturation can occur in the presence of stabilizing ionic ligands, demonstrating the link between protein structure and function (see, e.g., Yan et al. 2018). Protein denaturation can be

quantified by the time taken for the amount of proteins with well-formed quaternary structure to decrease by half, the definition of the aforementioned half-life of a protein. Protein half-lives decrease with increasing temperature, as increased thermal Brownian motion disrupts the bonds which stabilize the quaternary structure. Half-lives can also decrease with decreasing temperature in the case of cold denaturation (see Figure 1A, B and C). Note that different proteins have different half-lives and the same protein can have a different half-life in different environments (see, e.g., Kuhar 2009; Figure 1D and E).

3) The quaternary structure of proteins is also stabilized by hydrophobic interactions, which consist of short-range attractive van der Waals interactions among protein nonpolar groups and long-range repulsive hydration of these nonpolar groups (Privalov 1990). Once disrupted, van der Waals interactions, being delocalized many-body interactions (Distasio et al. 2014), are not easily reformed. As a result, protein quaternary structure, once lost, is not easily repaired, i.e., it is thermodynamically possible, but kinetically improbable, as the stable structure requires achieving a conducive transition-state configuration of proteins. Thus, once they have lost their quaternary structure, proteins usually need to be re-synthesized, which requires an input of energy (ATP) to overcome the (high) activation barrier for the protein synthesis reaction.

4) The stability of globular proteins is maximal at the temperature at which the entropies (disorder) of the native and denatured states are equal and the structure is stabilized only by the enthalpy (configurational energy) difference between these states (Privalov 1990). In aqueous solution, proteins are stablest at 4° C, the temperature at which hydrogen bonding in water is the strongest and hydration of the protein polypeptide chain, which consist largely of nonpolar groups, is the weakest. At temperatures above and below this temperature, protein stability decreases, i.e., protein denaturation occurs with both heating and cooling, yielding a distinct protein stability curve with temperature (Figure 1D and E). Spontaneous heat and cold denaturation reflect the thermodynamic balance between the configurational energy or enthalpy stabilized largely by hydrophobic interactions and the conformational entropy or tendency of the universe to disorder.

5) Disruption of the native protein structure on heating, called heat denaturation, proceeds with heat absorption and, consequently, with increases in the molecular enthalpy. Disruption of the native structure upon cooling, called cold denaturation, proceeds with the release of heat and,

hence, with enthalpy decreases (Privalov 1990). Heat denaturation requires only a small input of energy, on the scale of thermal fluctuations in Brownian motion, whereas cold denaturation releases a small amount of energy as heat. Thus, no additional external source of chemical energy, such as from ATP, is required, as the activation barrier to protein denaturation is low. Consequently, the folded and unfolded protein states often occur in dynamic near-equilibrium, with more denaturation than renaturation.

6) Low temperatures (< 4° C) also can cause loss of quaternary structure or "cold denaturation" (Privalov 1990). Cold denaturation is a general phenomenon caused by the specific and strongly temperature-dependent interaction of protein nonpolar groups with water. Hydration of these groups is favorable thermodynamically, i.e., the Gibbs energy of hydration is negative and increases in magnitude, as temperatures decrease. As a result, the polypeptide chain, tightly packed in a compact native structure, unfolds at a sufficiently low temperature, exposing internal nonpolar groups to water.

The hydrophobic interactions stabilizing the quaternary structure of native proteins are an enthalpic balance between the temperature-dependent repulsive hydration of protein nonpolar groups and the temperature-independent attractive van der Waals interactions among these nonpolar groups. At low temperatures, the weakly attractive van der Waals interactions can be disrupted, leading to unfolding and denaturation. Indeed, Fraser et al. (2022), based on work on limpets and the notothenoid fish *Harpagifer antarcticus* state that *"[p]rotein metabolism data for Antarctic invertebrates show low rates of protein synthesis and unusually high rates of protein degradation. Additionally, in Antarctic fish, increasing evidence suggests a lower frequency of successful folding of nascent proteins and reduced protein stability."*

Figure 1, in its 5 panels, presents evidence for the effects of protein denaturation on fish growth. Figure 1A illustrates the common pattern of increasing oxygen consumption in a WBE (the goldfish *Carassius auratus*) as temperature increases from 4 to 30 °C, and the reverse pattern of increasing oxygen consumption (in polar fishes) as temperature decreases from 4 to -2 °C. This twist was derived by Pauly (1979) from various growth and respiration datasets, including that of Wohlschlag (1964) working on nototheniid fishes. However, Wohlschlag's work was highly contested at the time (see, e.g., Holeton 1973). Therefore, it was left "*in profound hibernation*" (Pauly 2019), though 'cold adaptation' also was also detected with natural mortality (Pauly

1980), which is related to growth and temperature, and thus to respiration and protein denaturation.

Figure 1B illustrates that Wohlschlag (1964) interpreted his own data as increased "variability" at low temperature. This interpretation, for which he did not provide a mechanism, is probably one reason why his notion of "cold adaptation" became controversial. Another reason was the suggestion that his experimental set up may have stressed the fish whose respiration he was measuring, and their elevated oxygen consumption was thus deemed an "artifact" (Holeton 1973).

Figure 1C presents new, if indirect, evidence for cold adaption, i.e., elevated oxygen consumption, in 10 fish surveyed in cold Norwegian waters (Lavin et al. 2022). There, only three species exhibited a decrease in maximum observed length with increasing temperature, as shown (here as an inverse relationship) in line 1 (spotted wolffish, *Anarhichas lupus*), 2 (cusk, *Brose brosme*) and 3 (Norway redfish, *Sebastes viviparus*), compared with five species whose maximum length *increased* with temperature (here shown again as an inverse relationship), i.e., lines 4 (capelin, *Mallotus villosus*), 5 (Greenland halibut, *Reinhardtius hippoglossoides*), 6 (Golden redfish, *Sebastes norvegicus*), 7 (daubed channy, *Leptoclinus maculatus*) and 8 (polar cod, *Boreogadus saida*), while two species, bold lines 9 (Atlantic wolffish, *Anarhichas lupus*) and 10 (beaked redfish, *Sebastes mentella*) spanned the range from -1 to 10 °C with the very U-shaped relationships that best reflect the underlying biochemical mechanisms of protein denaturation.

Figure 1D and 1E show two examples, among many that could be shown, of enzymes' stability *vs* temperature (modified from Brandts 1967); Figure 1D documents ribonuclease and Figure 1E chymotrypsinogen. Note U-shape of the protein stability curves (but also note inverted scale), which support the effects illustrated in Figure 1A-1C.

Lastly, we must clarify a generally misunderstood concept, whose relevance to the growth of WBEs is not even perceived. It is widely accepted that proteins in aqueous solutions undergo denaturation at very high temperatures or with very low or very high pHs (see, e.g., Cox and Nelson 2008). However, it is usually not realized that all proteins undergo spontaneous denaturation at *all* temperatures – even at a protein´s optimal temperature – and that this is why proteins even have half-lives, ranging from a few hours to several days, even in homeotherms.

This implies that even at their optimal temperatures, a fraction of the stock of proteins in a living animal spontaneously loses the quaternary structures required to fulfill their specific roles, e.g., as an enzyme to catalyze a reaction, or, as in the case of hemoglobin, to transport oxygen. However, denaturation processes should be minimized at a WBE's optimal temperature.

Discussion

From an evolutionary perspective, the considerations above make sense, as organisms perform best when they are within a narrow optimal thermal range, representing the optimal energy trade-off between their rates of anabolism and catabolism. The underlying chemistry to support this conclusion is a similar trade-off between short and long-range forces of attraction and repulsion within and between biological macromolecules and between these macromolecules and their surrounding environment in the organism, which consists largely of water, other organic and inorganic molecules, and ions. The observed structure-function dualism of proteins (Yan et al. 2018) exhibits this evolutionary adaptation of molecular processes within organisms to their biochemical environments, which include temperature, pH, ionic potential, solvents, etc.

The following points summarize what we believe is strong evidence for spontaneous protein denaturation being the essential contributor to the negative catabolic term ($-kW^m$) of Pütter's equation:

1) Proteins undergo spontaneous heat and cold denaturation in aqueous solution, exhibiting stability curves as a function of temperature with a minimum at 4 ˚C. Denatured proteins in living organisms typically must be resynthesized, leading to elevated metabolic rates, increased oxygen demand, and smaller maximum sizes.
2) In WBEs, oxygen consumption is a convex function of temperature, with a minimum at 4 ˚C. If oxygen is limiting to metabolic rates of WBEs *and* if spontaneous protein denaturation is responsible for catabolism, then their asymptotic growth (balance of anabolism and catabolism) will reflect this temperature dependence.
3) Oxygen demand in WBEs increases with temperature above 4 ˚C, reflecting elevated rates of protein re-synthesis in response to spontaneous protein denaturation. Above 4 ˚C, when temperatures increase, the maximum sizes of WBEs decrease.
4) Cold protein denaturation, which tends to occur below temperatures of 4 ˚C, also increases oxygen consumption and limits growth, owing to elevated metabolism

required to resynthesize denatured proteins. Arctic and Antarctic fish occurring at temperatures below 4˚ C are smaller than predicted by a projection of their size-temperature relationship to low temperatures (Pauly 1979, 1980; Lavin et al. 2022).

5) To our knowledge, no other growth-negating process has been described that is consistent with Equation (1) and does not lead to contradictory conclusions about growth processes in WBEs.

Different proteins have different half-lives, but if it can be assumed that the protein composition of a young WBE is roughly similar to that of an older WBE, the abundance ratio of different proteins will remain similar. This implies that the same denaturation processes are going on all over the body and that the exponent of the catabolic term in Pütter's equation $m = 1$, and thus can be neglected. That is, catabolism is proportional to the weight of WBEs, in contrast to anabolism.

The effect of increasing protein denaturation provides a causal mechanism for temperature-size rules (TSR) that state that temperatures higher than 4 °C lead to smaller maximum sizes for WBEs. Thus complex hypotheses to explain the smaller sizes of WBEs at high temperatures are largely superfluous (Pauly 2021), at least if Ockham's Razor applies.

At temperatures below 4 °C, such TSR are reversed because of cold denaturation, which forces WBEs to replace denatured proteins at rates similar to those required at much higher temperatures (Figure 1). This phenomenon was detected in Antarctic fishes by Wohlschlag (1964) and by Pauly (1979 and 1980) in meta-analyses of growth and natural mortality patterns, respectively, but their evidence was not strong. Now, given the contributions of Forster et al. (1987), Torres and Somero (1988), White et al. (2012), Fraser et al. (2022) and Lavin et al. (2022), we can assess why *"[n]either the careful experimental work of Holeton (1973, 1974) nor theoretical arguments (Clarke 1980, 1991, 1993) resulted in the demise of the concept of metabolic cold adaptation*" (Clarke and Johnson 1999). The reason for this resilience of the cold adaptation concept is that researchers continue to stumble on it effects while conducting empirical studies of fish occurring mainly in waters below 4 ^{0}C, and for which we also have, through the review of Privalov (1990), the mechanistic explanation that was so far lacking.

Indeed, cold denaturation not only affects WBEs in high latitudes, but also provides strong support for the hypothesis that it is the spontaneous denaturation of proteins that shapes the growth and limits the size of fish and other WBEs exposed to different temperatures.

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See Figure 1 below

Sloughing skin and other mechanical processes also contribute to losses of body substances and weight, but these generate minor ‘replacement cost’ are considered minor compared to spontaneous denaturation.

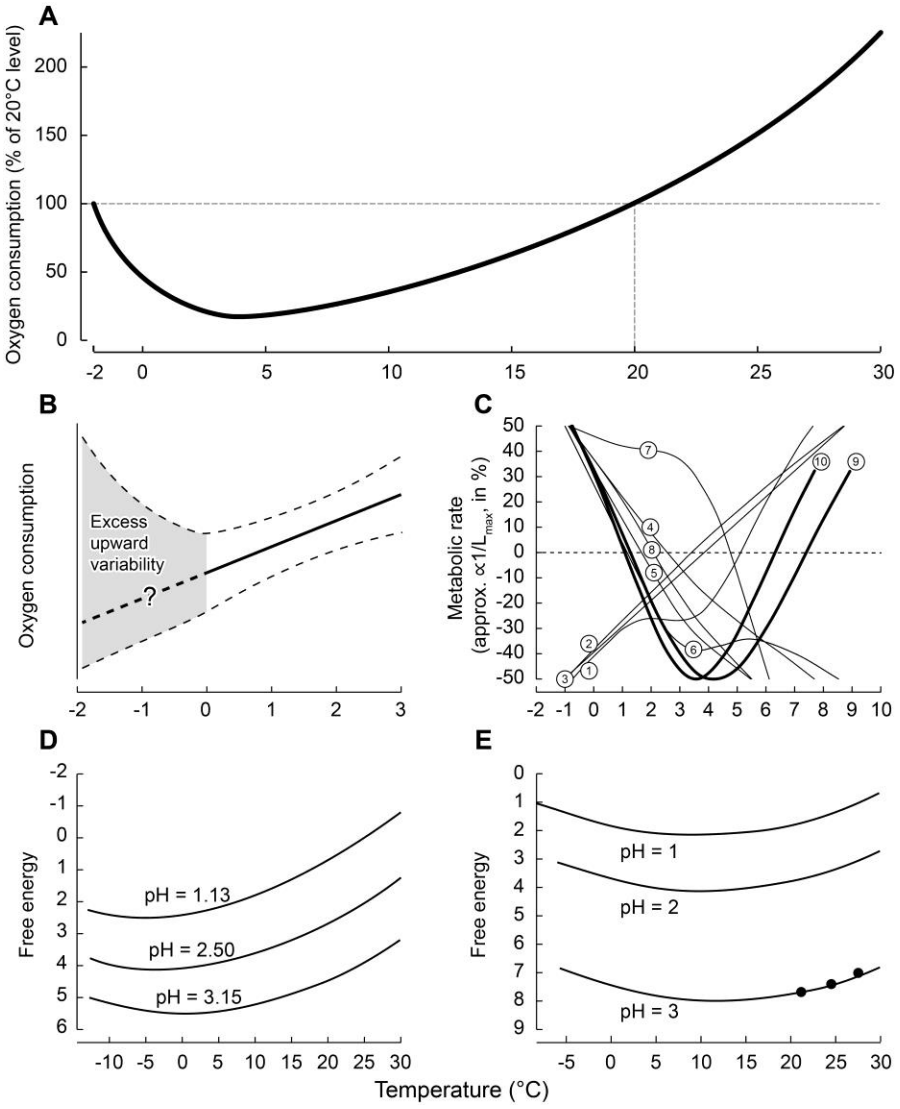

Figure 1. Relationships between oxygen consumption (and/or related processes) and temperature. **A**: Above 4 °C, the oxygen demand of WBEs increases with temperature, due to increasing Brownian motion leading to protein denaturation; below 4 °C, cold denaturation increases with decreasing temperature. Based on Ege and Krogh (1914) and Pauly (1979, 2021). **B**: Illustrating how Wohlschlag (1964) chose to present his data, with 'cold adaptation' interpreted as inducing 'variability.' **C**: Plots of non-linear regressions of maximum length *vs* water temperature of the 10 fish species in Norwegian trawl surveys (adapted from Figure 3 in Lavin et al. 2022), scale-inverted and rescaled as % deviation of their means, such as to make visible their relationships to plots of oxygen consumption *vs* temperature. **D** and **E**: plots of the stability (as a function of the Gibbs free energy) of two examples of enzymes *vs* temperature (modified from Brandts 1967), *viz.*, ribonuclease (**D**) and chymotrypsinogen (**E**). Note U-shape of the plots (but also note inverted scale), consistent with **A**-**C**.