10 Studies of metabolic rate and other characters across life stages

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10.1 Introduction

Environmental stressors like hypoxia, extreme temperature or elevated partial pressure of CO_2 as during ocean acidification scenarios may cause disturbances in acid-base status of an organism. Shifting pH values in different body compartments are widely accepted to affect individual physiological functions. Frequently, such effects are visible in changes in whole organism physiological rates, like growth rates and metabolic variables, but can also be detected at the level of protein/gene expression. Acid-base and metabolic regulation are interdependent processes such that changes in pH can affect metabolic rate, the mode of catabolism and energetic parameters (Pörtner, 1989).

Functional scope and thus metabolic features vary depending on the developmental stage and lifestyle of an organism. The role of acid-base regulation in metabolic regulation is likely species-specific, and within species, specific for the developmental and life stages of a species. Analyses and comparison of physiological processes and other functional characteristics between species and between life stages of a species over time therefore rely on the clear identification of the age, life stage or physiological status of compared individuals. Such comparisons frequently involve the study of metabolic rate, however, the principal approach when looking at the effect of pH on a process in different life stages is the same for all of these processes.

This chapter aims to provide keys on how to define the frame of reference for any process influenced by pH over time and, then, to provide methodology for metabolic studies, as an example. Other methodological aspects such as culturing methods and parameters that may influence any impact observed (e.g. food availability, maternal effect, intra-specific variations, see Qiu & Quian (1997)) are beyond the scope of this chapter. Experimental design and methods should be adapted to each particular species and question (see chapter 7). Note that the analysis of acid-base variables is addressed in chapter 9.

One key variable to consider when choosing the relevant methodology for studies of metabolic or other processes during ontogeny is a developmental signpost (e.g. age). Furthermore, it needs to be considered that mode of life and thus the level of spontaneous activity may change during ontogeny of the individuals analysed (e.g. pelagic larvae and benthic adults) as much as their age and body size change. Adequate precaution needs to be taken to assure that metabolic variables and other characters can be defined for specific life stages or developmental stages and also be compared between them.

10.2 Definition of a frame of reference: studying specific characters across life stages

The performance of an organism is dependent on intrinsic and extrinsic factors. Indeed, intrinsic processes such as metabolism or gene/protein expression are likely to vary with life stages (e.g. developmental stages) and body size or age and, in addition, will be highly influenced by environmental conditions such as temperature (e.g. during seasonal variations), food availability or CO_2 exposure. For example, there is a growing body of evidence indicating that when raised under conditions of ocean acidification expected in the near future, larval stages develop more slowly (Dupont & Thorndyke, 2008; Dupont *et al.*, 2010) while juveniles and adults can either grow slower (e.g. Shirayama & Thornton, 2005) or faster (e.g. Gooding *et al.*, 2009). This is complicated by interactions with other factors such as temperature, which is well known to impact growth and development

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processes in itself. The interaction of various factors can best be studied when using, for example, temperature sensitivity as a matrix and a unifying physiological concept such as the concept of oxygen- and capacity-limited thermal tolerance (OCLT) in animals to integrate the specific effects of temperature with those of other environmental factors like hypoxia or ambient hypercapnia (ocean acidification) (Pörtner, 2010).

The phenomenon of environmental effects on time-dependent functions can lead to a classic experimental design problem: how to assess the impact of a tested parameter (e.g. pH) on a given process (e.g. gene expression or metabolic rate) at a certain time or stage, while this parameter also influences the rates of growth and/or development and thereby leads to different stages at a given time in controls and experimental specimens? In other words, how can we discriminate the real effect of the parameter from phenomena which can result in differences due to differential growth or development?

Age (i.e. time post fertilisation) is still frequently used as the reference scale, for example in pH studies on larval development (see O'Donnell *et al.* (2009) and O'Donnell *et al.* (2010) for recent examples in the ocean acidification field). However, when the tested parameter influences growth and developmental rate, using *age* as the reference scale may introduce inaccuracies and even invalidate conclusions as a developmental stage defined in the controls may not be reached at the same age in exposed specimens, especially when only one time point is used.

For example, assume we want to see if low pH influences the expression of the gene X during the development of a sea urchin (Figure 10.1). In the control, this gene is activated only during a limited time window, post fertilisation (e.g. during 2 days when the larvae reach the pluteus stage). Under low pH conditions, development is slower and it will take one more day to reach the same pluteus stage.

In this theoretical example, using age as the reference frame, the investigator may decide to compare the expression of the gene X at day 2 in two tested conditions (control and low pH). S/he will conclude erroneously
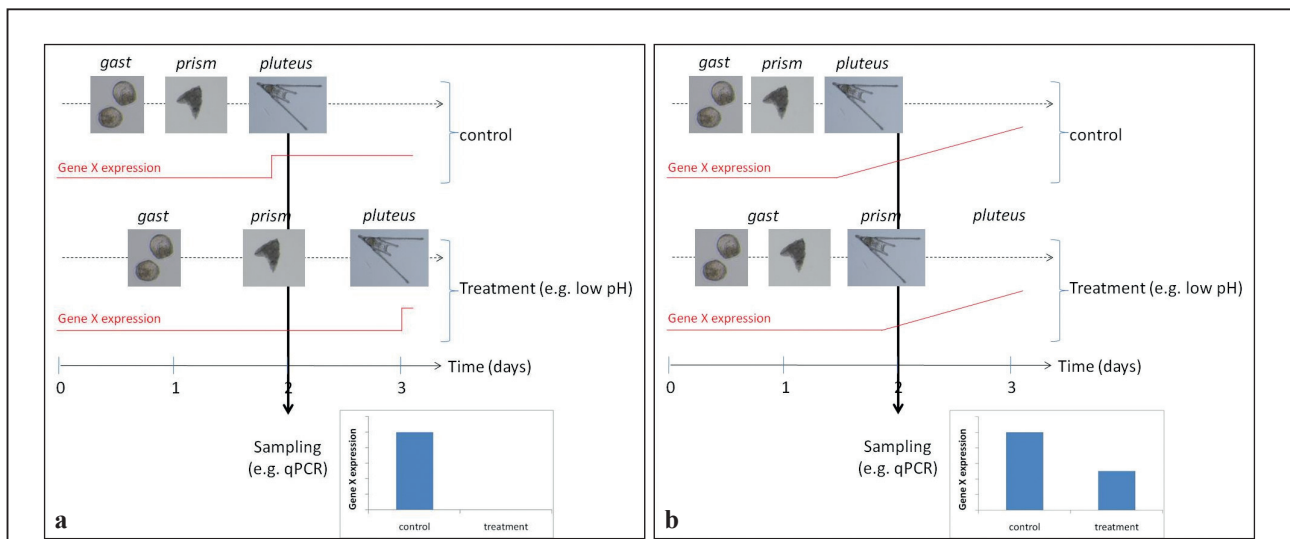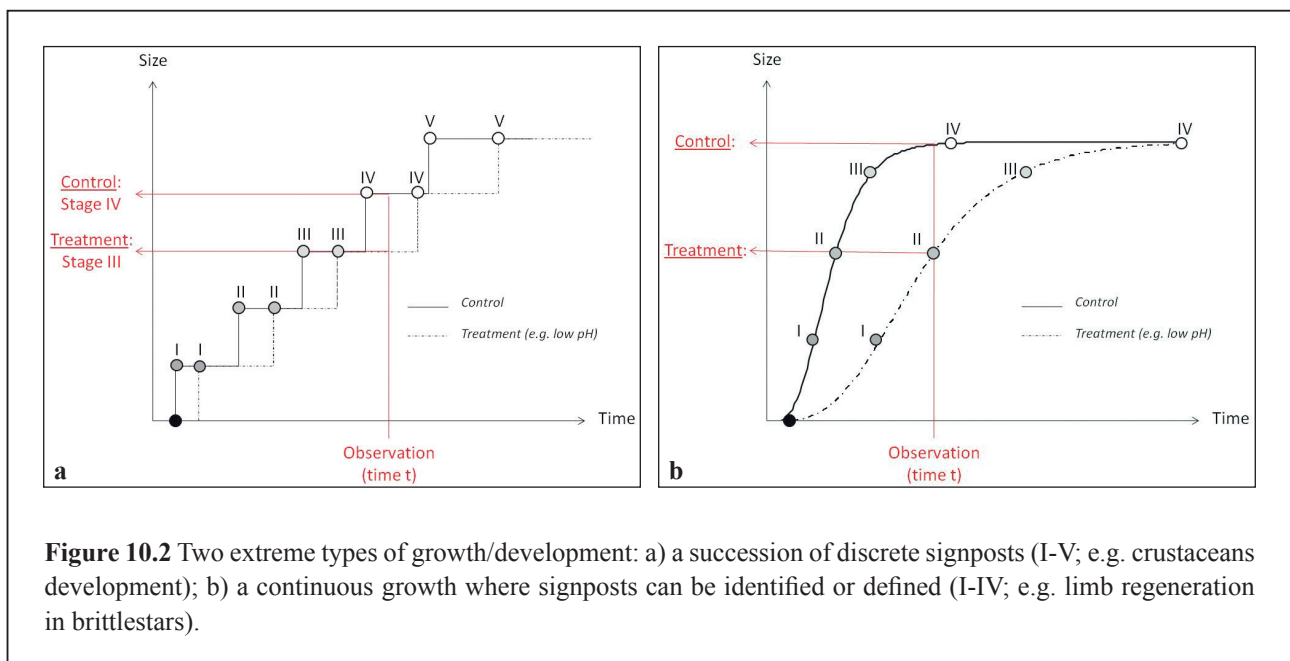

Figure 10.1 Theoretical experiment investigating the expression of a gene X during the development of a sea urchin under control or a treatment inducing a reduction in growth rate (e.g. low pH; Dupont & Thorndyke, 2009). Under the treatment, it takes more time to reach the same developmental signposts. In the scenario a), the gene is first expressed (on or off) when the larvae reach the pluteus stage (day 2 in control, day 3 in treatment). As a consequence, gene expression at day 2 is showing expression of the gene X only in the control. In the scenario b), the impact of the treatment is more subtle and, at day 2, the pluteus stage is reached in both conditions. However, pluteus larvae are slightly larger in the control. This small difference in size is translated into a small, quantitative difference in gene X expression. In both scenarios, the conclusion may be that the treatment induces a down-regulation of the gene X at day 2.

that the treatment induces a difference in gene expression (absence of, or lower, expression at low pH compared to "normal" expression in the control).

In conclusion, when the tested parameter influences growth and developmental rates, *age* is not the only relevant scale and one should rather use a reference frame of non-dimensional events (e.g. stages, *developmental signposts*) rather than time *per se*.

This could partly be resolved by following the dynamics of a given process (e.g. the evolution of physiological state over time; Meyer *et al.*, 2007) rather than choosing comparisons at discrete observation points. In order to be widely applicable in different research groups so as to allow comparison between studies, a *frame of reference* should be simple, easy to measure and provide a strong predictive capacity.

The definition of the frame of reference is often based on **developmental signposts** and can be straightforward for discrete processes (e.g. larval development in crustaceans; Figure 10.2a) but much more complicated for continuous (e.g. limb regeneration in brittlestars; Figure 10.2b) or semi-continuous ones (e.g. larval development in echinoderms).

Figure 10.2 Two extreme types of growth/development: a) a succession of discrete signposts (I-V; e.g. crustaceans development); b) a continuous growth where signposts can be identified or defined (I-IV; e.g. limb regeneration in brittlestars).

Crustaceans have a rigid exoskeleton, which must be shed to allow the animal to grow (ecdysis or molting). As a consequence, crustaceans usually develop through a sequence of planktonic larval forms. For example, the copepod *Acartia clausi* develops through a sequence of 6 nauplii and 5 copepodite stages before reaching the adult stage. These developmental stages are obvious and represent relevant developmental signposts classically taken into account during physiological and molecular studies (e.g. Calcagno *et al.*, 2003; Thatje *et al.*, 2004; Leandro *et al.*, 2006). The developmental clock is influenced by temperature as one of the main environmental parameters. Larvae develop faster at higher temperatures (e.g. shorter stage duration in *Acartia clausi*; Leandro *et al.*, 2006). To assess the impact of temperature on any given process (e.g. biomass in the stone crab *Paralomis granulose*; Calcagno *et al.*, 2003), the investigator should then compare the same *stage* rather than the same *age*.

The problem is more complicated when the studied process is continuous (no obvious signposts) or semi-continuous (signposts but continuous growth). Development in echinoderms is a good example of a semi-continuous process. In striking contrast with crustaceans, echinoderm larvae have either an internal skeleton (e.g. sea urchin) or no skeleton (e.g. sea star) allowing continuous growth. For example, the sea star *Asterias rubens* develops through few developmental stages (first, a bipinnaria larva, followed by a brachiolaria larva

that metamorphoses into a juvenile). However, each stage grows continuously and neither age nor obvious developmental signposts are sufficient to allow accurate comparison and assess the impact of tested parameters (see Figure 10.1b).

This is even more difficult with continuous processes, although for some of these processes it is possible to identify some developmental signposts. For example, larval growth of the pelagic tunicate *Oikopleura dioica* is continuous and includes some arbitrary developmental signposts: early hatchling, mid-hatchling, late hatchling and tailshift (Cañestro *et al.*, 2005). However, this simple staging method is not sufficient to assess the complexity of developmental progression at the cellular and molecular levels, and more accurate frames of reference have been recently developed (e.g. endostyle cell growth; Troedsson *et al.*, 2007). It is important to notice that such reference signposts are lacking for many taxa and processes.

Limb regeneration in the brittlestar *Amphiura filiformis* is another example of a continuous process. After amputation of an arm, new tissues arise from active proliferation of migratory undifferentiated cells (amoebocytes and coelomocytes), which accumulate in a blastema. The new arm extends from the tip with segmental maturity occurring in a temporally regulated fashion and proximal-distal direction until a full length and fully differentiated arm has regrown. The regeneration rate appears to be highly dependent on extrinsic environmental factors (e.g. the regenerated arm grows faster at higher temperatures (Thorndyke *et al.*, 2003) or lower pH (Wood *et al.*, 2008)), but also on intrinsic factors (e.g. length of the lost part of the arm (Dupont & Thorndyke, 2006)). In the regeneration field, time of regeneration is the classical parameter used in molecular, cellular, histological, dynamics and ecological studies. However, regenerates of the same size and/or same regeneration time can present very different characteristics in terms of differentiation and functional recovery, and vary according to the position of autotomy along the arm and/or environmental conditions. In consequence, the use of time of regeneration is inappropriate, especially in dynamic studies (Dupont & Thorndyke, 2006). One approach includes the definition of arbitrary signposts, such as level of differentiation of the regenerating arm (<10%, 50% and >90% of differentiation).
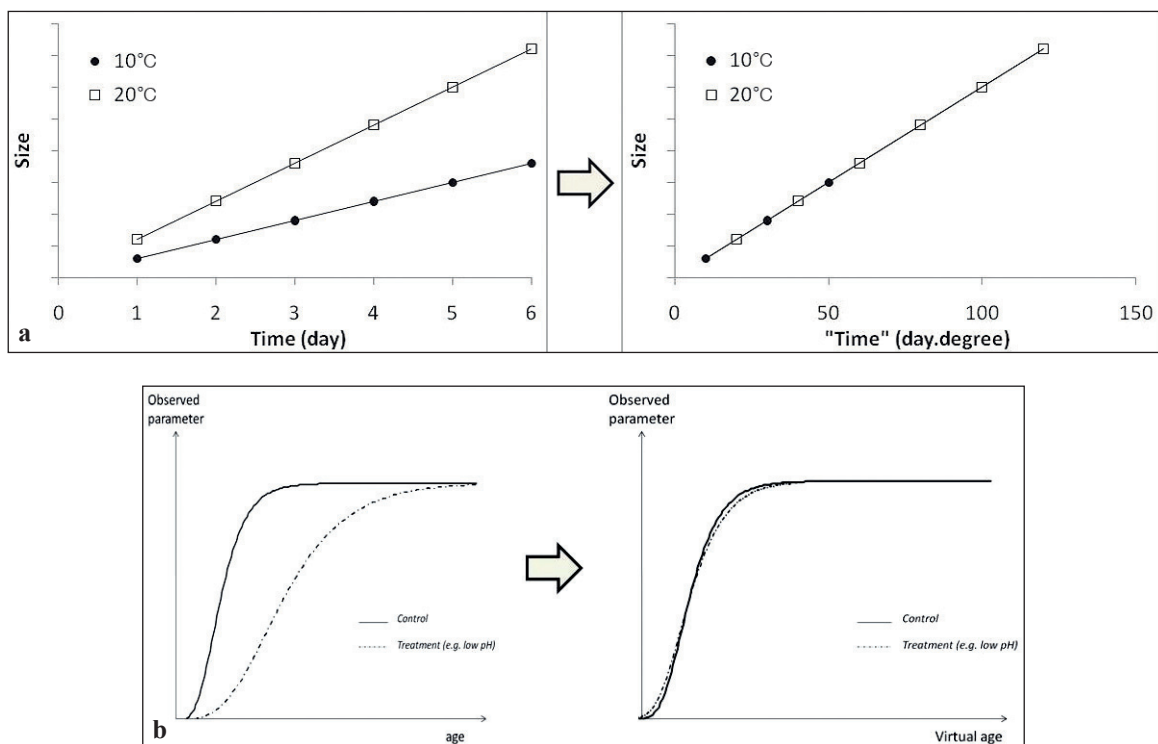
Figure 10.3 Theoretical example showing how to standardise data integrating the impact of a given parameter on growth and/or developmental rates: a) standardised time using a time × temperature scale; b) virtual age.
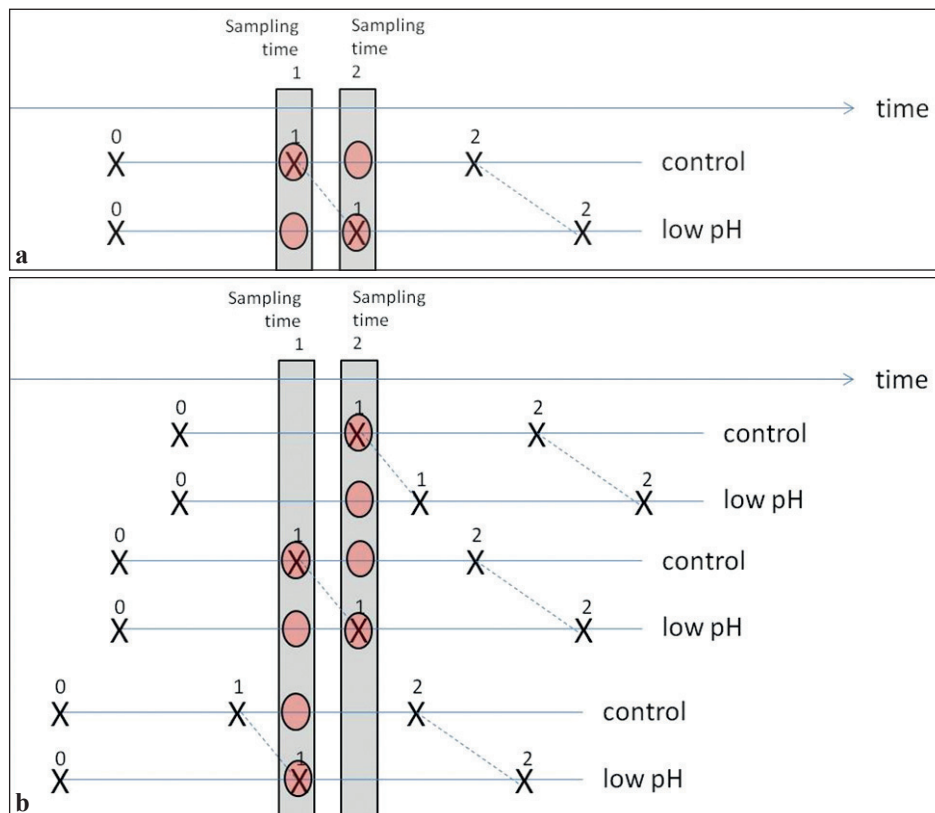
Figure 10.4 Example of good experimental design for a microarray experiment when developmental and/or growth rates are impacted by ocean acidification. a) Simple design (4 samples at 2 sampling times) allowing the comparison of the same developmental signpost (1) and same times. b) Complex design (8 samples at 2 sampling times) allowing comparison of the same developmental signpost (1) at the same time and allow revealing impact of any other confounding factor.

For continuous or semi-continuous processes with rates influenced by the given treatment, the ideal method consists in **following the whole process** by making multiple observations over time (e.g. respiration in sea urchin larvae under different feeding regimes; Meyer *et al.*, 2007). However, this is not always possible due to practical constraints. For example, some molecular techniques such as microarrays are costly, both financially and regarding biological material needed, and, in most cases the investigator is allowed only few sampling points (see below for questions concerning the experimental design in such experiments).

There are several ways to correct for "age" and take into account the impact of the tested parameter on growth or developmental rate including (1) using a **standardised time scale** that takes confounding parameters into account (e.g. temperature × day; Thorndyke *et al.*, 2003; see Figure 10.3a), (2) the use of the **virtual age** (i.e. the time required in controlled conditions to reach a precisely defined stage based on relevant morphometry and/or signposts; see Figure 10.3b), which can be a more efficient way to correct for the age, and (3) including relevant signposts into the analysis.

As an example, we can consider a theoretical design for a microarray experiment to assess the impact of ocean acidification on larval development using the hypothesis that low pH will have a negative impact on developmental rate.

Transcription profiling using suppression subtractive hybridisation or microarrays is an important approach in ecological genomics, for example when studying CO_2 effects (Dupont *et al.*, 2007; Deigweiher *et al.*, 2008). In this technique, RNA is isolated from two (or more) different samples derived from study subjects under

different conditions. The easiest and classic experimental design comprises exposure of organisms to two different treatments for a given time (see O'Donnell *et al.* (2009, 2010) for recent examples in the ocean acidification field).

However, in our theoretical example, pH affects developmental rate, and comparing individuals from the treatments at a given time is not relevant (see discussion above). Therefore, a more complex experimental design is needed. The easiest way to solve this problem is to include developmental signposts in the sampling. Rather than sampling at given times (sampling time 1), another sample should be taken (sampling time 2) when the larvae reach the same developmental signpost at low pH as those in the control conditions at sampling time 1 (Figure 10.4a). This design will then allow comparison of both the same time (but different developmental signposts) and the same developmental signpost (but different time), and then allow discrimination between the impact of the treatment on specific gene expression patterns from the impact on developmental rate. Ideally, to assess the impact of other potentially confounding factors, samples should also be taken at all developmental stages under the experimental pH at the same sampling times (Figure 10.4b).

10.3 Approaches and methodologies: metabolic studies

Performance capacity of an organism is one basic link between environmental challenges like ocean warming and acidification and ecosystem level consequences (Pörtner & Farrell, 2008). Performance capacity is closely linked to metabolic capacity. Therefore, the study of metabolic rate under various environmental conditions and during various behaviours provides a basis for addressing performance and is one of the most common physiological characters investigated. Both the capacity of performance and associated metabolic capacity may change during life history and among various larval stages. The discussion above is therefore most relevant for analyses of metabolic rate, which changes not only depending on life stage but also, within a life stage, on body size and age or under the influence of environmental parameters. This section will briefly address the principles of metabolic studies across life stages.
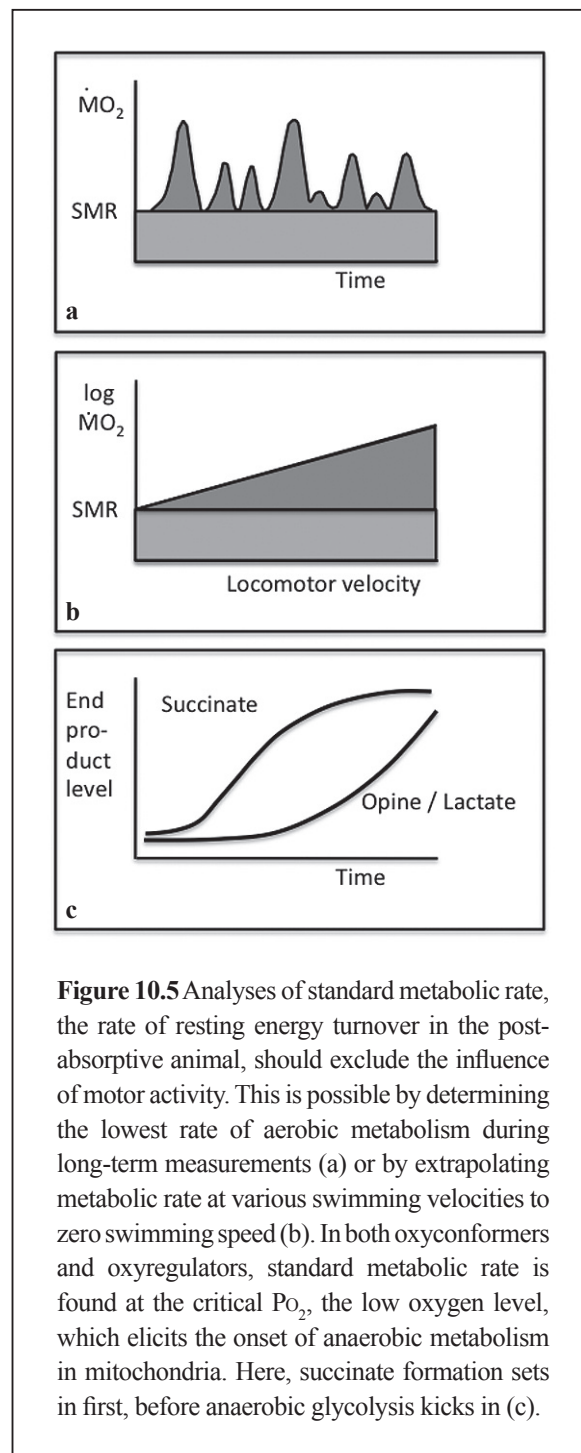
In animals, aerobic scope is a proxy for performance capacity. The excess in oxygen availability is reflected in an animal's aerobic scope, which supports a performance curve with an optimum close to an upper thermal limiting threshold (see below). These considerations match earlier definitions by Fry (1971) who classified environmental factors based upon their influence on aerobic metabolism and aerobic scope (the difference between the lowest and highest rates of aerobic respiration). Aerobic scope is the difference between maximum aerobic metabolic rate (displayed, for example, under maximum sustained exercise conditions) and standard metabolic rate (the rate associated with maintenance in the resting, conscious and unfed animal). Feeding, growth, behaviour and muscular exercise exploit the range of aerobic scope. Net aerobic scope is restricted to a species-specific thermal window (see below).

The study of standard metabolic rate requires excluding the effects of stress, food consumption or spontaneous activity on metabolic rate. It also requires consideration of whether an animal is an oxyregulator or an oxyconformer (Figure 10.5). It has been recognised earlier that animals may show different patterns of oxygen consumption in response to changes in ambient partial pressure of oxygen (P$_{O_2}$). Some keep their oxygen consumption more or less constant in a wide range of P$_{O_2}$ and are called oxyregulators. Others reduce their oxygen uptake with decreasing oxygen tensions and have, consequently, been termed oxyconformers. Intermediate responses exist (Mangum & van Winkle, 1973) which do not support such clear categorisation. It is nonetheless useful to help identify metabolic patterns of oxyconformity and the underlying mitochondrial mechanisms. Circumstantial evidence for the presence of an alternative mitochondrial oxidase (cytochrome o) exists in lower marine invertebrates, for example sipunculids, annelids or bivalves (Pörtner *et al.*, 1985; Tschischka *et al.*, 2000; Buchner *et al.*, 2001). This oxidase might represent an ancient mechanism of oxygen detoxification used in animals that live in hypoxic environments. Only these aerobic oxyconformers, which display oxyconformity at cellular and mitochondrial levels should be considered as "true" oxyconformers, whereas the progressive drop
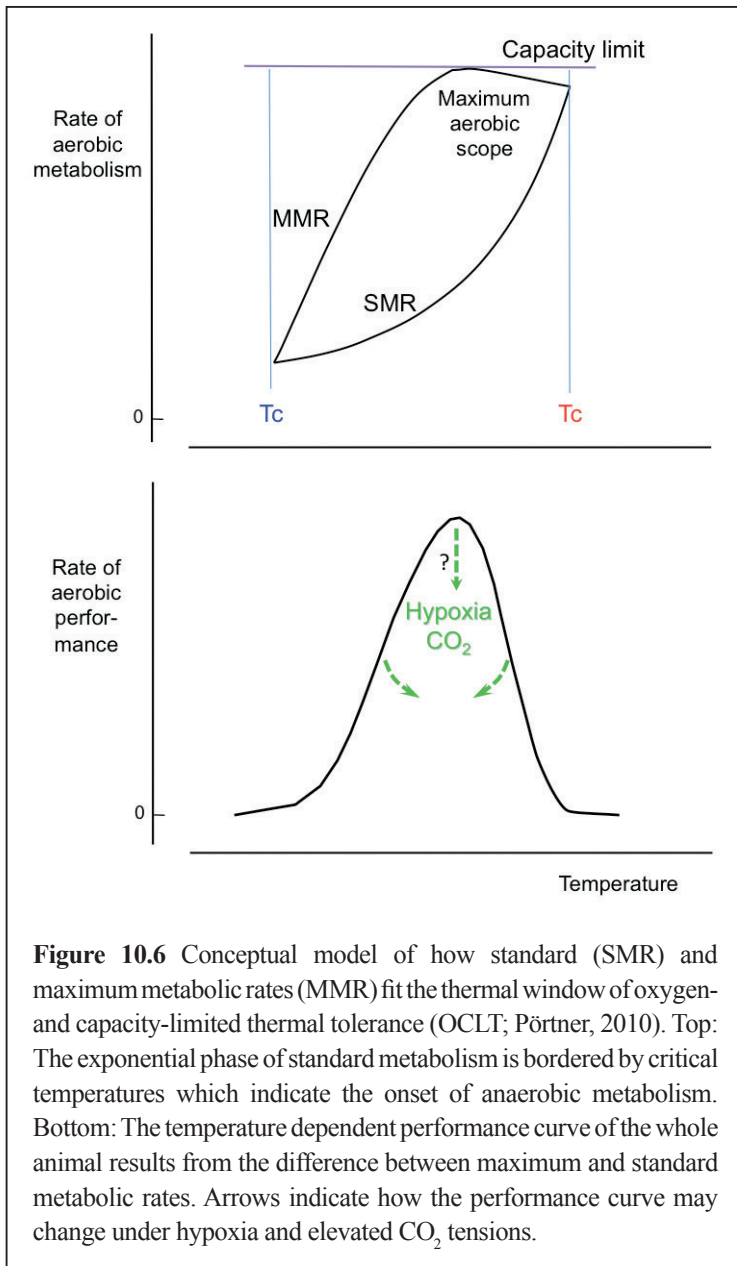
in oxygen consumption seen during extreme hypoxia in both oxyconformers and oxyregulators is caused by oxygen supply being insufficient to completely cover energy demand. Variable intracellular oxygen levels drive variable rates of oxygen consumption in oxyconformers, whereas this rate remains constant above a critical threshold (critical P_{O_2}) in oxyregulators. The critical P_{O_2} characterises the degree of hypoxia sensitivity of a species or life stage.

The terms oxyconformity and oxyregulation have been defined for quiescent animals, which display a baseline or a "standard" metabolic rate. Standard metabolic rate (SMR) is defined as the lowest rate of oxygen consumption for oxyregulators, obtained when all organs are at rest. The data available for several oxyregulators suggest that this rate is maintained down to the critical P_{O_2} (P_c), below which anaerobiosis starts (see below). This clear physiological definition of SMR excludes the "perturbing" effect of spontaneous muscular activity on the pattern of oxygen consumption during hypoxia. SMR is analysed by: (1) correcting for the influence of spontaneous activity during long-term measurements, (2) extrapolating to zero activity during analyses of aerobic metabolism at various activity levels, or (3) determining metabolic rate at or slightly above the P_c, to exclude the effect of oxyconformity mechanisms (Figure 10.5). Standard metabolic rate is determined in the post-absorptive organism considering that food-induced changes in metabolic rate (specific dynamic action) may last between hours and weeks, depending on metabolic performance of the animal or on ambient temperature.

Ideally, SMR is determined in a flow-through system, simulating natural conditions of the animal in terms of ambient light or temperature. A constant metabolic rate is reflected in a clearly measurable steady-state drop in oxygen tensions (e.g. by around 10%, i.e. 2 kPa, or 20,000 µatm when starting from normoxia), while a constant flow of water is maintained. It requires consideration that similar molar quantities of CO_2 will accumulate in the

Figure 10.5 Analyses of standard metabolic rate, the rate of resting energy turnover in the post-absorptive animal, should exclude the influence of motor activity. This is possible by determining the lowest rate of aerobic metabolism during long-term measurements (a) or by extrapolating metabolic rate at various swimming velocities to zero swimming speed (b). In both oxyconformers and oxyregulators, standard metabolic rate is found at the critical P_{O_2}, the low oxygen level, which elicits the onset of anaerobic metabolism in mitochondria. Here, succinate formation sets in first, before anaerobic glycolysis kicks in (c).

respirometer. At 20°C and with a respiratory quotient (RQ) of 1 (carbohydrate catabolism), the about 30-fold higher solubility of CO_2 in water will cause a rise in CO_2 tensions 30-fold lower than the drop in oxygen tension (e.g. by around 0.066 kPa or 660 µatm). This value is a maximum estimate under the assumption that the greatest proportion of CO_2 remains fugacious and does not form H_2CO_3. At seawater pH, the rate of formation of CO_2 hydrate is minimal (Pilson, 1998). This rise will then be reduced by the response of seawater buffers (see chapter 9). Net proton excretion by the organism would further increase CO_2 tensions. Somewhat lower levels of CO_2 accumulation result with RQs of 0.7 (lipids) or around 0.85 (proteins or mixed diets). Overall, such fluctuations are in the order of magnitude of ocean acidification scenarios and require consideration during studies of effects on metabolism and also when setting perfusion rates in experimental systems to relatively low rates.

Figure 10.6 Conceptual model of how standard (SMR) and maximum metabolic rates (MMR) fit the thermal window of oxygen- and capacity-limited thermal tolerance (OCLT; Pörtner, 2010). Top: The exponential phase of standard metabolism is bordered by critical temperatures which indicate the onset of anaerobic metabolism. Bottom: The temperature dependent performance curve of the whole animal results from the difference between maximum and standard metabolic rates. Arrows indicate how the performance curve may change under hypoxia and elevated CO_2 tensions.

Prior to analysis, a sufficiently long acclimation period (typically around a day in temperate zone invertebrates or fish) is necessary to ensure the resting state of the organism, visible from a minimisation of steady-state metabolic rate. Before measuring, any flow-through system must reach equilibrium washout. Measurement periods usually last more than 3 h for a clear analysis of steady state metabolism. Acute changes in metabolic rate in response to changing experimental conditions (temperature, CO_2 additions) will have to be quantified by correcting for the washout characteristics of the system, until a new steady-state is reached. Alternatively, oxygen consumption can be derived from the oxygen depletion in closed systems. Here, stoichiometric CO_2 accumulation also occurs, as outlined above, but not to a steady-state level. Sufficient flushing between measurements should make sure that oxygen depletion and CO_2 accumulation always start from normoxic and normocapnic levels (380 ppm), respectively. Long-term analyses can still be carried out in an intermittent flow system, which is flushed and replenished with oxygenated water and then closed for recordings of oxygen depletion at regular intervals.

SMR displays an exponential phase between critical temperatures (Figure 10.6). Aerobic scope is limited to this range of temperatures, and thereby defines a species-specific temperature window, which forms the basis for the temperature dependent biogeography of a species. A functional optimum characterises the thermal optimum. Building on the concept of oxygen- and capacity-limited thermal tolerance (OCLT), experiments should identify this range, both acutely, to identify the sensitivity of animals to short-term temperature fluctuations within a specific season, and after long-term acclimation to a (seasonal) change in temperature, which allows animals to shift their thermal window as between seasons (Wittmann *et al.*, 2008). This enables addressing the specific effects of other factors, like ocean acidification, over a relevant range of environmental temperatures (cf. Pörtner, 2010) and, thereby, identify more comprehensively which factors are causing functional limitations, alone or in combination.

As outlined above, not only SMR, but also and independently, aerobic scope and thus aerobic exercise capacity might be affected by ocean acidification. The study of metabolism can be carried out during periods of sustained activity in animals on treadmills or in swim tunnels, for example in the case of fish or squid. Aerobic exercise capacity needs to be clearly distinguished from exercise bouts supported by anaerobic metabolism, such as during attack or escape responses. For a complete understanding, the mechanistic background of metabolic and functional scopes for performance requires exploration.

Technology and approaches for studying aerobic exercise metabolism are most advanced in the field of fish physiology (see e.g. Hammer (1995) and Plaut (2001) for comprehensive reviews), whereas only a few attempts have been made to study oxygen consumption during aerobic exercise in invertebrates (e.g. Booth *et al.,* 1984 (decapod crustacea); Wells *et al.*, 1983, 1988 (cephalopoda)). In order to study aerobic scope for exercise, fish or cephalopods are forced to swim against a current in a sealed chamber of a swim tunnel respirometer. Oxygen consumption rates are then calculated from linear declines in oxygen partial pressure within the chamber (no more than 1-2 kPa), which is measured using an oxygen electrode or optode (see below). Swimming speed is increased in a stepwise mode and the water in the otherwise sealed chamber is replaced between oxygen consumption measurement runs in order to maintain high oxygen partial pressures (typically >18 kPa) at all times (= intermittent closed respirometry). Typically, fish are maintained at a given swimming velocity for 5 to 60 minutes (e.g. Plaut, 2001). Once the fish cannot swim against the current at high velocities for this entire preset time period, the critical swimming speed (U_{crit}) is reached. It can be calculated according to Brett (1964):

$$U_{crit} = u_i + \left(\frac{t_i}{t_{ii}} u_{ii} \right); \qquad\qquad (10.1)$$

with u_i being the highest swimming velocity (m s^{-1}) sustained for the entire preset time interval t_{ii}, t_i the time interval shorter than t_{ii}, spent at exhausting velocity (min), t_{ii} the time interval at each swimming speed (min) and u_{ii} the velocity increment between steps (m s^{-1}). Oxygen consumption values M_{O_2} can then be plotted against swimming speed. Active metabolic rate (AMR) can be approximated as the metabolic rate at the highest sustainable swimming speed. Standard metabolic rate (SMR) can be estimated from M_{O_2} vs. swimming velocity relationships by extrapolating to zero swimming speed (cf. Reidy *et al.,* 2000). Previous studies have established that U_{crit} and M_{O_2} during U_{crit} trials are significantly reproducible traits for individual fish (e.g. Nelson *et al.*, 1994; Reidy *et al.*, 2000). Aerobic scope (AS) can be estimated by subtracting SMR from AMR. Automated swim tunnel respirometers can be purchased in various sizes from a few selected companies (e.g. Loligo Systems ApS, Denmark; Qubit Systems, Canada).

10.4   Study of early life stages

Most studies of metabolism focus on juveniles and young adults, since these are sufficiently large and easy to maintain in the laboratory. However, one crucial field of future study comprises larval responses to ocean acidification. In the marine realm, most ectothermal animals pass through complex life cycles comprising planktonic larval phases, which might be among the most sensitive to expected scenarios (Pörtner & Farrell, 2008) despite the presence of mechanisms that can improve overall resistance to stressful ambient conditions (Hamdoun & Epel, 2007). Metabolic measurements will help to unravel effects of ocean acidification on larvae and larval development. Larval respiration rates represent a comprehensive parameter to characterise metabolic rates. Metabolic processes underneath comprise carbohydrate, lipid and protein metabolism. There is a huge amount of literature on changes in the biochemical composition of early life stages and how to measure them. Covering these methods is beyond the scope of this chapter.

Determining metabolic rates of larval invertebrates and fish involves a number of technical difficulties because such larval stages are small, they are usually swimming actively in the water column and they are developing (section 10.6). All of these factors influence respiration rates. Furthermore, non-feeding and feeding stages have to be distinguished during the early life history of a species as these display strong differences in respiration rates (Marsh & Manahan, 1999; Anger, 2001; Meyer *et al.*, 2007). Animals may have either non-feeding larvae (lecithotrophic) or feeding (planktotrophic) larvae. For the planktotrophic ones, the onset of feeding will cause a strong rise in respiration rates. The respiration rate associated with feeding has been termed specific dynamic action, SDA (e.g. Kiørboe *et al.*, 1987).

One problem during the actual measurement centres on the need to confine a small pelagic organism to a small volume as needed for oxygen depletion measurements. Minimising handling stress and controlling tiny sizes and locomotor activity are the biggest challenges (Childress, 1977) to ensure accurate measurements of metabolic rates in marine invertebrate and fish embryos and larvae (for an extensive discussion see Hoegh-Guldberg &

Pearse (1995), Marsh & Manahan (1999), Marsh *et al.* (2001), Glazer *et al.* (2004) and Storch *et al.* (2009)). Depending on the sensitivity and stability of the oxygen sensors, tiny individuals may need to be pooled for a single measurement, leading to crowding and potential stress effects. The optode (Marsh *et al.*, 2001; Thatje *et al.*, 2003; Szela & Marsh, 2005; Storch *et al.*, 2009a) and the couloximeter (Marsh & Manahan, 1999; Peck & Prothero-Thomas, 2002) most conveniently measure individual respiration rates in embryonic and larval life stages of marine invertebrates beyond a minimal size. In others, however, the sensitivity of the technique chosen needs to be maximised by appropriate adjustments in experimental design. For instance, in sea urchins, one may need several hundreds of larvae to obtain a sufficient signal when using classical optodes. Alternatively, one may convert a 384-well microtiter plate into a 384-chamber (50µl) micro-respirometer using a plate-reading fluorometer for continuous, real-time data acquisition (Szela & Marsh, 2005; Strathmann *et al.*, 2006).

In their natural habitat, larvae swim permanently in the water column and measurements, therefore, include the effect of motor activity and yield routine metabolic rates. The costs for locomotor activity can result in more than threefold higher oxygen consumption rates, seen, for example, in megalopae of the kelp crab *Taliepus dentatus* (Storch *et al.*, 2009b). Activity costs of crustacean larvae can be quantified from pleopod beat rates. SMR can then be calculated by subtracting these costs from measured rates. Respiratory costs of swimming in fish larvae can be calculated from the relationship between swimming speeds and respiration rates (Kaufmann, 1990). Larval maintenance, swimming and metamorphosis all use energy from a common pool, such that increased allocation to maintenance or swimming occurs at the expense of growth and/or development (Hunter *et al.*, 1999; Marshall *et al.*, 2003). Ocean acidification might affect each of these processes differently, such that the organismal energy budget needs to be analysed (Storch *et al.*, 2009a).

10.5 Techniques for oxygen analyses

The most common techniques of measuring oxygen are (1) Winkler titration (Winkler, 1888; Meyer, 1935), (2) polarographic oxygen sensors or Clark-type electrodes (after Clark (1956), see also Gnaiger & Forstner (1983)), (3) coulometric oxygen sensors (Peck & Whitehouse, 1992; Hoegh-Guldberg & Manahan, 1995) and (4) optical oxygen sensors (optrodes or optodes) (Stokes & Somero, 1999; Frederich & Pörtner, 2000; Marsh *et al.*, 2001; Gatti *et al.*, 2002; Glazer *et al.*, 2004; Lannig *et al.*, 2004; Szela & Marsh, 2005), e.g. O_2 PSt1, Presens. Temperature needs to be finely controlled as metabolic rate varies with temperature. Adequate mixing of the chamber volume ensures precise recordings and elimination of boundary layer effects. For convenience, metabolic rate should be given in molar units of oxygen consumption per unit of metabolically active (soft) body or tissue weight, for clear access to stoichiometries and the effective concentrations of substrates and other biochemical components.

1. The Winkler titration and its modifications are often denoted the "gold standard" for measuring dissolved oxygen. More recent methods are usually validated by comparing their results with those of the Winkler technique (Wilkin *et al.*, 2001; Glazer *et al.*, 2004). However, the Winkler titration is laborious and is not suitable for continuous non-invasive monitoring of oxygen concentrations. Further disadvantages of Winkler titrations include the use of toxic chemicals (e.g. sodium azide) for some modifications and the requirement of tedious wet chemistry techniques with multiple steps. Large sample volumes are needed, and handling and transport of the apparatus can be cumbersome.

2. Clark-type electrodes have been widely and reliably used in flow-through or closed system analyses of respiration. Measurements need to be corrected for the inherent rate of oxygen consumption by the electrodes. Furthermore, they are susceptible to disturbance of the signal when stirring.

3. Most coulometric oxygen sensors are based on redox indicators and are pH sensitive, which has to be considered when measuring in seawater with varying CO_2 concentrations and, thus, pH values.

4. Optical oxygen sensors (optrodes or optodes) depend on dynamic quenching of an oxygen-sensitive fluorophore. These sensors do not consume oxygen, have predictable temperature responses, have fast response times and long-term stability, and can be made inert to interfering chemicals like acids. The decay of the fluorophore over time requires repeated calibration and causes sensitivity losses.

10.6 Overall suggestions for improvements

Potential pitfalls in experimental design and metabolic studies include the following:

– The age of the individual or the time periods chosen for exposure may not be relevant for comparative –omic or physiological studies when growth or developmental rates are affected by ocean acidification and change the relative time scale of crucial processes.

– The metabolic rate measured may not fully reflect resting, routine or active states of the organism as defined by the experimenter. Stress phenomena or feeding effects may persist longer than anticipated before the organism displays standard metabolic rate or clearly defined parameters such as active metabolic rate, net or factorial aerobic scopes.

Suggestions to alleviate these problems include the following:

– Methods and frame of reference (preferably according to developmental signposts) should be clearly defined.

– Methods should be used that allow work at the level of the individual in order to assess the variability in the population.

– The physiological or acclimation state of the organism should be clearly definable, as demonstrated by stable characters, for example based on long-term recording of oxygen consumption.

10.7 Data reporting

Investigators should report:

– Information required to fully describe the experimental procedure and exposure regime, length of exposure, water physicochemistry values (levels of e.g. pH, bicarbonate, carbonate, calcium) on physiologically relevant scales.

– Information required to fully describe the physiological state of the animals (e.g. acclimation and treatment), and their age (post-fertilisation) and life or developmental stage (preferably according to developmental signposts).

10.8 Recommendations for standards and guidelines

1. When pH is affecting the rate of any investigated process, the experimental design should be adapted to discriminate the real impact on the parameter from other confounding factors linked to differential growth or development. Specifically, comparative studies on developmental stages should comprise experiments monitoring changes progressively over time to clearly distinguish the effects of developmental delay from those of the stressor itself at certain time points of ontogeny.

2. Metabolic rate studies should make sure to use gas-tight material for chambers and tubings.

3. Metabolic rate analyses should allow the animal to reach a steady-state rate of oxygen consumption clearly defined for its physiological situation. Experimental animals should be unrestrained and allowed to resume unstressed resting rates before starting any trials.

4. Upon changes in metabolic rate in flow-through systems, consider the washout characteristics of your system before attributing a reading to a specific experimental condition.

5. Intermittent flow systems should be fully washed out between measurement periods in order to minimise the interference and accumulation of respiratory CO_2.

6. Periods and levels of spontaneous activity levels (steady-state and non steady-state) should be recorded and associated increments in metabolic costs determined.

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