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HYPOXIA ACCELERATES THE DEVELOPMENT OF RESPIRATORY REGULATION IN BRINE SHRIMP – BUT AT A COST

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Summary

The ability to regulate O_2 uptake during exposure to acutely declining O_2 tensions developed early (stage 6) in the brine shrimp *Artemia franciscana* and co-occurred with the appearance of a functional heart and gills. Culture under chronic hypoxia (P_{O_2}=10 kPa) resulted in this regulation being brought forward both in development (to stage 3) and in time (hypoxia stimulated early growth), but still before heart and gill formation took place. Consequently, it was suggested that the hypoxia-related early appearance of respiratory regulation is most probably linked to an increase in haemoglobin concentration that occurred at this time. Brine shrimp cultured under conditions of intermittent hypoxia exposure (16 h of normoxia, 8 h of hypoxia) showed a pattern of regulation development intermediate between that of individuals reared in normoxic and chronically hypoxic culture. This occurrence of hypoxia-related, physiological 'heterochrony' in brine shrimp resulted in a decrease in Darwinian fitness (as indicated by a decrease in individual lifetime reproductive output), indicating that, in some cases at least, relatively small alterations in the expression of physiological traits may well have major ecological, and ultimately evolutionary, consequences.

Key words: development, hypoxia, heterochrony, Darwinian fitness, brine shrimp, *Artemia franciscana.*

Introduction

Induction, *via* tissue interactions, is the basis of the epigenetic developmental signals and cascades that determine and modify basic patterns of tissue/organ development (Hall and Hörstadius, 1988; Hall, 1990, 1992). From an ecophysiological perspective, a comparable situation would be the onset (timing) and development of physiological regulations and functions (de Beer, 1958; Adolph, 1968; Burggren, 1992; McCormick, 1994; Johnston et al., 1996; Spicer and Gaston, 1997, 1999; Burggren and Keller, 1998) and, in particular, how this itinerary is triggered or modified (permanently or otherwise) by environmental factors. Such potential for environmentally induced variation, as there is at the level of the developing individual, is critical because it is likely to have consequences for variation at higher levels of biological organisation, i.e. population, species or community (Spicer and Gaston, 1997, 1999).

Consequently, we studied the onset and development of one particular regulation, namely respiratory regulation and, in particular, the ability to maintain rates of O_2 uptake ($\dot{V}_{O_2}$) during exposure to acutely declining O_2 tensions (P_{O_2}). The brine shrimp *Artemia franciscana* was chosen for investigation because its comparatively short generation time and its amenability to large-scale culture in the laboratory make it tractable for studying physiological development. Furthermore, we already have some understanding of key physiological functions and when they appear during the ontogeny of this species. Numerous authors have investigated the respiration of brine shrimp nauplii and the associated physiological mechanisms employed by individuals at different stages of their life cycle (Eliassen, 1952; Engel and Angelovic, 1968; Bowen et al., 1969; Bernaerts et al., 1981; Varo et al., 1991, 1993; Hemamalini and Munuswamy, 1994; Spicer, 1994, 1995a,b; Spicer and Morritt, 1996). In particular, the ability to regulate $\dot{V}_{O_2}$ during exposure to declining P_{O_2} present in adults (Gilchrist, 1954; Vos et al., 1979; DeWachter and van den Abbeele, 1993) seems to appear very early in the development of *Artemia* (Varo et al., 1991, 1993; Spicer, 1995a). However, the complete story must be pieced together using a large number of disparate studies. Even having done so, we still know little of the timing of key events and the ability of individual brine shrimp to control, or alter, the timing and nature of the onset of this regulation. Certainly neither the timing, nor the pattern, of the onset of cardiac function was altered by culture under different temperature or O_2 regimes (Spicer, 1994, 1995a; J. I. Spicer, unpublished observations), being constrained by the onset of segmentation (the heart cannot form before there is somewhere to put it). There is some evidence, however, that the ontogenetic shift in haemoglobin O_2-affinity that co-occurs with the appearance and development of thoracic

segments in brine shrimp (Moens and Kondo, 1976; Heip et al., 1977, 1978a, 1980) can be 'brought forward' in development (Heip et al., 1978b) and that Hb structure and function can be modified in the adult stages by exposure to environmental hypoxia (Gilchrist, 1954; Heip et al., 1978a; Ferry et al., 1983; Moens et al., 1991; Trotman, 1991; DeWachter et al., 1991). Given that the timing of the onset of particular physiological functions during development may, or may not, be open to modification, the question arises as to the influence such functions have on the appearance of respiratory regulation (which represents the integration of a number of such physiological functions) during development. Consequently, we examined the effect of culturing brine shrimp under different oxygenation regimes on the pattern of respiratory regulation and how such regulation changes with different stages of development encompassing the entire life cycle. Furthermore, we attempted to link any hypoxia-related alterations in the development of respiratory regulation with some indices of Darwinian fitness, namely survival and lifetime reproductive output. Thus, it should be possible to speculate on the ecological implications of such physiological variation. As well as investigating chronic hypoxia, we also present the results of some preliminary work on exposure to periodic hypoxia as this, arguably, is more likely to mirror the situation in nature (e.g. Carpelan, 1957).

Materials and methods

Origin and culture of animal material

Individuals of the brine shrimp *Artemia franciscana* Kellogg were obtained from dried cysts purchased from King British Aquarium Accessories Co. Ltd, Bradford, UK. These cysts were collected from the South Arm of the Great Salt Lake, Utah (personal communication to J. I. Spicer from the manager, King British Aquarium Supplies, UK). Unless stated otherwise, all cysts were hatched in artificial sea water (ASW; Tropic Marine, salinity 35‰) under conditions of continuous illumination (60–100 μmol m^2 s^{-1}) and aeration (using a free-standing Aquarium air pump). All ASW solutions were autoclaved and filtered before use in any culture. Culture vessels and aeration equipment were also autoclaved. A large number of culture vessels (1.5 l) were maintained at a nominal temperature of 28 °C (measured range 27.3–28.2 °C).

All culturing took place at 28 °C. After hatching, the nauplii were separated from their shells, and the remaining unhatched cysts (<40 %) were discarded. Newly hatched nauplii were then washed thoroughly using filtered (0.2 μm, Whatman WCN1 filter) ASW. Newly hatched individuals (2000–3500 individuals per flask) were transferred to culture flasks (2 l) containing ASW. The exact number to be introduced into each culture flask (approximately 2500) was determined from the results of preliminary experiments on the effect of culture density on growth and development in which different numbers of individuals were cultured in identical vessels and conditions. There were always at least three replicate flasks for each set of culture conditions. The different culture conditions were defined as follows: normoxia, the water was vigorously aerated with air supplied from a bench air line; periodic hypoxia, the water was vigorously aerated with air for 16 h each day, and for the remaining 8 h the water P_{O_2} (Pw_{O_2}) was reduced to 10 kPa; chronic hypoxia, the Pw_{O_2} was maintained continuously at 10 kPa.

The gas mixture used to produce and maintain hypoxic conditions was produced from mixtures of N_2 (O_2-free) and air, using precision gas-mixing apparatus (Wöstoff, Bochum, Germany). Pw_{O_2} was checked periodically using an O_2 electrode (E5046, Radiometer, Copenhagen Denmark) coupled to an O_2 meter (Strathkelvin Instruments, Glasgow, UK).

Individuals in each of the culture flasks were fed every 2 days on Liquifry (0.4 ml) until stage 8. After this time, the volume of Liquifry was increased to 0.8 ml, until approximately stage 10. From stage 10 to the late stages, cultures (each consisting of approximately 100 individuals) were supplied with 0.6 ml of Liquifry every 2 days. This feeding regime, worked out in detail in initial experiments, ensured that growth was not resource-limited and that ASW quality was not compromised. The culture medium was replaced every 4 days.

Individuals were removed from culture vessels at a number of different times and used for either (a) the determination of the Hb concentration of the haemolymph ($[Hb]_h$) or (b) the measurement of $\dot{V}_{O_2}$ and/or the determination of dry body mass. The developmental stage and length of each individual sampled were determined as described below.

Haemolymph sampling and treatment

Prior to haemolymph sampling, individuals were rinsed briefly using deionised double-distilled water and then carefully, but quickly, dried using a tissue (Mediwipe). Haemolymph was collected under immersion oil (Sigma, St Louis, MO, USA) from the bases of excised limbs, using silicone-coated microcapillary tubing (Drummond microcaps, 2 μl capacity) and transferred to a microcentrifuge tube (Eppendorf, 1.6 ml) kept on ice.

Haemoglobin concentration was estimated by measuring the iron content of pooled haemolymph samples after appropriate dilution with double-distilled deionised water using furnace atomic absorption spectrophotometry (Perkin-Elmer M2100, HGA 700). Samples were prepared following closely the method of Blust et al. (1988). The concentration of Hb was calculated assuming an iron:Hb ratio of 1:16 (Moens and Kondo, 1976).

The O_2-binding properties of Hb resuspended in buffered physiological saline [Pantin's (1948) crustacean saline with Tris-HCl (0.1 mmol l^{-1}) adjusted to pH 7.40] were examined using a spectrophotometric technique. The solution containing the Hb was equilibrated with the desired gas mixture in a tonometer coupled to a quartz cuvette (0.3 ml, 4 mm pathlength). Absorbance was measured at 415 nm using a spectrophotometer (Shimadzu). Gas mixtures were blended using a pair of precision gas-mixing pumps (Wöstoff, Bochum, Germany).

Measurement of individual length, mass and stage of development

Developmental stage was determined using the scheme devised by Weisz (1946, 1947) in which stages are numbered 0–19, depending on when the last thoracic (0–13) or abdominal (14–19) segment rudiment first appears. Stage 19 is taken as the final stage and covers all of the remaining life cycle of the sexually mature adult. Total body length was measured (±5 μm) using an ocular micrometer. Body length was taken as the distance from the front of the median eye to the posterior margin of the telson (excluding cercopods).

Dry body mass was measured for all individuals, with the exception of those used in haemolymph sampling, as follows. Individuals were counted and briefly washed in distilled water, to remove salt deposits, before they were gently blotted dry on filter paper. After being placed on small pre-weighed aluminium foil squares, individuals were dried to constant mass at 60 °C for 24 h. Individual animals were then weighed to an accuracy of ±1 μg on a micro balance (Mettler ME30).

Measurement of $\dot{V}_{O_2}$

The $\dot{V}_{O_2}$, during acutely declining P_{O_2}, of individuals was examined using a closed respirometer technique. The technique and apparatus employed (RC 300 microrespirometer, model 781 O_2 m, readability 13.3 Pa, microcathode O_2 electrode repeatability 26.7 Pa, Strathkelvin Instruments, Glasgow) is now well established and has been used previously for examining the $\dot{V}_{O_2}$ of *Artemia* individually and in groups of 100 or more (Varo et al., 1991; Spicer, 1995b).

Groups of individuals (of equivalent mass and developmental stage) or individual specimens (in the case of adults) were placed into the respirometer chamber and left to acclimatise to experimental conditions for 5 min in each case. The ASW in the chamber was sterile, being identical to that used in the appropriate culture technique. The chamber was sealed, and the individual(s) was allowed to deplete available O_2. The rate of O_2 depletion was followed on a chart recorder down to approximately 5 % of normoxic saturation. After this time, the experiment was terminated and the dry mass of the individual animal(s) was determined as described above. Rates of oxygen uptake under conditions of acutely declining P_{O_2} were then calculated and expressed as either $\mu l\, O_2\, h^{-1}$ or $\mu l\, O_2\, mg^{-1}\, h^{-1}$. Each run lasted less than 1.5 h, the exact time being dependent on the chamber volume used. After the completion of each run, the respirometer chamber was cleaned using absolute ethanol and then rinsed with distilled water to reduce microbial contamination. The critical P_{O_2}, or P_c, was defined as the point (expressed in kPa) at which the ability to maintain a constant $\dot{V}_{O_2}$ ceased. This was readily estimated from a visual examination of the chart recorder trace.

Effects of culture under hypoxia on survival and lifetime reproductive output

To assess the ecological implications of the hypoxia-related acceleration of the development of respiratory regulation detected in this study, the following experiment was carried out. Cultures of brine shrimp under normoxia and chronic hypoxia (50 % normoxic saturation; 10 kPa) were carried out using the techniques described above, but with the following modification; five replicates of 50 individual new hatchlings (<2 h after hatching) per treatment were used to seed the cultures, and the supply of food was adjusted accordingly. A third treatment was established in which brine shrimp hatchlings were exposed to hypoxia for only the first 6 days, i.e. until the 'adult' pattern of respiratory regulation was established in most individuals, and thereafter they were maintained under normoxic conditions. Without incorporating such a treatment into the experimental design, it is difficult to disentangle any possible effects of chronic hypoxic culture from the effect of accelerating respiratory development using hypoxia. Even then, we know from the experiments described above that development *per se* is affected by hypoxia. Consequently, it is not possible to differentiate between effects due to acceleration of respiratory development linked with faster development and effects resulting from the earlier onset of respiratory mechanisms, independent of developmental stage. This said, the experiment provides information about whether there are ecological implications associated with accelerated respiratory development, however it is achieved.

Five individuals at stage 3 and a further five different individuals at stage 6 were removed from each of the three culture treatments, and their rates of O_2 uptake in response to acutely declining P_{O_2} tensions were examined exactly as described above. This was performed merely to confirm that the data on the respiratory physiology of the individuals used in this experiment were comparable with results obtained in the earlier experiment. After 20 days (around the time that most of the individuals in each of the experimental treatments became sexually mature), the number of individuals surviving in each replicate was noted, and the lengths of 16 individuals chosen at random were measured as described above. Around this time, ten pairs of brine shrimp (i.e. when males had clasped females) were separated from each treatment as soon as they were noticed. Individual pairs were then transferred to a culture flask (0.1 l) containing ASW (35 ‰, 28 °C) maintained under their appropriate oxygenation regime as described above. Brine shrimp pairs were then supplied with 0.2 ml of diluted Liquifry (1:3 Liquifry:culture medium) every 2 days, and the water was changed after each brood. Each day, offspring (as nauplii or cysts) were removed from each of the experimental flasks and counted. Recovery of offspring only ceased when the mother died.

Results

Effects of hypoxic culture on growth and development

For *Artemia franciscana* from the control and experimental cultures, there were significant relationships between body length and developmental time (after double-logarithmic transformation of the data) (Fig. 1A). There was a significant difference between the control and the chronic hypoxia cultures (analysis of covariance, ANCOVA, $F_{1,477}$=4104; P<0.05) but not between the control and the periodic hypoxia

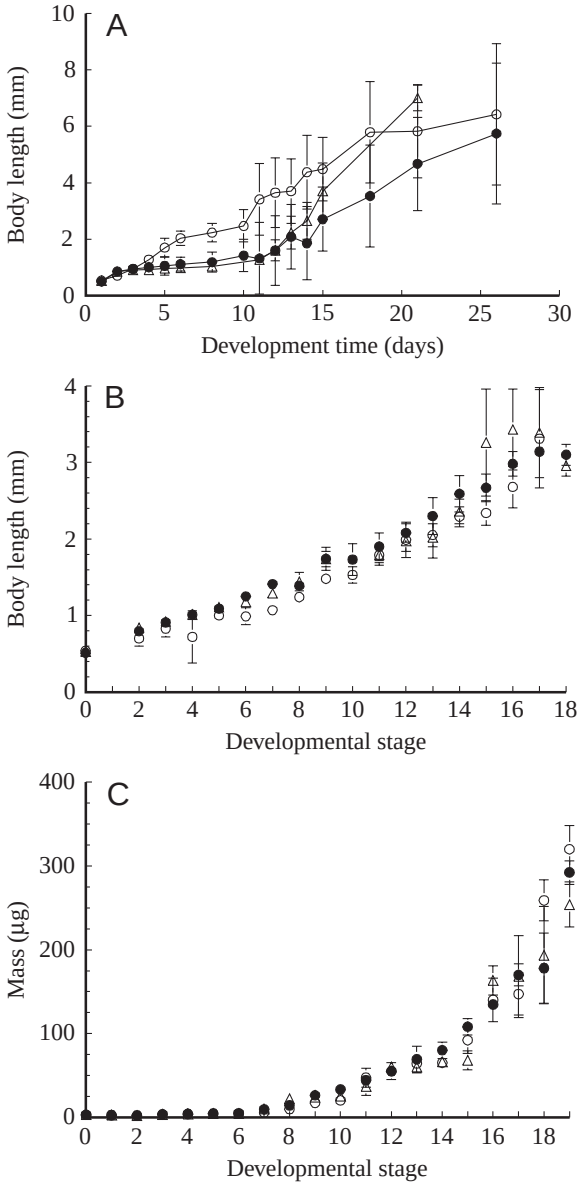

Fig. 1. Relationships between growth and development for *Artemia franciscana* cultured under different oxygenation regimes. ●, normoxia; ○, chronic hypoxia; △, periodic hypoxia. Each value represents the mean ±1 S.D. of 15–45 determinations. (A) Body length (mm) and developmental time (days); (B) body length (mm) and developmental stage; (C) body mass (µg) and developmental stage.

cultures (ANCOVA, $F_{1,366}$=2.7; P>0.05). Closer observation of the graphs, followed by the use of Student's t-test, indicated, however, that there was only a significant difference between normoxic and chronic hypoxic cultures for each interval examined between days 6 and 15 of culture (P<0.05 in each case), and not before or after that time, i.e. culture under chronic hypoxia resulted in individuals being of a greater body length than the controls, although this difference was not detectable by day 15 of culture.

There were, however, no significant effects of culture under

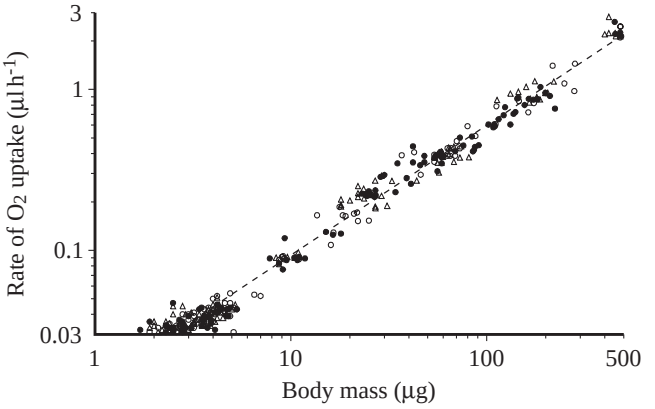

Fig. 2. The relationship between rate of O_2 uptake ($\dot{V}_{O_2}$) and body mass in individuals of *Artemia franciscana* (at 28 °C, salinity 35 ‰) cultured under different oxygenation regimes: ●, normoxia; ○, chronic hypoxia; △, periodic hypoxia. The broken line is the relationship for the three conditions combined (see text for further details).

periodic or chronic hypoxia on either the relationship between body length and developmental stage (ANCOVA, $F_{2,660}$=0.97; P>0.05) (Fig. 1B) or the relationship between body mass and developmental stage (ANCOVA, $F_{2,660}$=1.31; P>0.05) (Fig. 1C). This means that the increase in length of hypoxia-cultured individuals, noted above, could be accounted for solely by an increase in developmental rate. Culture under chronic hypoxia resulted in individuals apparently 'rushing through' the first four developmental stages more rapidly; although the first sexually mature brine shrimp were noted earlier (9 days) in the chronic hypoxia treatment than in the other culture conditions, it was not until day 15 that all the individuals in the culture were sexually mature.

Development and $\dot{V}_{O_2}$

The relationship between $\dot{V}_{O_2}$ and body mass, after double-logarithmic transformation, is presented in Fig. 2 for *A. franciscana* cultured under each of the experimental conditions: normoxia, chronic hypoxia and periodic hypoxia. In each case, there was a significant relationship between $\dot{V}_{O_2}$ and body mass (for normoxia, r^2=0.971, d.f.=1,122; for chronic hypoxia, r^2=0.988, d.f.=1,104; for intermittent hypoxia, r^2=0.915, d.f.=1,113; all significant at P<0.001). There was no significant difference between these relationships that could be attributed to culture conditions (ANCOVA, $F_{2,341}$=2.04, P>0.05). The overall relationship between body mass (x, expressed as µg) and $\dot{V}_{O_2}$ (y, expressed as µl h^{-1}) is described by the equation y=0.80x–1.83 (r^2=0.987, d.f.=1,341, P<0.001). Therefore, the hypoxia culture regimes employed here had no detectable effect on $\dot{V}_{O_2}$.

While there was a decrease in $\dot{V}_{O_2}$ with each successive developmental stage from stage 1 onwards, there was an increase between stages 0 and 1 from 12.74±1.84 to 14.49±0.89 µl O_2 mg^{-1} h^{-1} (means ± S.D.; Student's t-test, t=–3.12, d.f.=18, P<0.01), and $\dot{V}_{O_2}$ values for stages 1 and 2

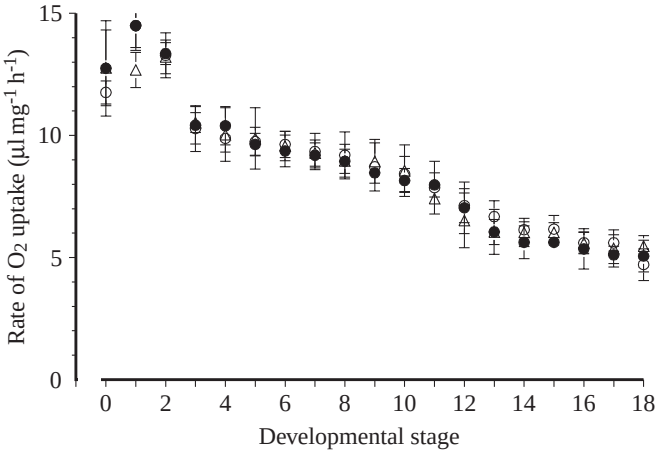

Fig. 3. Mass-specific rates of O_2 uptake ($\dot{V}_{O_2}$) for *Artemia franciscana* (at 28 °C, salinity 35 ‰) of different developmental stages, cultured under different oxygenation regimes: ●, normoxia; ○, chronic hypoxia; △, periodic hypoxia. Values are means ±1 S.D. of 7–16 determinations.

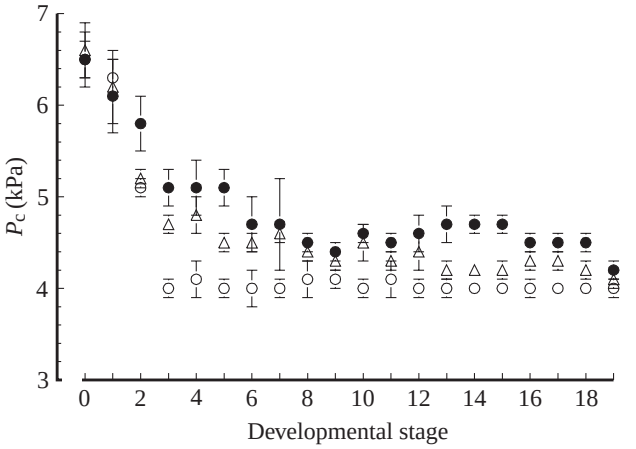

Fig. 4. Critical O_2 tensions (P_c) for *Artemia franciscana* (at 28 °C, salinity 35 ‰) of different developmental stages, cultured under different oxygenation regimes: ●, normoxia; ○, chronic hypoxia; △, periodic hypoxia. Values are means ±1 S.D. of 7–16 determinations.

appear to be much greater than we might have predicted from the general trend (Fig. 3). This is due to the fact that stages 1 and 2 did not weigh as much as stage 0; that is, over these first three stages, mass actually decreased with development from 2.8±0.4 to 1.9±0.2 μg (Student's *t*-test, t=17.86, d.f.=65, P<0.001). There was, however, a small but significant difference between $\dot{V}_{O_2}$ values of stage 1 nauplii cultured under different O_2 regimes (ANOVA, $F_{2,18}$=20.9, P<0.001) that could not be explained by differences in mass: there was no significant difference in the masses of stage 1 individuals cultured under each of the experimental conditions (ANOVA, $F_{2,18}$=1.68, P>0.05).

Development of respiratory regulation

The effects of culture under different hypoxia regimes on how the ability to regulate $\dot{V}_{O_2}$ over a wide range of environmental P_{O_2} changed during development are presented in Fig. 4. The overall pattern of change during development was similar for individuals from each of the culture regimes. There was a steady increase in regulatory ability, i.e. a decrease in P_c, during early development (stages 1–6). However, from stage 7 onwards, the established regulatory pattern varied little (with the possible exception of some stages, e.g. stages 14 and 15 cultured under normoxic conditions). Although the general response was the same, there were also important and distinctive differences as a result of different culture conditions. Under normoxic culture conditions, P_c decreased from 6.5±0.3 kPa for newly hatched individuals (stage 0) to 4.6±0.3 kPa (means ± S.D., N=8) in individuals at stage 6. Thereafter, there were no significant changes noted in P_c with development (ANOVA, $F_{13,91}$=1.01, P>0.05, mean P_c=4.5±0.2 kPa) except that the adult stage had a slightly lower P_c value of 4.2±0.1 kPa (P<0.05, N=12). However, in individuals cultured under chronic hypoxia, the establishment of the 'adult' pattern of regulation occurred much sooner: P_c decreased from 6.5±0.2 kPa (N=8) for newly-hatched individuals (stage 0) to 4.0±0.1 kPa (N=8) in individuals at stage 3 (P<0.05), and did not change significantly thereafter (ANOVA, $F_{15,84}$=1.13, P>0.05) until the adult stage was reached, when there was another small improvement (P<0.05) in respiratory performance (P_c=3.7±0.1 kPa, N=16). Furthermore, individuals cultured under chronic hypoxia possessed a greater regulatory ability (i.e. consistently lower P_c values) than controls once the 'adult' pattern of regulation had been established. Interestingly, individuals cultured under periodic hypoxia displayed a pattern of response (i.e. the time to establish the 'adult' pattern of regulation and the breadth of the ability to maintain $\dot{V}_{O_2}$ during exposure to declining P_{O_2}) intermediate between those of individuals cultured under chronic hypoxia and the normoxic controls. In nearly all cases, after P_c was reached, there was a marked decrease in swimming activity.

Changes in [Hb] and Hb O_2-affinity during development

Adult individuals, of both sexes, cultured under chronic hypoxia were a pronounced red colour compared with individuals cultured under either periodic hypoxia or normoxia. Females cultured under chronic hypoxia were invariably more strongly pigmented than males of equivalent size. Normoxic individuals showed little red pigmentation. Brine shrimp cultured under periodic hypoxia were intermediate in colour between normoxic and hypoxic cultured individuals. These differences were observed consistently throughout the experimental work.

A more quantitative approach, based on actual measurements of $[Hb]_h$, was used to study changes very early in development. There was a significant effect of both culture conditions and development on $[Hb]_h$ of brine shrimp (ANOVA, $F_{2,45}$=253.06 and $F_{1,45}$=1061.77, respectively, P<0.001 in each case) (Fig. 5). Although $[Hb]_h$ increased significantly in normoxic controls from stages 0/1 to stage 6,

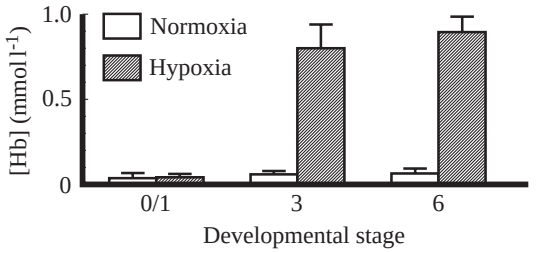

Fig. 5. Developmental changes in the concentration of haemoglobin (Hb) in the haemolymph of *Artemia franciscana* cultured under normoxia and chronic hypoxia. Values are means +1 S.D. of 8–10 determinations.

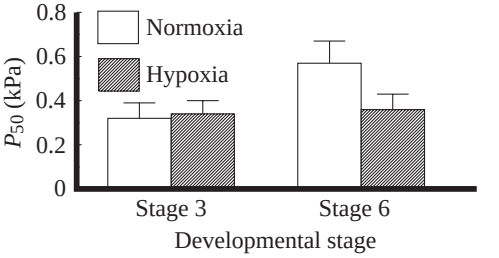

Fig. 6. The half-saturation values (P_{50}) for pooled haemoglobin samples from stage 3 and stage 6 individuals of *Artemia franciscana* cultured under either normoxic or chronically hypoxic conditions. Values are means +1 S.D. of 7–16 determinations.

this increase was relatively modest (0.028 mmol l^{-1}) compared with that observed for individuals cultured under chronic hypoxia. Stage 0/1 individuals possessed a $[Hb]_h$ of 0.037±0.019 mmol l^{-1}, irrespective of culture conditions, although by stage 6 the $[Hb]_h$ of hypoxia-cultured individuals was 0.895±0.090 mmol l^{-1}, almost 25 times greater than that of normoxic controls.

There was a significant difference in Hb O_2-affinity (increase in P_{50}) between stage 3 and 6 individuals (Student's *t*-test, t=–4.49, d.f.=5, P<0.01) cultured under normoxic conditions, with there being a decrease in Hb O_2-affinity from 0.32±0.07 kPa (stage 3) to 0.57±0.10 kPa (stage 6) (Fig. 6). However, no such difference was detected in individuals cultured under chronic hypoxia (Student's *t*-test, t=–0.10, d.f.=4, P>0.05); the high-affinity pigment present in stage 3 individuals was retained by stage 6 individuals.

Survival and lifetime reproductive output

The effects of acute (6 days of exposure to hypoxia immediately post-hatch) and chronic (20 days of exposure to hypoxia immediately post-hatch, i.e. well into sexual maturity) hypoxia on mortality and individual lifetime reproductive output are presented in Table 1. There was no significant difference in mortality as a result of either of the experimental treatments (ANOVA, $F_{2,47}$=1.82, P>0.05). There were, however, pronounced differences in the lifetime reproductive output of individuals among the treatments. No free-swimming offspring were produced by individuals cultured under chronic hypoxia, only cysts. In the normoxic control and acute hypoxia treatments, however, both free-swimming offspring and cysts were produced. There was no significant difference in the number of cysts produced among the three treatments (ANOVA, $F_{2,29}$=0.18, P>0.05), but the number of free-swimming offspring from individuals from the control treatment was significantly greater than that from individuals exposed to hypoxia only in early life (Student's *t*-test, t=4.26, d.f.=17, P<0.001).

Also given in Table 1 are the P_c values for stage 3 and stage 6 individuals and the lengths of sexually mature individuals 20 days after the experiment commenced. There was a significant difference between the P_c values of stage 3 and stage 6 individuals cultured under normoxia (Student's *t*-test, t=4.21, d.f.=6, P<0.01), but not between equivalent P_c values for individuals in either of the hypoxic treatments (Student's *t*-test, t<1.09, d.f.=6, P>0.05 in each case). The values are very close to those reported above for similar developmental stages, kept under similar culture conditions. Similarly, as described above, there was no significant difference in body length (20 days

Table 1. *The effects of early (hypoxia for 6 days post-hatch only followed by normoxia) and chronic (20 days) hypoxic culture on mortality, growth and lifetime reproductive output of* Artemia franciscana

		N	Normoxia	Early hypoxia	Chronic hypoxia
Hatchling mortality out of 50 (% in parentheses)		5	18.8±3 (37.6 %)	19.6±4.8 (39.2 %)	19.2±7.0 (38.4 %)
Adult body length (mm)		16	3.38±0.61	3.35±0.64	3.78±0.83
Individual reproductive output (no. of offspring produced)	Live	10	382.1±94.1	211.8±84.4	None
	Cysts		109.9±63.4	108.1±52.9	95.20±67.0
P_c (kPa)	Stage 3	5	4.96±0.14	4.04±0.08	4.02±0.05
	Stage 6	5	4.53±0.19	3.99±0.03	4.00±0.04

The critical P_{O_2} values (P_c) for individuals at stage 3 or 6 cultured under each of the experimental conditions are also given.
All values are expressed as means ±1 S.D.

after hatching) as a result of either of the experimental treatments (ANOVA, $F_{2,14}$=0.03, P>0.05).

Discussion

Patterns in respiratory regulation during development

Artemia franciscana, cultured under normoxic conditions, displayed a dramatic 'improvement' in respiratory regulation (evident when exposed to acutely declining P_{O_2}) as development proceeded. There was, however, no difference in $\dot{V}_{O_2}$ related to the oxygenation status of the culture medium, as was found for adult *Artemia* by Decleir et al. (1980). Furthermore, the regulatory ability of the individuals examined during the present study was much better developed than in the study of Decleir et al. (1980). The ability of *A. franciscana* to regulate $\dot{V}_{O_2}$ (when exposed to acutely declining P_{O_2}) developed early in ontogeny (stages 4–6) and had attained the 'adult pattern' by the time the gills and heart had formed in the thoracic stage of development. Newly hatched brine shrimp may have a greater capacity for anaerobic metabolism than adults (compare Ewing and Clegg, 1969; Decleir et al., 1980). If so, this could allow newly hatched nauplii to survive hypoxic exposure even before respiratory regulation is established.

Some data on the development of respiratory regulation during ontogeny are available for four other crustacean species, the lobsters *Panulirus interruptus* (Belman and Childress, 1974) and *Nephrops norvegicus* (Spicer, 1995b; S. P. Eriksson and J. I. Spicer, cited in Eriksson, 1998), the crab *Cancer productus* (Belman and Childress, 1974) and the amphipod *Echinogammarus pirloti* (Spicer, 1995b). As with brine shrimp, individual lobsters and crabs show little capacity for respiratory regulation at hatching. The 'adult' pattern of regulation seems to develop later (e.g. just before metamorphosis in *N. norvegicus*; S. P. Eriksson and J. I. Spicer, cited in Eriksson, 1998). In contrast, the amphipod *E. pirloti* hatches with the 'adult' pattern of respiratory regulation already established. However, individuals of this species do hatch at a much more advanced state of development than those of the three other species, and *A. franciscana* in particular.

For *A. franciscana* cultured under chronic hypoxia, the onset of respiratory regulation was seen in a much earlier developmental stage than in normoxic controls. In other words, physiological development and morphological development were proceeding at different rates. Furthermore, the effects of hypoxic culture of bringing the whole of development forward in time, i.e. hypoxia-cultured brine shrimp developed faster than normoxic controls (at least during early ontogeny), meant that the hypoxia-induced early onset of respiratory regulation was brought even further forward in real time. This 'telescoping' of respiratory physiological development meant that the 'adult' pattern of respiratory regulation was achieved considerably sooner than was found for normoxic controls. To our knowledge, data from only one other crustacean study have suggested that the onset of an 'adult' pattern of respiratory regulation can be 'brought forward' *via* environmental influence: exposure to chronic hypoxia appears to result in respiratory regulation being established at an earlier developmental stage in *N. norvegicus* (Spicer, 1995b; S. P. Eriksson and J. I. Spicer, cited in Erikkson, 1998). A change in developmental timing is one source of variation upon which selection can act. Exactly how common this within-individual 'heterochrony', i.e. the amount of variation possible within ontogenic trajectories, is for physiological functions in crustaceans (and even animals generally) has yet to be established.

The effect of periodic hypoxia on both growth and respiratory regulation in *A. franciscana* was shown to be intermediate between that of the normoxic control and the chronic hypoxic experimental treatment. In other words, it may be that it is not the pattern of hypoxic/normoxic exposure that matters quite so much as the duration of the hypoxia. If this is true, it is possible that many of the acclimation effects elicited in aquatic invertebrates as a response to either periodic or chronic hypoxia are only quantitatively, not qualitatively, different. Thus, we could legitimately extrapolate from physiological studies that examine exposure to chronic hypoxia to what would happen under conditions of periodic hypoxia. The generality of this finding, however, remains to be tested.

Mechanisms underpinning changes in respiratory regulation during development

Having established the patterns in the development of respiratory regulation, it is now pertinent to turn to the mechanisms underlying the improvement in respiratory regulation in *A. franciscana* during development as well as those underlying the hypoxia-related telescoping of the onset of that regulation. Unfortunately, much of the evidence is circumstantial. A more manipulative approach using carbon monoxide to block O_2 binding by haemoglobin generated data that were difficult to interpret, and even those data could not be repeated (J. I. Spicer, unpublished observations).

The 'improvement' in respiratory regulation recorded during normoxic development co-occurred with the development of gills in *A. franciscana*. This has been noted before (Spicer, 1995a). It is not unreasonable then to link the appearance of respiratory regulation and gill formation. What is certain is that the further (hypoxia-related) improvement in respiratory regulation that takes place even earlier in development cannot be attributed to early gill formation. The gills of hypoxia-cultured individuals developed earlier in time than those of normoxic controls, but not at an earlier developmental stage. Indeed, this is physically impossible because the gills require limbs to bear them and the limbs require thoracic segments to bear them. Similarly, while the development of cardiac function may be implicated in the late stages of the development of respiratory regulation in normoxia-cultured individuals, it is unlikely to be involved in the early appearance of respiratory regulation in hypoxia-cultured animals, i.e. you cannot develop a heart before you develop somewhere (thoracic segments) to keep it (Spicer, 1994, 1995a,b; Spicer and Morritt, 1996). Thus, the developmental itinerary is clearly an important constraint in the ability to bring some structural features forward in ontogeny.

That there are changes in O_2 affinity of brine shrimp Hb during development is not new and is relatively well documented (see Introduction for references). As suggested by Spicer (1995b), after piecing together information derived from a number of different but relevant studies, it appears that the shift in affinity takes place at the same time as the gills and heart develop. This has been confirmed in the present study. Also shown here, the shift from a high- to a low-affinity respiratory pigment co-occurs with the improvement in respiratory performance that takes place at this time in *A. franciscana*. However, the developmental shift in the Hb O_2-affinity curve observed here, and in previous studies, appears to be going in the wrong direction (from high to low affinity) if such a shift is to be implicated in the development of respiratory regulation that appears during the early period of thoracic development. In those crustaceans possessing haemocyanin as their respiratory pigment, there is a shift from a low- to a high-affinity pigment during early development (Terwilliger et al., 1986; Terwilliger and Brown, 1993; Spicer, 1995b), and this presumably co-occurs with an improvement in respiratory regulatory ability (e.g. Spicer, 1995a,b). This makes sense because a pigment with a high O_2 affinity is generally seen as being more advantageous for maintaining $\dot{V}_{O_2}$ under conditions of declining P_{O_2} than a low-affinity pigment (Weber, 1980; Mangum, 1983, 1990a, 1997). Consequently, it is difficult to see how the shift in Hb O_2-affinity in *A. franciscana*, although it co-occurs with the development of respiratory regulation, is actually responsible for the 'improvement' of that regulation.

There was a small increase in $[Hb]_h$ coinciding with the development of respiratory regulation in individuals cultured under normoxia. An increase in $[Hb]_h$ with increasing body size (from hatching to sexual maturity) has also been recorded for the waterflea *Daphnia magna* (Kobayashi, 1982; Kobayashi and Nezu, 1986). This said, the values for $[Hb]_h$ recorded here, and previously (Gilchrist, 1954), for normoxic cultured brine shrimp are still comparatively very low. Commenting on Gilchrist's data, Mangum (1997) notes that the [Hb] is so low that one wonders why Hb is present at all. However, Hb does seem to be involved in O_2 transport even in individual small waterfleas *Daphnia magna*, whether they have a high or a low [Hb] (Kobayashi and Tanaka, 1991).

When *A. franciscana* were cultured under hypoxia, the total $[Hb]_h$ of individuals increased visibly and dramatically compared with that of normoxic controls. Also, the observed developmental shift from a high- to a low-affinity Hb characteristic of individuals cultured under normoxia is negated in hypoxia-cultured individuals. Hypoxia-related increases in [Hb] have been recorded previously for *Artemia* species (Fox, 1949; Gilchrist, 1954; Bowen et al., 1969) and for other crustaceans such as *Daphnia* species (Kobayashi, 1982, and references therein; Kobayashi and Nezu, 1986; Tokishita et al., 1997; for a review, see Mangum, 1990b). However, this present study has shown that such marked induction of respiratory pigment is possible as early as stage 3 in *A. franciscana*. Furthermore, the dramatic increase in $[Hb]_h$ in hypoxia-cultured brine shrimp coincides with the early appearance of respiratory regulation. As this improved regulation takes place before the appearance of a functional heart and gills, alterations in Hb concentration (if not O_2 affinity) must be a strong candidate as the mechanism underlying the improvement.

Physiological 'heterochrony' and ecology

That there is a programmed physiological itinerary in *A. franciscana* with respect to the development of respiratory regulation is clear. What is also clear, however, is that this itinerary can be altered, in this case brought forward, both in real time and also during the developmental process *per se*. The term heterochrony is usually reserved for phylogenetic differences in the timing of (most commonly morphological) development (McKinney and McNamara, 1991; Gould, 1992; McNamara, 1995), but there is no *a priori* reason why this should be so (Reilly et al., 1997; Spicer and Gaston, 1999). Unfortunately, physiological 'heterochrony' both between and within species (Spicer and Gaston, 1999) has not attracted the attention that it deserves. Indeed, patterns of within-individual variations in the timing of physiological events may account for more physiological variation than has yet been recognised; for example, the altering of the ontogeny of thermoregulation in birds (Thomas et al., 1993) or the ontogeny of digestive and nutrient transport processes (Henning, 1985; Buddington and Diamond, 1989). Investigating the extent to which alterations in the timing of physiological events are either plastic or genetically fixed may enable us to test the generality of the hypothesis (based mainly on morphological traits) that heterochrony is the principal phenomenon producing all developmental change (de Beer, 1958; Gould, 1977; Hall, 1992).

One of the most important findings of the present study is that, associated with the physiological 'heterochrony' (or whatever we are to term the scope for within-individual variation in the timing of physiological events) described above, there are changes in Darwinian fitness (measured as individual lifetime reproductive output). It is not uncommon when physiological patterns or mechanisms are examined for investigators to refer to, and discuss, their adaptive significance. However, such an assumption of adaptive significance for physiological traits is rarely tested and, when it is, it is not always supported (Huey and Berrigan, 1996; Huey et al., 1999; Spicer and Gaston, 1999). Brine shrimp living in unpredictable environments are know to 'bet-hedge', i.e. they produce both live young and cysts that can remain dormant for some considerable time (Hildrew, 1985; Saiah and Perrin, 1990). The brine shrimp cultured under continuous hypoxia in this present study only produced dormant cysts, perhaps indicating that individuals are no longer treating such an environment as unpredictable. More interesting from the point of view of obtaining information about any costs associated with physiological 'heterochrony' is the fact that, even for those individuals exposed to hypoxia only during the development of the 'adult' pattern of respiratory regulation, there was a reduction in individual lifetime reproductive output.

The inference is that there are fitness costs associated with rushing through early development in a hypoxic environment and that part of these costs may be associated with the early development of an adult respiratory regulatory capacity. The exact physiological mechanism(s) underlying any such trade-off are, at present, far from clear but, given their ecological and ultimately evolutionary importance, they certainly warrant further study.

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