Stress response of a freshwater clam along an abiotic gradient: too much oxygen may limit distribution

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Summary

1. Distribution and abundance patterns are influenced by cellular responses to abiotic stressors.
2. The freshwater clam Sphaerium sp. inhabits swamp/stream systems in Uganda, in which dissolved O₂ (DO), pH and water transparency form ecotonal gradients along tributary streams. Along a swamp/stream transect, clam abundance was negatively related to DO, pH and transparency; clams were most abundant in the hypoxic/acidic swamp, less abundant in the ecotone, and absent from stream sites (neutral pH and higher DO).
3. We tested the hypothesis that individuals living in low abundance at the distribution edge have increased stress by assessing cellular-level stress indicators in clams from four sites: dense swamp interior (reference site), swamp margin, ecotone and stream. Compared with swamp interior clams, stream clams had decreased RNA : DNA ratio (decreased protein synthesis and growth) and increased DNA and RNA oxidation (free radical damage). Of seven stress proteins related to general damage and free radical detoxification, expression of nearly all were higher in margin and ecotone clams than in swamp interior or stream clams.
4. This pattern, which includes the first measurement of DNA and RNA oxidation in a natural population, demonstrates that stress increases along the transect, inversely to clam abundance. Additionally, this pattern is consistent with free radical stress, which suggests that inhabiting swamp conditions minimizes oxidative damage in this species.

Key-words: 8-oxodGuo, distribution, free radical, hypoxia, stress protein.

Introduction

The size and location of a species’ range are dynamic and influenced by evolutionary constraints (Holt 2003), biotic interactions (Case et al. 2005) and abiotic factors (Brown, Stevens and Kaufman 1996), acting either singly or in combination. Abiotic factors may influence a species’ distribution by stressing organisms beyond their physiological limits (Parsons 1991). Near the edge of a species’ distribution, particularly if that edge is influenced by abiotic factors, we expect to find decreased abundance as well as decreased body condition of individuals. Condition has traditionally been assessed using whole-organism characters such as body size and fecundity (Caughley et al. 1988), but cellular-level indicators may be at least equally useful for assessing condition. Such cellular-level indicators include: RNA : DNA ratio as an indicator of protein synthesis and growth (Dahlhoff 2004); DNA and RNA oxidation as indicators of cellular damage from free radicals (Halliwell and Gutteridge 1999); and up-regulated expression of specific stress proteins, such as heat shock proteins, as indicators of a homeostatic cellular response to a stressor (e.g. Hofmann and Somero 1995; Downs et al. 2001; Abele and Puntarulo 2004). These indicators provide information about cellular metabolic and homeostatic responses to stress, and thus may be more sensitive than whole-organism characters for determining how abiotic factors affect the condition and distribution of individuals in a population.

In the current study we used cellular-level indicators to test the following hypothesis: if a population’s distribution overlies a stressful environmental gradient, then abundance should decrease near the distribution edge, and edge individuals should have increased stress and lower condition. In selecting the appropriate
system, we looked for a stable population of sessile animals in an environment with a stable gradient of a small number of potentially stressful abiotic factors. For this study, we examined the distribution and physiology of a population of small freshwater clam *Sphaerium* sp., which inhabits a swamp/stream system in western Uganda. This habitat contains a relatively stable dissolved O\(_2\) (DO) gradient, from freshwater tributary streams with normoxic water (equilibrated with atmospheric O\(_2\) levels) to a papyrus swamp with very hypoxic water (DO less than 10% of fully aerated water), and a pH gradient from the neutral stream water to acidic swamp water. A 2-year, broad-scale survey of macroinvertebrates in this system (Chapman et al. 2004) revealed that the clams were very abundant in the swamp but largely absent from regions of tributary streams with normoxic water and neutral pH, despite the absence of any apparent physical or biotic barriers limiting access to stream sites. As sphaerid clams are ooviviparous (i.e. they internally brood their young; Mackie 1978), it appears that these clams do not spend any part of their life cycle in normoxic, neutral pH water. This distribution pattern could arise because hypoxic and acidic conditions are correlated with other factors that make the swamp a habitat of high suitability, or alternatively because the hypoxic and/or acidic conditions are favourable for these clams. This latter possibility is especially intriguing because it is typically assumed that marine and freshwater invertebrates avoid hypoxic and/or acidic conditions when possible, or utilize physiological mechanisms to compensate for the resulting hypometabolism and acidosis when the conditions cannot be avoided (Burnett 1997).

To test whether the sphaerid clams, which are abundant in the hypoxic and acidic swamp, show evidence of physiological stress in the normoxic and neutral pH conditions of the tributary streams, we measured clam distribution and cellular-level indicators along a gradient from the swamp into a tributary stream. In addition, we measured the limnological characters of DO, conductivity, pH, temperature and transparency, and we quantified the relationship between these characters and clam density. To examine how the abiotic gradient along this transect affects the physiological condition of the clams, we sampled clams from a reference site from the dense swamp interior and from three sites along the transect, including the extreme edge of the population’s distribution. We assessed RNA : DNA ratio, oxidized DNA and RNA bases, and stress protein expression levels, including several stress proteins that indicate generalized cellular stress, as well as those that specifically respond to increased free radical production, which has been linked to fluctuations in DO and pH (Boveris and Chance 1973; Li, Wright and Jackson 2002; Veselá and Wilhelm 2002). Our goal was to determine whether the patterns of cellular indicators were consistent with increased stress in clams near the edge of their distribution and furthermore whether the pattern was consistent with increased oxidative damage.

### Materials and methods

#### Study site

This study was conducted in a swamp/stream system in Kibale National Park in western Uganda (0°13′–0°41′N, 30°19′–30°32′E). The park is an equatorial moist evergreen forest, transitional between lowland rainforest and montane forest, with distinct bimodal wet and dry seasons (Chapman et al. 1997). The Rwembaita Swamp is a large (approximately 6·5 km in length) papyrus *Cyperus papyrus* swamp, with dense papyrus stands that can reach 5 m in height and form a closed canopy. The low rates of mixing and low incident light induced by the heavy forest of papyrus sedge, and high rates of organic decomposition produce hypercapnic (elevated dissolved CO\(_2\)) and hypoxic water (Chapman et al. 2001). Over a 3-year period, Chapman et al. (2000) reported DO in the Rwembaita Swamp averaging 0·0466 ± 0·0072 mmol L\(^{-1}\) across dry and rainy seasons. [To convert mmol O\(_2\) L\(^{-1}\) to mg O\(_2\) L\(^{-1}\), multiply by 32 (molecular weight of O\(_2\)).] The swamp is fed by several small streams that have intermediate DO, which can vary among streams from 0·118 ± 0·0056 mmol L\(^{-1}\) to 0·222 ± 0·0059 mmol L\(^{-1}\) (Chapman et al. 2004). The streams form ecotonal gradients of decreasing DO concentration as they enter the swamp.

In May 2004, at the beginning of the summer dry period, we measured limnological characters and clam abundance along a transect from the Rwembaita Swamp, through an ecotonal region and into a tributary of the swamp, Inlet Stream West (see Fig. 1). Data from the broad range surveys conducted by Chapman et al. (2000, 2004) were used to design our sampling regime. Sites on the transect were separated by 10 m. Five sites (sites 1–5) with papyrus canopy were designated as swamp sites; five sites (sites 6–10) with emergent (nonpapyrus) vegetation mixed with forest understory vegetation were designated as ecotone sites; and six sites (sites 11–16) were designated as stream sites. Water depth was shallow (10·2 ± 4·3 cm, \(n = 48\)) across all 16 sites. The width of the water stream at ecotone and stream sites (\(n = 11\)),

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SAMPLING METHODS

Triplicate readings of all limnological characters were collected between 09.00 and 12.00 h at each site along the transect, following previous protocols (Chapman and Liem 1995). DO, water temperature and conductivity readings were collected using YSI meters, and pH was measured using an Oaktron meter. Water transparency was measured using a transparent tube marked off in centimetres, with a miniature Secchi disc at the bottom; all measurements were taken by the same individual. At each site we recorded maximum water depth and water velocity (categorical variables ranked on a scale of 0–3; 0 = no current, 1 = low, 2 = medium, 3 = high; Chapman 1995).

At each site along the transect, clams were sampled using triplicate scoop nets (frame size 30·5 × 40·6 cm, mesh size of 0·32 cm), with triplicates separated by approximately 0·5 m. The samples were taken by disturbing and scooping the bottom substrate. Scoop net samples were sorted directly in the field. We calculated an index of clam density (catch per unit effort; CPUE) as the average number of clams per scoop. All clams collected in the initial survey were used for the size/frequency distribution analysis. For size measurements, shell length was measured from umbo to ventral margin using digital calipers.

SAMPLING FOR RNA: DNA RATIO, NUCLEIC ACID OXIDATION AND STRESS PROTEINS

Clams used for nucleic acid analyses and stress proteins were collected from four sites, three of which were on the transect: a swamp site (site 2), an ecotone site (site 8) with comparable clam abundance with the swamp site but higher DO, and the stream site (site 11) with the highest DO of any site with clams. The fourth site (our reference site) was in the swamp interior, approximately 500 m downstream from stream water input. At this site, multiyear surveys indicated presence of clams in all seasons (Chapman et al. 2004). DO at this site averaged 0·0337 mmol L\(^{-1}\) over a 2-year period (Chapman et al. 2004). Clams were collected with a scoop net, briefly rinsed in water from their habitat, pierced with a dissecting needle to release water held in the mantle cavity, and flash-frozen in a liquid N\(_2\) dry shipper (CX100, Taylor Wharton Cryogenics, Theodore, AL, USA). The time from scoop netting to flash-freezing was less than 1 min. Clams were collected on two consecutive days between 09.00 and 12.00 h. Clams were maintained in the dry shipper during transport back to the University of Florida, where they were stored at −80 °C. All clams used for nucleic acid and stress protein analyses were 5–7 mm in shell length.

Amounts of total tissue DNA, RNA, oxidized DNA and RNA bases were determined in four to five clams per site. DNA guanine base oxidation produces 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoGuo), and RNA guanine base oxidation produces 8-oxo-7,8-dihydroguanosine (8-oxoGuo). Samples consisting of 80–100 mg whole clam homogenate were processed as described previously (Hofer et al. 2006). Briefly, we simultaneously extracted both DNA and RNA from whole clam homogenates using guanidine thiocyanate and phenol/chloroform at neutral pH, after which nucleic acids were hydrolysed with Nuclelease P1 and alkaline phosphatase. Hydrolytic enzymes were then removed by filtration, and the hydrolysate was analysed by high performance liquid chromatography coupled to electrochemical detection (HPLC-ECD; ESA Inc., Chelmsford, MA, USA).

Stress protein expression levels were determined using mono-specific, ELISA-grade polyclonal antibodies generated by and donated by EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). The following antibodies were used: manganese superoxide dismutase (MnSOD; catalogue no. AB-1976), copper/zinc superoxide dismutase (Cu/Zn SOD; catalogue no. AB-SOD-1516), glutathione peroxidase (GPx; catalogue no. AB-GPX-1433), mitochondrial 8-oxoguanine DNA glycosylase (OGG1-mito; lot 2916), heat shock protein 60 (Hsp60; catalogue no. AB-H100-IN), heat shock protein 70 (Hsp70; catalogue no. AB-Hsp70-1519), and invertebrate small heat shock protein homologues (sHsp; catalogue no. AB-H105). A summary of the functions of these proteins is presented in Table 1. The antibodies were raised in rabbits against 8–15 amino-acid polypeptides (conjugated to bovine serum albumin) derived from each target protein sequence of the bivalve Mya arenaria (Downs et al. 2002). Stress proteins were analysed in whole clam homogenates (Joyner-Matos, Downs and Julian 2006) from 10 clams from each of the four sites, including those clams used for nucleic acid analyses. Briefly, whole clams (with shells) were homogenized in liquid N\(_2\). Homogenized tissue from each clam was suspended in protein denaturing buffer, and total soluble protein concentration was determined (Ghosh et al. 1988). Each clam was processed individually, and all clam samples were prepared on the same day using the same buffer. Antibody specificity of seven stress proteins was verified by polyacrylamide gel electrophoresis (PAGE) and Western blotting, as described in the Results section. Expression levels were analysed in triplicate by enzyme-linked immunosorbent assay (ELISA).

STATISTICAL ANALYSES

The nonparametric Spearman’s rank correlation was used to test for a significant relationship between each limnological variable and distance along the transect, and the index of clam density (CPUE) and distance. Linear regression was used to evaluate the relationship between clam CPUE and the following variables: DO,
conductivity, pH and transparency. Multiple regression was used to quantify the relationship between clam CPUE and the same suite of independent variables when entered into the regression model together. RNA oxidation data and stress protein expression levels were analysed by one-way ANOVA with Fisher’s Least Significant Difference post-hoc multiple comparison test. The DNA oxidation data were not normally distributed (Shapiro–Wilks’ test $W = 0.77764$ and $P < 0.0007$) and were therefore analysed using a Kruskal–Wallis one-way ANOVA with Conover–Inman pairwise comparisons, using StatsDirect (v. 2.4.5, www.statsdirect.com). To better understand the relationship between overall stress protein expression pattern and site, a principal components analysis was performed on the stress protein data. Factor scores resulting from an analysis with two unrotated factors were then analysed by one-way ANOVA as above. Except as noted all statistical analyses were performed with Statistica (v. 7.1, Statsoft Inc., Tulsa OK, USA). Except as indicated, all values are reported as mean ± standard error. Statistical significance was accepted at $\alpha = 0.05$.

**Results**

**LIMNOLOGICAL SURVEY**

DO in the swamp sites (sites 1–5) averaged 0.023 mmol L$^{-1}$, which is less than one-tenth that of aerated stream water (0.25 mmol L$^{-1}$). DO increased along the transect from the swamp into the stream ($r_s = 0.944$, $P < 0.0001$, Fig. 2a), reaching 0.19 mmol L$^{-1}$ at the most upstream site (site 16). The surface water temperatures averaged 17.5 ± 1.5 °C ($n = 48$; data not shown) across all sites. Water transparency increased with distance along the transect ($r_s = 0.743$, $P = 0.0021$, Fig. 2b), as did pH ($r_s = 0.889$, $P < 0.0001$, Fig. 2c), whereas conductivity did not change with distance ($r_s = 0.329$, $P = 0.226$, Fig. 2d). At all sites (with the exception of site 16), water velocity was low with ranks between 0 and 1, and

![Fig. 2. Limnological features of the stream-swamp transect. Mean (±1 SE) of triplicate measures of (a) dissolved O$_2$ (mmol L$^{-1}$), (b) transparency (cm), (c) pH, and (d) conductivity (mΩ cm$^{-1}$). Sites correspond to the survey sites shown in Fig. 1. We were unable to collect a sufficient volume of water from site 4 to measure conductivity, pH or transparency.](image)
with most sites ranked as 0.5, indicating sluggish water flow (data not shown).

### CLAM DENSITY AND SIZE-FREQUENCY PATTERN

The catch per unit effort (CPUE) of clams was high but ‘patchy’ in the swamp (range from 17 to 144, corresponding to c. 140–1200 clams m⁻²) and decreased slightly in the ecotone sites (Fig. 3a). CPUE decreased along the transect (r = −0.889, P < 0.0001), with clams absent from stream sites with DO level higher than 0.17 mmol L⁻¹ (i.e. sites 13–16). Clams were buried below the sediment surface.

We examined the degree to which DO, conductivity, pH and transparency explained variation in CPUE of clams along the transect. In a multiple regression, only DO was marginally significant (whole model, R² = 0.4168, P = 0.0005; DO, partial r = −0.308, P = 0.0596); however, all four factors exhibited collinearity (VIF ranging from 1.11 to 3.65). We therefore examined the relationship between clam CPUE and each independent variable using least-squares linear regression. DO explained 52.9% of the variance in clam CPUE (P = 0.0014), pH explained 48.2% (P = 0.0041), and transparency explained 39.8% (P = 0.0117). In contrast, the relationship between conductivity and clam abundance was not significant (P = 0.34).

The umbo-to-margin length of the clams was quantified to examine variation in size and size frequency among sites. For this analysis we grouped the data according to site type (Fig. 3b). All size classes from 2 mm to 7 mm were present in samples from the swamp (n = 1209) and the ecotone (n = 545). In the swamp and ecotone, the distribution of clams into size classes roughly matched a normal distribution. In the stream site, the sample population (n = 15) was heavily skewed towards the larger (> 5 mm) size classes, with only one (7%) of the 15 clams having a shell length of less than 5 mm. In the swamp and ecotone, clams with a shell length of less than 5 mm represented 40% and 59% of the samples, respectively.

### RNA : DNA RATIO

To investigate whether clams near the edge of the distribution exhibited decreased protein synthesis or growth in comparison with clams from the swamp interior, we analysed RNA : DNA ratio in four to five clams per site. The RNA : DNA ratio of the clams in the ecotone and stream was 25% lower than that of clams in the swamp margin (F = 5.283, P = 0.012; swamp margin vs. ecotone, P = 0.049, swamp margin vs. stream, P = 0.054, Fig. 4a).

### NUCLEIC ACID OXIDATION

To determine whether clams experienced cellular oxidative damage, which results from increased production or decreased detoxification of free radicals, we measured the levels of two forms of oxidatively damaged nucleosides: the DNA oxidation product 8-oxodGuo and the RNA oxidation product 8-oxoGuo. DNA yield was consistent across sites (0.472 ± 0.07 µg mg⁻¹ tissue; F = 0.246, P = 0.863, data not shown), indicating that nucleoside extraction was equivalent in all samples. Clams in the ecotone and stream sites had significantly more DNA and RNA oxidation products than clams from the swamp interior: 8-oxodGuo, Kruskal–Wallis ANOVA H₁ = 8.456, P = 0.037, Conover–Inman pairwise comparisons t₁₄ = 2.145, P = 0.0077 (Fig. 4b), and 8-oxoGuo, F = 5.871, P = 0.008, for all comparisons, P = 0.036 (Fig. 4c). At each site, RNA oxidation was proportionally more extensive than DNA oxidation.

### STRESS PROTEIN ANALYSES

We analysed the expression levels of seven stress proteins (Table 1), which we categorized according to function as antioxidants (MnSOD, Cu/ZnSOD and GPx), oxidative repair (OGG1m) and chaperones (Hsp60, Hsp70, sHsp).

- **Antioxidants**: MnSOD levels were higher in clams from the swamp margin and ecotone sites than in clams from either the swamp interior or stream sites (Fig. 5a; n = 40, F = 2.83, P = 0.052; swamp margin vs. stream P = 0.014, ecotone vs. stream P = 0.020). Cu/ZnSOD levels were lower in the clams from the stream site than in clams from the other three sites (Fig. 5b; n = 40, F = 4.21, P = 0.012; stream vs.
Stress response along a natural oxygen gradient

Other sites $P = 0.012$). There were no significant changes in GPx expression levels (Fig. 5c; $n = 40$; $F = 1.39$, $P = 0.262$).

- **Oxidative repair**: Clams from the swamp margin expressed more OGG1m than did clams from the swamp interior or stream sites (Fig. 5d; $n = 40$; $F = 3.82$, $P = 0.018$, swamp interior vs. swamp margin $P = 0.013$, swamp margin vs. stream $P = 0.003$).

- **Chaperones**: Clams from the swamp margin and ecotone expressed more Hsp60 than clams from the dense interior, with clams from the swamp margin expressing more Hsp60 than clams from the stream (Fig. 5e; $n = 40$; $F = 5.333$, $P = 0.004$, for all significant comparisons, $P = 0.036$). Clams from the stream site had lower Hsp70 levels than clams from the swamp margin or ecotone site, but comparable expression levels with clams from the swamp interior (Fig. 5f; $n = 40$; $F = 3.647$, $P = 0.021$, stream vs. swamp margin and ecotone, $P = 0.0125$). Clams from the swamp margin had higher sHsp expression levels with clams from the other three sites (Fig. 5g; $n = 40$; $F = 3.87$, $P = 0.017$, swamp margin vs. other sites $P = 0.014$).

We conducted a principal components analysis to investigate whether the stress protein expression showed evidence of a co-ordinated response. We extracted two factors that explained a total of 63.53% of the variance, with the first factor explaining 45.13% of the total variance. The first factor was composed of four proteins with factor loading scores greater than 0.7: GPx, Hsp60, Hsp70 and MnSOD, all of which had positive loading scores ranging from 0.718 to 0.827. The second factor, consisting only of OGG1m, explained 18.4% of the variance in stress protein expression. OGG1m loaded heavily on the second factor with a score of $-0.762$. We next examined whether the mean factor scores (assigned to each individual on the basis of factor 1) differed among sites. Factor 1 loading scores were significantly different among sites ($F = 4.034$, $P = 0.0153$, Fig. 5h), with clams from the swamp margin having a higher score than clams from the dense interior or the stream ($P = 0.035$ and $P = 0.039$, respectively).

**Discussion**

The ‘abundant centre’ distribution pattern (Sagarin and Gaines 2002) predicts that when a population overlies a strong environmental gradient, abundance will be highest where conditions are the least stressful and/or the most beneficial. Furthermore, those individuals living where abundance is highest are expected to be in better condition than those living where abundance is low (Caughley et al. 1988). Although the Sphaerium sp. distribution pattern matches the abundant centre pattern, the distribution appears to indicate that the hypoxic, acidic swamp water is less stressful (or more beneficial) than the normoxic, neutral pH stream water.

**LIMNOLOGICAL CHARACTERS AND RELATIONSHIP WITH CLAM DENSITY**

A previous characterization of the Rwembaita Swamp showed that the water is extremely hypoxic and mildly acidic relative to the tributary streams feeding into it (Chapman et al. 2000), consistent with abiotic conditions in other papyrus swamp systems in Africa (Carter 1955; Chapman et al. 2001). The ecotonal region of intermediate pH and DO is always present along the tributary streams, but the exact location of the ecotone varies with season and other local habitat characteristics (e.g. stream volume), and it can be disturbed by large animals such as elephants (Chapman et al. 2001). We conducted a fine-scale survey of a
transect extending from within the Rwembaita Swamp into one of its tributary streams (Fig. 2a). We found that clams were abundant in the swamp, less abundant along the ecotone, and absent from stream sites with DO above 0·17 mmol L$^{-1}$ and a pH above 6·9. In a multiple regression of our measure of abundance (CPUE) vs. DO, pH, transparency and conductivity, DO was the only significant predictor. However, all four factors were collinear and when examined by least-squares linear regression, DO, pH and transparency each explained significant variation in CPUE.

Fig. 5. Antibody specificity tests and stress protein expression levels presented as relative units per nanogram of total soluble protein (RU ng TSP$^{-1}$; $n = 10$ from each site). (a) Manganese superoxide dismutase (MnSOD), (b) copper/zinc superoxide dismutase (Cu/ZnSOD), (c) glutathione peroxidase (GPx), (d) the DNA repair enzyme mitochondrial 8-oxoguanine DNA glycosylase (OGG1-m), (e) heat shock protein 60 (Hsp60), (f) heat shock protein 70 (Hsp70), and (g) small heat shock proteins (sHSP). Clams were collected from the swamp interior, swamp margin (site 2), ecotone (site 8), and stream (site 11). Bars depict mean ± 1 SE. Similar letters indicate statistically indistinguishable samples according to Fisher's Least Significant Difference post-hoc multiple comparison test. For antibody specificity tests, two random samples of *Sphaerium* sp. were pooled, subjected to SDS–PAGE, and assayed with the appropriate antibodies. The positions of known molecular weight standards are indicated in kilodaltons. (h) Factor 1 loading scores from a principal components analysis of stress protein expression levels presented in box-and-whisker plots with the box defining data in the 25th to 75th percentiles, the line indicating the median, and the whiskers defining the 10th and 90th percentiles.

The population of clams at the distribution edge was skewed towards larger size classes (5–7 mm). As this is the first assessment of a size-frequency pattern in this system, and it was only done at one season, these results must be interpreted with caution. None the less, these data suggest that demographic differences exist along this physicochemical gradient. *Sphaerid* life spans range from less than 1 year to 5 years (Burky 1983), and all known species internally brood their young, which are released as shelled juveniles (Mackie 1978). The lack of smaller size classes in stream sites...
Stress response along a natural oxygen gradient

could reflect fluctuations in the position of the ecotone; for example, a period of low rainfall could have led to abiotic conditions in these sites that were transiently similar to the swamp, allowing successful recruitment.

Other physicochemical characters, as well as biotic interactions, likely influence clam distribution and abundance in this swamp-stream system. For example, substrate type typically influences the distribution of burrowing bivalves, although a relationship between substrate type and habitat preference was not supported in several studies of sphaerid clams (reviewed in Burky 1983). In the Rwembaita Swamp-stream system, substrate type (mud mixed with vegetation) was similar across all sites with clams and no clams were observed on the surface of the substrate, which is consistent with a previous survey in which clams were sampled from a depth of 2–3 cm (Osborne et al. 2001).

The extent to which the increased sandiness in the stream might negatively influence burial and thus influence dispersal to these sites is unknown. Currently, we cannot address the potential influence of biotic factors such as competition and predation on clam distribution. However, previous studies (Chapman 1995; Chapman et al. 1999, 2004; Osborne et al. 2001) have additionally highlighted the importance of considering effects of physicochemical gradients when assessing how biotic interactions shape this community structure (Menge and Sutherland 1987; Case et al. 2005).

CELLULAR-LEVEL INDICATORS

To test whether clams at the distribution edge were under more stress and whether the condition of the clams changed along the abiotic gradients, we investigated several cellular-level indicators of stress: RNA : DNA ratio, nucleic acid oxidative damage, and number of different stress proteins. Compared with clams in the swamp interior and swamp margin, we expected clams from the ecotone and stream site (distribution edge) to have lower RNA : DNA ratio, higher oxidative damage, and higher stress protein expression, all consistent with higher stress.

Decreased RNA : DNA ratio indicates reduced levels of protein synthesis, as could occur with stress or decreased growth rate (Elser et al. 2000; Dahlhoff 2004). We found that clams from the ecotone and stream sites had lower RNA : DNA ratio in comparison with clams from the swamp margin. Although statistically significant, the difference in RNA : DNA ratio was small in comparison with studies examining changes across seasons or following experimental manipulations (Dahlhoff 2004). None the less, to our knowledge comparable studies of RNA : DNA ratio across a population do not exist, so it is unknown whether even small differences are functionally significant in natural populations.

Oxidative damage, including damage to DNA and RNA, occurs when free radical production overwhelms a cell’s ability to detoxify the free radicals (Halliwell and Gutteridge 1999). The deleterious effects of DNA oxidation on mutation accumulation and ageing are well-established (Hartman et al. 2004; Sanz, Pamplona and Barja 2006), but the effects of RNA oxidation have not been well characterized (Seo et al. 2006). We found that clams from the ecotone and stream sites had significantly elevated levels of oxidatively damaged DNA and RNA in comparison with clams from the dense interior of the swamp, and furthermore that RNA oxidative damage was proportionally higher than DNA damage at all sites. To our knowledge, this is the first quantification of DNA and RNA oxidation in a natural population.

Stress proteins are produced by nearly all eukaryotes, and patterns of stress protein expression and activity serve as cellular-level indicators of stressor type (e.g. Willmore and Storey 1997; Abele and Puntarulo 2004; Magalhães et al. 2005) and indicators of how stressors influence habitat preferences (e.g. Feder and Hofmann 1999; Downs et al. 2002). We found patterns of stress protein expression in clams along the transect indicating that clams in the swamp margin and ecotone were experiencing more stress than those in the dense interior of the swamp. The elevations in chaperone proteins (Hsp60, Hsp70 and sHsp) in clams in the intermediate sites indicate generalized cellular stress, particularly that which results in protein misfolding, protein aggregation, or heightened need for protein degradation (Krege 2002). Furthermore, elevations in antioxidants (MnSOD) and the repair enzyme OGG1-m in clams in the swamp margin and ecotone compared with the dense interior suggests that these clams experienced elevated free radical production and oxidative damage (Halliwell and Gutteridge 1999). Similar relative changes in protein expression were detected in bivalves exposed to abiotic stressors in controlled laboratory conditions (Joyner-Matos et al. 2006) and in bivalves at sites impacted by a crude oil spill (Downs et al. 2002). However, these data on relative changes do not allow comparisons of absolute stress protein expression levels between different organisms nor of comparisons between expression levels and enzyme activity.

Principal components analysis, which provides a composite view of a co-ordinated stress response, showed that clams in the swamp margin and ecotone had a larger stress response than did clams in either the dense interior or stream sites, but that the response of clams in the swamp margin did not differ from that of clams in the ecotone. This integrated stress response was dominated by two antioxidants, GPx and MnSOD, and two chaperone proteins, Hsp60 and Hsp70, indicating that clams at the intermediate sites were experiencing stress at the cellular level and that at least some of that stress was due to elevated free radical production.

THE EXTREME EDGE OF THE DISTRIBUTION

Comparison of clams in the swamp margin with those in sites closer to the edge of the distribution followed the expected pattern: as abundance decreases, RNA : DNA
ratio decreases, oxidative damage increases, and, for the swamp margin and ecotone sites, stress protein expression increases. Surprisingly, however, stress protein expression in clams from the stream site was equivalent to, or lower than, that of clams from the dense interior. One interpretation of this pattern is that clams from the stream site were less stressed than clams from the swamp margin and ecotone, but this is not consistent with the RNA : DNA ratio and nucleotide oxidative damage results, which both indicate that clams in the stream were under more stress. The alternate interpretation is that clams from the stream site were so stressed that they were not capable of maintaining high stress protein expression (Werner and Hinton 1999).

Therefore, the clams at the extreme edge of the distribution show a stress response pattern that might otherwise be interpreted as pathological loss of cellular homeostatic stress response and repair mechanisms. It is unknown whether this pattern of extreme physiological stress in organisms at the extreme distribution edge is present in other systems with stable population distributions overlying stressful abiotic gradients.

RELATIONSHIPS BETWEEN LIMNOLOGICAL CHARACTERS AND CELLULAR-LEVEL STRESS INDICATORS

The distribution data demonstrated that the swamp is less stressful (or more beneficial) than the stream, consistent with the abundant centre distribution pattern. Of the limnological variables measured along the transect, DO, pH and transparency were significant predictors of clam CPUE, and all showed a negative correlation. When examining cellular-level stress indicators, we found a pattern consistent with increasing oxidative stress towards the distribution edge. While any of the three limnological variables could contribute to oxidative stress, we believe the evidence is strongest for DO.

Oxygen availability, particularly hypoxia, affects a wide variety of ecological processes in aquatic organisms (Baker and Mann 1992; Diaz and Rosenberg 1995; Rosenberger and Chapman 2000). The ability of the sphaeriid clams in the Rwembaita Swamp to tolerate hypoxia is not surprising, given the well-documented ability of clams from this cosmopolitan family to tolerate DO fluctuations (Waite and Neufeld 1977) and to aestivate during seasonal dry periods in ephemeral ponds (McKee and Mackie 1983). However, the hypoxia tolerance of the sphaeriid clams in the Rwembaita Swamp appears to be greater than that demonstrated by congeners and closely related pisidial clams. For example, a European congener showed significantly decreased valve-movement behaviour at DO levels comparable with that of the ectocote sites (Heinonen et al. 1997), and a North American sphaeriid and European pisidial had critical O2 tensions comparable with the DO level at swamp sites (Waite and Neufeld 1977; Hamburger et al. 2000).

While it is still unclear whether hypoxia causes elevated free radical production (e.g. Li and Jackson 2002; de Oliveira et al. 2005), it is well established that hyperoxia (typically defined as O2 concentrations higher than atmospheric levels) is linked to increased free radical production and oxidative damage (Boveris and Chance 1973; Abele and Puntarulo 2004). However, the concentration of O2 necessary to cause oxidative damage may be much less than ‘hyperoxia’ for isolated cells and small animals (Packer and Fuehr 1977; Saito, Hammond and Moses 1995; Gray et al. 2004). Given that the sphaeriid clams in the Rwembaita Swamp system are adapted to hypoxia and appear to spend their entire life cycle in hypoxic conditions, we propose that clams living in the intermediate sites and distribution edge experience the equivalent of hyperoxic stress. The pattern of cellular-level indicators in our study is consistent with biomedical studies of hyperoxia (Wong et al. 1998; Freiberger et al. 2004; Roper et al. 2004; Olsvik et al. 2005). Therefore, ‘hyperoxia’ stress may occur in any animal exposed to oxygen levels higher than those to which it is adapted, whether this is a normoxia-adapted animal exposed to hyperoxia, a hypoxia-adapted animal exposed to normoxia, or even, in the case of Sphaerium sp., an animal adapted to extreme hypoxia exposed to moderate hypoxia.

We cannot, however, rule out the possible influence of pH or transparency on clam distribution or cellular-level indicators of stress. In aquatic habitats, pH is influenced by CO2 concentration, and the co-occurrence of hypoxia and acidic/hypercapnic conditions is widespread (Burnett 1997). In the papyrus swamps of East Africa, both DO and pH are strongly influenced by organic matter decomposition, with 81% of the variance in DO explained by the concentration of free, dissolved CO2 (Chapman et al. 2001). Interestingly, CO2 may either promote or inhibit oxidative damage through a variety of reactions (Veselá and Wilhelm 2002). To our knowledge, there are no studies linking oxidative stress to environmental alterations of pH or CO2.

Water transparency, which is influenced by concentrations of tannins (Labieniec, Gabryelak and Falcioni 2003) and suspended materials (Ward and Shumway 2004), also was inversely correlated with clam abundance. As tannin levels in the Rwembaita Swamp system are unknown, we are unable to evaluate their potential influence on clam physiology. Suspended materials, which may be inorganic or organic, also decrease water transparency, but the composition of suspended materials in this system is unknown. As bivalve feeding efficiency decreases in water with high concentrations of inorganic suspended materials (Ward and Shumway 2004), we would expect clam abundance to be lowest in the swamp, where water transparency is lowest, which is not consistent with our results or multiyear surveys (Chapman et al. 2004). However, when the suspended materials are primarily organic, clam abundance would be lowest where transparency is highest, as observed in this study. Nevertheless, the observed size/frequency distribution is inconsistent with nutrient limitation influencing the population.
Stress response along a natural oxygen gradient

Conclusions

In the current study of *Sphaerium* sp. in the Rwembaita Swamp system, we found that the hypoxic, low-transparency, and acidic conditions of the swamp minimize oxidative damage, while the elevated DO, high transparency and neutral pH conditions of the stream increase oxidative damage. We propose that in this clam, DO above the extreme hypoxia of the swamp causes a physiological condition similar to that of other animals exposed to hyperoxia. A critical test of this hypothesis would entail long-term transplant experiments, coupled with direct manipulation of the physical environment. However, such experiments would be challenging at this site due to frequent disturbance by large animals and substantial fluctuations in water level during dry and rainy seasons. In addition, we found that clams at the extreme edge showed decreased stress protein expression, despite decreased condition and elevated oxidative damage, which we propose is an indication of extreme stress. This demonstrates that analysis of stress protein expression alone may be a misleading index of stress and highlights the importance of sampling the extreme distribution edge. The integration of ecological and biochemical responses provides a useful approach to understanding the role of the physicochemical environment in structuring population distributions.

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Stress response along a natural oxygen gradient

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