Cadmium-dependent oxygen limitation affects temperature tolerance in eastern oysters (Crassostrea virginica Gmelin)


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Lannig G, Cherkasov AS, Pörtner H-O, Bock C, Sokolova IM. Cadmium-dependent oxygen limitation affects temperature tolerance in eastern oysters (Crassostrea virginica Gmelin). Am J Physiol Regul Integr Comp Physiol 294: R1338–R1346, 2008. First published February 13, 2008; doi:10.1152/ajpregu.00793.2007.—Marine ectotherms, including oysters are exposed to variable environmental conditions in coastal shallow waters and estuaries. In the light of global climate change, additional stressors like pollution might pose higher risk to populations. On the basis of the concept of oxygen- and capacity-limited thermal tolerance in aquatic ectotherms, we show that a persistent pollutant, cadmium, can have detrimental effects on oysters (Crassostrea virginica). During acute warming from 20 to 28°C (4°C/48 h) standard metabolic rate (SMR) rose in control and cadmium-exposed (50 μg Cd/l) animals, with a consistently higher SMR in Cd-exposed oysters. Additionally, Cd-exposed oysters showed a stronger temperature-dependent decrease in hemolymph oxygen partial pressures. This observation indicates that the effect of temperature on aerobic metabolism was exacerbated due to the additional Cd stress. The oxygen delivery systems could not provide enough oxygen to cover Cd-induced elevated metabolic demands at high temperatures. Interestingly, cardiac performance (measured as the heart rate and hemolymph supply to tissues) rose to a similar extent in control and Cd-exposed oysters with warming indicating that cardiac output was unable to compensate for elevated energy demand in Cd-exposed oysters. Together with the literature data on metal-induced reduction of ventilatory capacity, these findings suggest that synergistic effects of elevated temperatures and cadmium exposure led to oxygen limitation by impaired performance in oxygen supply through ventilation and circulation. Overall, cadmium exposure resulted in progressive hypoxemia in oysters at high temperatures, suggesting that the thermal tolerance window is narrowed in marine ectotherms inhabiting polluted areas compared with pristine environments.

aerobic metabolism; cardiac performance; magnetic resonance imaging, hemolymph PO2; anaerobiosis

Pollution is a widespread phenomenon, and contamination of lakes and coastal regions by heavy metals deposited by the atmosphere or by industrial and urban activities is well documented (16, 25). Among trace metals, cadmium is a major aquatic pollutant (16, 25, 43). The average cadmium concentration in unpolluted coastal waters is about 0.05 μg Cd/l, but it can reach up to 10–35 μg Cd/l or higher in very polluted areas (16, 25, 43, and references therein). Cadmium exerts toxic effects on all organisms studied so far, and mechanisms of these effects encompass a broad range of critical physiological and cellular functions. Cadmium represses uptake and metabolism of essential metals such as zinc or copper (46) and affects gene expression in multiple cellular pathways (3, 35, 44, 49, 57). Cadmium exposure in vivo or in vitro also leads to cellular energy disturbances due to the limitation of mitochondrial functioning (11, 21, 48) and elevated oxidative stress (15, 33, 53). Trace metals, including cadmium, are known to interfere with vital physiological processes such as ventilation (50), oxygen consumption (33, 34), blood oxygen transport (34, 54), and activity/growth (22, 27). As a result, profound “ripple” effects of trace metals on organisms’ performance and fitness are expected.

Recent models of global climate change predict an increase in mean global temperature (by 1.8–4°C by the year 2100, according to different scenarios) paralleled by a rise in the frequency and magnitude of seasonal thermal fluctuations (http://www.ipcc.ch/). This increase is expected to strongly affect physiology and metal toxicity in ectotherms such as oysters due to the fact that their body temperature changes with the temperature of the environment, resulting in the corresponding alterations of the rates of all physiological and biochemical reactions. Metabolic adjustment may partially counteract the temperature-dependent effects on energy metabolism, allowing an organism to maintain positive scope for growth, activity, and reproduction. However, if thermal acclimation is incomplete or impossible, stress and reduced fitness will ensue. Given the strong effects of temperature on ectotherm metabolism and the fact that exposure to toxic metals such as cadmium is often associated with energy costs (13, 33), one can expect that toxic effects of metals will increase with elevated temperatures and will be especially pronounced at temperatures outside the optimal range. Moreover, earlier studies, including those in our laboratories, show that the relationship between temperature and metal toxicity is often nonadditive and cannot be reliably predicted from the effects of single stressors. Our earlier studies in a model marine ectotherm, the eastern oyster Crassostrea virginica showed strong synergism between the effects of temperature and cadmium stress on survival, physiology, and metabolic function (15, 33, 48).

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ities were also considerably more susceptible to deleterious effects of Cd at elevated temperatures (15, 48). Thus, to understand factors affecting the tolerance limits, survival, and productivity of ectotherm populations and to model the effects of global climate change on these populations (especially in areas with high anthropogenic pressure), it is critical to understand the physiological mechanisms involved in interactions between temperature and pollutants (such as cadmium) and their relevance for the organisms’ performance and survival.

According to the concept of oxygen- and capacity-limited thermal tolerance in aquatic ectotherms (40), the temperature range in which an animal exhibits its highest capacity for growth and reproduction is mainly determined by optimized oxygen supply. Beyond this optimal temperature range, oxygen supply and thus aerobic scope decline until temperatures are reached (called upper and lower critical temperature) where insufficient oxygen delivery results in the onset of anaerobic metabolism and survival becomes time limited (40). Therefore, any factor that affects aerobic capacities would also influence thermal tolerance. We proposed that the capacity for oxygen supply was overridden by the synergistic effects of cadmium and high temperature in oysters accounting for their inability to provide enough oxygen to meet elevated energy demand (33). In aquatic invertebrates, including bivalves (41) and crustaceans (24), impaired oxygen supply at elevated temperatures has been shown to result from the temperature-dependent failure in both ventilatory and circulatory performance. In aquatic ectotherms, perturbation of aerobic metabolism by heavy metals has been linked to the damage of gill structure surface and/or increased mucus production associated with reduced efficiency of gas exchange (34, 39, 50, 51), and high metal levels resulted in a severe disruption of cardiac performance with alternating periods of tachycardia and bradycardia (12, 17, 20, 36). It has not been previously explored how combined temperature and heavy metal stress may affect oxygen supply in aquatic ectotherms.

To obtain a more comprehensive mechanistic picture of the interactive effects of elevated temperature and metal pollution on aerobic metabolism of marine ectotherms, we challenged cadmium-exposed oysters, C. virginica, with an acute rise in temperature and investigated their metabolic energy demand and circulatory performance by measuring standard metabolic rate (SMR), heart rate, hemolymph oxygen partial pressure (PO2) and hemolymph supply to tissues using in vivo MRI and NMR spectroscopy (MRS), as well as noninvasive laser-Doppler-flowmetry.

MATERIALS AND METHODS

Animal collection and maintenance. Experiments were carried out at the University of North Carolina at Charlotte (USA) and the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven (Germany). Wild-cultured adult individuals (2- to 3-yr-old diploids, 80–120 mm mean shell length) of C. virginica were obtained from J & B Aquafood (Stump Sound, NC). All experimental oysters were sexually mature. For determinations of hemolymph PO2, oysters were obtained in May 2005 and August 2006, and for all other experiments, they were obtained in August 2006. Average water temperature at time of collection varied between 25 and 30°C, and salinity was 29–30 psu.

All animals were maintained in closed recirculating aerated water tanks that were filled with either artificial seawater (Instant Ocean, Kent Marine, Acworth, GA) or natural seawater from the North Sea delivered by ship at 20 ± 1°C and 30 ± 1 psu. Our previous studies have shown that this temperature and salinity are close to the optimum for oysters (unpublished data). Oyster density in the tanks was maintained to ensure at least 6 liters of water per animal. After a recovery period of at least 7 days, oysters were divided into a control group and a Cd-exposed group, with two or three tanks per group. Cadmium was added to the tanks with Cd-exposed group to a nominal concentration of 50 μg Cd2+/l as CdCl2. Oysters were exposed for 20 days to the respective experimental conditions and haphazardly selected for each experiment from different replicate tanks. Because it was not possible to measure all parameters in the same oyster, the experiment was repeated four times: for determination of standard metabolic rate, hemolymph PO2, heart rate, and the NMR studies.

Oysters were fed three times a week ad libitum with a commercial algal blend containing *Nannochloropsis*, *Tetraselmis*, and *Isochrysis spp.* (PhytoPlex, Kent Marine, Acworth, GA) or *Nannochloropsis*, *Phaeodactylum tricornutum* and *Chlorella* (DT’s Live Marine Plankton, Coralsands, Germany, www.coralsands.de). The water was changed 8–10 h after feeding. Before experimentation, oysters were removed from the maintenance tanks, and surgery was performed if needed. Oysters were then placed into respirometer chambers at 20°C and 30 PSU and left for 12–24 h to recover from surgery (if applicable) and/or handling stress (see details of experiment-specific setups below). To avoid interference with postprandial metabolism and feces excretion, animals were kept unfed for 12–24 h before the start of data collection. Measurements were taken at the control temperature (20°C), and then temperature was increased by 4°C once every 48 h. Animals were allowed to acclimate at the new temperature for at least 12 h and were measured continuously during a 24- to 36-h period prior to the next temperature increment. Measurements were performed at 20, 24, and 28°C. The temperature in the experimental setups was maintained within ± 0.1°C from the set temperatures by means of recirculating cooling water baths. At the end of the experiments, animals were killed.

Hemolymph oxygen partial pressure (PO2) was monitored online by implanted needle-type oxygen microsensors with integrated temperature compensation (Tx-Type, PreSens, Regensburg, Germany, www.Presens.de). To insert the sensor while avoiding injury to the mantle tissue, a small hole was drilled through the right shell valve directly above the pericardium. Position of the pericardium was estimated by drawing an imaginary line perpendicular to the longest shell dimension and crossing it through point A until it reached point B, where A = total valve length (measured from the hinge) + 0.6 (mm) and B = total width (measured from the ventral side) – 0.45 (mm).

For optimum positioning of the microsensor (optode) inside the pericardial cavity, the syringe was placed on the right shell valve and fixed with dental periphery wax. This way an angle of the oxygen microsensors (encased in a needle) was maintained constant during insertion into the pericardial cavity thus avoiding disruption of mantle and pericardium. Once the needle was inserted, the sensor tip was gently extended, and sensors values were checked to confirm that the sensor tip was not damaged during the procedure. Finally, SuperGlue was used for final firm positioning of the syringe, and the hole in the shell was covered with dental periphery wax. Prior to insertion, oxygen sensors were calibrated in oxygen-free (0%, using sodium sulfite, Na2SO3) and air-saturated (100%) seawater. The measurements were done following the procedures described by Lannig et al. (32). Values of hemolymph PO2 were recorded as % air saturation and converted to PO2 as follows: Hemolymph PO2 (kPa) = (P atm – P H2O) × 0.2095 (% air saturation/100), where P atm is atmospheric pressure (kPa), P H2O is temperature-specific water vapor pressure (kPa), calculated after Dejours (18) and 0.2095 is the proportion of oxygen content in the air.

SMR referring to resting postprandial oxygen consumption (M02) was determined by online recordings using oxygen microsensors with integrated temperature compensation (Tx-Type, PreSens). Two-point
calibration of sensors was performed at each temperature. Oyster shells were carefully scrubbed and cleaned of fouling organisms. Oysters were placed into flow-through respiration chambers and allowed to recover overnight. Water flow (30–80 ml/min) was adjusted so that animals consumed less than 25% of O$_2$ at all times to avoid potential inhibitory effects of low oxygen levels on respiration rate. Oxygen consumption was continuously monitored for 24–36 h. After measurements, oysters were dissected and tissue dry mass determined. SMR was calculated as described elsewhere (33): SMR = ($\Delta$P$_{O2}$·$\beta$O$_2$·$V_h$)/M$^{0.8}$, where SMR is oxygen consumption (µmol O$_2$·h$^{-1}$·g dw$^{-1}$) normalized to a standard dry weight of 1 g, $\Delta$P$_{O2}$ is the difference in partial pressure between inflowing and outflowing water (kPa), $\beta$O$_2$ is temperature-specific oxygen capacity of water (µmol O$_2$·l$^{-1}$·kPa$^{-1}$), $V_h$ is flow rate (l/h), M is oyster dry tissue mass (g), and 0.8 is the allometric coefficient (9). Because of the limited size range of oysters used in the present study, the allometric coefficient for scaling of MO$_2$ on body mass could not be determined, and the allometric coefficient from a closely related oyster species C. gigas was used (9) to correct for potential size effects on metabolism in C. virginica. Earlier studies have shown that the allometric relationship between MO$_2$ and body mass is highly conserved in marine bivalves (4).

Heart rate was monitored using a noninvasive laser Doppler perfusion monitor (LDPM PeriFlux System 5000, Perimed AB, Järfalla, Sweden, www.perimed.se). Before experiments, a two-point-calibration was performed according to the manufacturer’s instructions. After drilling a small hole into the right shell, the probe was positioned directly above the heart in close proximity of the pericardium to ensure optimal light path. The probe was fixed with dental periphery wax, thereby sealing the hole. Heart rate was determined by counting the changes in the laser-Doppler signal, indicating hemolymph flow changes through the pericardium during heart contraction. At each temperature, 6–10 intervals of 2 min each were randomly selected, and the number of heart beats was counted. The number of beats was averaged between the intervals for each individual at each temperature, and values were expressed as beats/min.

Hemolymph supply and tissue metabolites were determined by MRI and spectroscopy, respectively. All MRI/MRS experiments were conducted using a 4.7-T magnet with a 40-cm horizontal side bore and actively shielded gradient coils of 50 mT/m field strength (Bruker Biospec 47/40 DBX System, www.bruker-biospin.com), according to the MRI techniques previously developed for marine animal research at the AWI (6, 8). Animals were placed inside the magnet using the same flow-through animal chamber, as described for SMR determinations. Seawater was recirculated continuously from a temperature-controlled and aerated water reservoir (~50 liters). Temperature was measured inside the chamber using a fluoroptic fiber probe (Luxtron, Polytect Brinkmann Instruments, Westbury, NY), controlled with a recirculating cooling water bath (Lauda, Waldheim, Germany) within ±0.1°C from the set value and continuously recorded on a computer using a PowerLab system (ADInstruments, Colorado Springs, CO). A 5-cm diameter 1H-31P-13C surface coil was placed directly under the chamber close to the anticipated location of the adductor muscle of the animal. Standard gradient echo pilot scans were collected in all three directions before the start of the experiments to confirm the position of the adductor muscle relative to the center of the sensitive volume of the coil for optimized signal-to-noise ratios and defined MR parameters (see Fig. 1A). Four different MR techniques were used to collect a multiparameter data set: 1) In vivo 31P-NMR spectroscopy was used for observations of tissue energy metabolism and acid-base status. Spectroscopy parameters were as follows: pulse shape, bp32; pulse length, 200 µs; pulse angle, 45°; repetition time (TR), 1 s; number of scans, 1,800; and resulting acquisition time, 30 min. Spectra were processed and analyzed according to Bock et al. (7, 8). Because of time limitations, these experiments were performed at control temperature (20°C) only. 2) Hemolymph supply to the adductor muscle was measured using a flow-weighted gradient echo snap-

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**Fig. 1. Anatomical and flow-weighted MR images of eastern oysters, Crassostrea virginica.**

- **A:** anatomical MR image of an oyster showing different organs: 1, muscle separated in fast (a) and catch (b); 2, pericardium and heart; 3, hepatopancreas; 4, gills; and 5, shell material. **B** and **C:** flow-weighted MR images of closed (**B**) and open (**C**) oysters, C. virginica at 20°C. In the open oyster with beating heart the bright spots in the pericardium indicate hemolymph flow (see arrows). In contrast, no flow is evident in the closed oyster with nonbeating heart.
shot flash sequence (37). The adductor muscle of oysters can be divided into catch and fast muscles (Fig. 1A) that differ in structure and function. Catch adductor contracts slowly and can maintain contraction for long periods of time, whereas the fast muscle also referred to as phasic, or quick adductor, is capable of rapid response (closure as a response to disturbance) but incapable of prolonged contraction. One coronal slice was positioned showing heart and adductor muscle in the center of the MR image. The vertical slice position was placed through the muscle types on the basis of optimized visible heart function. Parameters were as follows: matrix size, 128x128; field of view (FOV), 8x6 cm; slice thickness (one slice), 2.74 mm; pulse shape, sinc3; pulse length, 2,000 μs; pulse angle, 22.5°; TR, 12.9 ms; TE, 4.3 ms; 1 average; 12 repetitions; and 64 dummy scans. 3) Hemolymph flow through the ventricles was measured after Bock et al. (7) using the same sequence and parameters as described in 2), except that 12 averages and 1 repetition were taken to determine mean flow ratios over several heart cycles. 4) T1- and T2-weighted spin echo MR images were collected using RARE for anatomical images (see Ref. 8) and used to account for possible temperature and/or cadmium effects on T1 or T2, respectively [parameters: excitation pulse sinc3, pulse length 2,000 μs, refocusing pulse ma04_180, pulse length 5,000 μs, TR = 6,000 (2,000) ms, TE = 20.9 (9.6) ms, 8 averages, 1 repetition, rare factor 64 or 32]. The same geometry (as described for cardiac flow) was used in all MR images.

**Data processing.** Mean signal intensities were calculated by an operator-controlled pixel by pixel analysis of various regions of interest (ROIs) for the determination of relative hemolymph flow changes. To account for any potential effects of temperature and cadmium exposure on signal intensity unrelated to hemolymph flow, two noise frames were selected inside the shell material of oysters. There were no significant differences in noise signal intensity at different temperatures or between control and Cd-exposed oysters (data not shown), confirming that changes in the flow-weighted signal with temperature and/or Cd were due to the changes of hemolymph flow rate.

In the pericardium, an increased hemolymph flow resulted in strongly elevated signal intensity in the snapshot FLASH images. This difference can be particularly easily seen from the comparison of images taken from oysters with nonbeating and beating hearts (Fig. 1, B and C). Thus, mean signal intensities of two flow areas in the pericardium (see arrows in Fig. 1C) corresponding to ventricular and atrial flow, respectively, were used to quantify the cardiac flow in oysters.

In muscles, hemolymph lacunae appeared as bright meanders against the dark background of the muscle tissue (Fig. 1A) and average intensity of the muscle tissue decreased when hemolymph supply to the muscle was high. Therefore, mean signal intensity of muscles in the closed control animals (with the lowest hemolymph supply) was taken as a background, and a normalized hemolymph supply index (HSI) was calculated for catch and fast muscle as follows: HSI (AU) = MSl1 - MSlc, where MSl1 is the mean intensity of flow-weighted signal in the catch or fast muscle in closed control animals with a nonbeating heart, and MSlc, the mean intensity of flow-weighted signal in the respective muscle type in animals under all other conditions (open/closed, control/Cd-exposed and/or exposed at different temperatures). This transformation changed the scaling of the data without affecting its variation, and thus allowed us to obtain a normalized index, which monotonically increased with increasing hemolymph supply to the muscle. Repeated measurements for ROIs obtained from the same oyster were averaged within each experimental temperature, and the means were used for statistical analyses to avoid pseudoreplication.

**Statistics.** Statistical analysis was performed using ANOVA (SigmaStat or InStat, SAS). Differences between tissue metabolite levels were tested by paired (open/closed oysters) and unpaired (control/Cd-exposed oysters) t-test, respectively. Effects of temperature and cadmium exposure on experimental end points were tested with repeated-measures ANOVA (with individual oyster used as a repeated-measures variable) followed by post hoc procedures [Tukey’s honestly significant difference (HSD) test for unequal N]. Factor effects and differences between the means were considered significant if the probability of Type II error were less than 0.05. Data are presented as means ± SE.

**RESULTS**

Metabolism was strongly affected by open/closed status of the oysters as shown by the alterations in the levels of muscle phosphagen (phospho-1-arginine) and a product of high-energy phosphate hydrolysis, inorganic phosphate (Pi), measured in control and Cd-exposed oysters at 20°C (Fig. 2). Compared with open oysters, closed animals had significantly elevated levels of inorganic phosphate (Pi) correlating with significantly lower levels of phospho-1-arginine (PLA) (P < 0.001 for both parameters). Intracellular pH was decreased by =0.3 units (data not shown). Cadmium exposure significantly affected energy metabolism in oysters as indicated by significantly lower PLA levels in open Cd-exposed oysters compared with the respective controls (P < 0.01). Differences in inorganic phosphate and intracellular pH values between control and Cd-exposed oysters were not significant (P > 0.05).

Temperature significantly affected oxygen consumption rate (MO2) in oysters (ANOVA: F2,27 = 23.95, P < 0.001), with MO2 increasing with rising temperatures (Q10 = 1.8–2.0 between 20 and 28°C; Fig. 3). Oxygen consumption rates were significantly higher in Cd-exposed oysters than in their control counterparts (ANOVA: F1,14 = 6.16, P = 0.026), and there was no significant interaction between the effects of temperature and Cd exposure on MO2 (ANOVA: F2,27 = 1.46, P = 0.25).

Both temperature and Cd exposure had significant effects on hemolymph oxygen partial pressure (PO2) in oysters (ANOVA: F2,27 = 18.31, P < 0.0001 and F1,16 = 9.63, P = 0.0007, respectively), whereas the interaction between these two factors was not significant (ANOVA: F2,27 = 1.38, P = 0.27; Fig. 4A). During the acute temperature rise (20 to 28°C) hemolymph PO2 decreased significantly from 13.8 ± 0.4 to 11.1 ± 0.6 kPa in controls (P < 0.01) and from 12.4 ± 0.7 to 7.6 ± 1.3 kPa in Cd-exposed oysters (P < 0.05). In contrast to control animals in which hemolymph PO2 leveled off at 24°C...
showing a decrease of ~20% compared with 20°C, hemolymph PO2 continued to decrease with increasing temperatures in Cd-exposed oysters and dropped by ~30% and 40% at 24°C and 28°C, respectively (Fig. 4A). Hemolymph PO2 was lower in Cd-exposed animals at all temperatures; this difference became significant at 28°C (P < 0.05) compared with the respective controls.

Comparison of heart rate traces, video recordings, and observer notes showed that both heart beat rate and amplitude dropped considerably when oysters closed. In most cases, there was no detectable heartbeat after just a few minutes of closure (data not shown). Therefore, in all future analyses, only traces for open oysters with actively beating hearts were analyzed. As expected, an acute rise in water temperature resulted in a significant increase of heart rate in oysters (ANOVA: \( F_{2,17} = 132.88, P < 0.0001 \), Fig. 4B). The temperature-dependent rise in heart rate was steeper when temperature increased from 24 to 28°C (\( Q_{10} = 3.2–3.4 \)) than when it rose from 20 to 24°C (\( Q_{10} = 1.8–2.3 \)). There was no difference in heart rate between control and Cd-exposed oysters (ANOVA: \( F_{1,10} = 0.15, P = 0.70 \) for the effect of Cd exposure), and no significant interactions were found between the effects of temperature and Cd exposure (ANOVA: \( F_{2,17} = 2.23, P = 0.14 \)).

Flow-weighted MR imaging showed that a temperature rise from 20 to 28°C led to a significant increase in cardiac flow by a factor of ~1.2 as indicated by elevated signal intensity for atrial and ventricular flow (Fig. 5). Although the magnitude of increase was similar for atrial and ventricular flow, it was only significant for the former (ANOVA: \( F_{1,6} = 7.36, P = 0.03 \) and \( F_{1,6} = 2.71, P = 0.15 \), respectively). In contrast, cadmium exposure had no effect on cardiac flow in oysters (ANOVA: \( F_{1,6} < 0.001, P = 0.98 \) and 0.97, for ventricular and atrial flow, respectively), which goes hand in hand with the similar heart rates in control and Cd-exposed oysters.

During the periods when the heart was not beating, the hemolymph supply index (HSI) in both catch and fast muscles was low and increased in open animals with actively beating hearts (cf. Fig. 1, B and C). A temperature increment from 20 to 28°C resulted in elevated hemolymph supply to both muscle types in open oysters; however, this increase was statistically significant in the catch muscle only (Fig. 6, A and B; ANOVA: \( F_{1,6} = 5.86, P = 0.05 \) and \( F_{1,6} = 3.59, P = 0.11 \) for the effects of temperature in catch and fast muscles, respectively; differences between 20°C and 28°C were significant by post hoc tests in Cd-exposed oysters only, \( P < 0.05 \)). The differences between control and Cd-exposed oysters were not statistically significant (ANOVA: \( F_{1,6} = 0.69, P = 0.44 \) and \( F_{1,6} = 1.28, P = 0.30 \)).
dependency observed between the two groups \((Q_{10} \approx 2)\). Oxygen consumption rates in this study \((47.9 \pm 4.5 \mu\text{mol} \ O_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1} \cdot \text{d}^{-1} \text{ for control and Cd-exposed oysters, respectively})\) were within the wide range of values reported for \(C. \ virginica\) acclimated at 20–22°C \((11–92 \mu\text{mol} \ O_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1} \cdot \text{d}^{-1} ; 47, 55)\), although somewhat higher than \(MO_2\) rates recorded in our earlier study \((30.0 \pm 6.3 \mu\text{mol} \ O_2 \cdot \text{h}^{-1} \cdot \text{g}^{-1} \cdot \text{d}^{-1} \text{ for control and Cd-exposed oysters, respectively; 33})\). Although we observed slightly different \(MO_2\) values likely due to the different seasons when experiments were performed, cadmium exposure \((20 \text{ days at } 50 \mu\text{g} \text{ Cd}^{2+}/l)\) resulted in a similar magnitude of \(MO_2\) increase \((\text{by } \sim 50\%)\), indicating a common metabolic response to cadmium.

Recorded hemolymph oxygen partial pressures \((P_{O_2})\) of 12–14 kPa in \(C. \ virginica\) under resting conditions were in the range of earlier published data for oysters and other marine invertebrates: \(\sim 125 \text{ mmHg } (=16.6 \text{ kPa})\) for Pacific oyster, \(C. \ gigas\) at 12.5°C \((30)\), \(\sim 85 \text{ mmHg } (=11.3 \text{ kPa})\) for \(C. \ gigas\) at 16°C \((31)\) and \(\sim 92 \text{ mmHg } (=12 \text{ kPa})\) for spider crab, \(Maja \ squinado\) at 10/12°C \((24)\). Hemolymph \(P_{O_2}\) did not differ significantly between control and Cd-exposed animals at 20°C, indicating that the observed Cd-induced rise in energy demand was more or less balanced by oxygen supply mechanisms at this temperature. The situation changed considerably during acute warming. Although hemolymph \(P_{O_2}\) decreased in both control and Cd-exposed groups, the drop was much stronger in cadmium-exposed oysters, resulting in significantly lower values compared with controls at 28°C. The decline in hemolymph \(P_{O_2}\) suggests earlier onset of hypoxemia in tissues of Cd-exposed oysters at elevated temperatures, which, in turn, could trigger a progressive shift from aerobic to anaerobic metabolic pathways and elevated levels of anaerobic end-products. Indeed, we observed a proportional increase in succinate levels in gill tissue of Cd-exposed oysters compared with the respective controls at 28°C but not at 20°C, 210 ± 78% \(\text{compared with } 82 \pm 27\% \text{ (means } \pm \text{ SE; G. Lannig and I. M. Sokolova, unpublished data.})\). Considering decreased energy reserves \((\text{PLA levels})\) in muscle tissue of Cd-exposed oysters compared with the controls \((\text{which was already observed at } 20^\circ\text{C})\), warming may accelerate cadmium-dependent deficiency of high-energy phosphates. This putative severe impact on cellular energy resources at high temperatures may result in an earlier demand for additional energy production, for example, by use of anaerobiosis. This hypothesis, however, needs to be rigorously tested by future studies in which levels of PLA and anaerobic end products are measured at different temperatures and the period that oysters spend open is carefully monitored to unequivocally confirm the earlier onset of anaerobiosis during cadmium exposure at elevated temperatures. Although tissue samples from control and cadmium groups used for succinate determinations in this study comprised similar numbers of open and closed oysters at the time of sampling \((3:3 \text{ and } 4:2 \text{ open:closed at } 20^\circ\text{C and } 28^\circ\text{C, respectively})\), the previous history of opening/closure during cadmium and temperature exposure has not been tracked in sufficient detail. As shown by Michaelidis et al. \((38)\) during air exposure, closed oysters \((C. \ gigas)\) depend on anaerobic metabolism and their tissue succinate levels are significantly elevated after 4 h in air.

**DISCUSSION**

As expected, partial anaerobiosis was found in closed oysters, indicated by the depletion of steady-state phospho-L-arginine \((\text{PLA levels})\), an increase in inorganic phosphate levels, and a decrease in intracellular \(pH\) values compared with open oysters. An effect of cadmium exposure was detectable in both control and Cd-exposed groups, the drop was much stronger in cadmium-exposed oysters, resulting in significantly lower values compared with controls at 28°C. The decline in hemolymph \(P_{O_2}\) suggests earlier onset of hypoxemia in tissues of Cd-exposed oysters at elevated temperatures, which, in turn, could trigger a progressive shift from aerobic to anaerobic metabolic pathways and elevated levels of anaerobic end-products. Indeed, we observed a proportional increase in succinate levels in gill tissue of Cd-exposed oysters compared with the respective controls at 28°C but not at 20°C, 210 ± 78% \(\text{compared with } 82 \pm 27\% \text{ (means } \pm \text{ SE; G. Lannig and I. M. Sokolova, unpublished data.})\). Considering decreased energy reserves \((\text{PLA levels})\) in muscle tissue of Cd-exposed oysters compared with the controls \((\text{which was already observed at } 20^\circ\text{C})\), warming may accelerate cadmium-dependent deficiency of high-energy phosphates. This putative severe impact on cellular energy resources at high temperatures may result in an earlier demand for additional energy production, for example, by use of anaerobiosis. This hypothesis, however, needs to be rigorously tested by future studies in which levels of PLA and anaerobic end products are measured at different temperatures and the period that oysters spend open is carefully monitored to unequivocally confirm the earlier onset of anaerobiosis during cadmium exposure at elevated temperatures. Although tissue samples from control and cadmium groups used for succinate determinations in this study comprised similar numbers of open and closed oysters at the time of sampling \((3:3 \text{ and } 4:2 \text{ open:closed at } 20^\circ\text{C and } 28^\circ\text{C, respectively})\), the previous history of opening/closure during cadmium and temperature exposure has not been tracked in sufficient detail. As shown by Michaelidis et al. \((38)\) during air exposure, closed oysters \((C. \ gigas)\) depend on anaerobic metabolism and their tissue succinate levels are significantly elevated after 4 h in air.

**Fig. 6.** Changes in hemolymph supply index (HSI) in fast (A) and catch (B) muscle of control and cadmium-exposed \((20 \text{ days at } 50 \mu\text{g} \text{ Cd}^{2+}/l)\) oysters, \(C. \ virginica\) during acute warming \((4^\circ\text{C/48 h})\). HSI was normalized so that in control closed oysters \(\text{HSI} = 0.\) Significant difference from the respective data at 20°C. Data are presented for open oysters only and are means ± SE; \(n = 5 \text{ (Control) and } n = 4 \text{ (Cadmium).}

\(P = 0.30\) for Cd effects in catch and fast muscle, respectively. Factor interactions were not significant for cardiac flow or HSI \((P > 0.10 \text{ in all cases}).\)
Interestingly, cadmium showed no direct effect on temperature-dependent changes in circulatory performance. The temperature-induced increase in heart rate of both control and Cd-exposed oysters was well within the range observed in other studies on oysters, with reported Q_{10} values of ~3.6 (ΔT = 13.5°C) for C. virginica (23) and ~2.1 (ΔT = 8.5°C) for Isognomon alatus (52). In contrast to the temperature effect, effects of trace metals on heart rate are not uniform, indicating species- and/or metal-specific responses. De Pirro and Marshall (19) observed no change in heart rate in copper-exposed patellogastropod limpets but a copper-induced depression in heart rate in Siphonaria limpets. Bradycardia, as well as tachycardia, was observed in copper-exposed marine crustaceans and mollusks (12, 17, 36, and references therein). Also for cadmium exposure, opposing effects on heart rate were reported. Schuwerack et al. (45) observed an increase in heart rate after 21 days of cadmium exposure in the freshwater crab, Potamonautes warreni, whereas no change in heart rate was observed for cadmium-exposed oysters (this study) or zebrafish embryos (26). The observation that the hemolymph supply index tended to be higher (although not significantly so) in Cd-exposed oysters compared with controls might indicate elevated hemolymph supply, independent of heart rate frequency. A dilation of hemolymph lacunae to improve tissue perfusion or an increase of free water by metal-induced edema [similar to that hypothesized by Brouwer et al. (10) in the blue crab, Callinectes sapidus] may be possible but is not detectable by our MR measurements due to high interindividual variation.

Earlier investigations on the synergistic effects of high temperature and cadmium on mitochondria revealed impaired mitochondrial capacities due to the combined effects of these stressors (13, 14, 21, 49). Our previous studies showed that cadmium interferes with mitochondrial aerobic capacities in oysters resulting in reduced ATP synthesis and decreased efficiency due to elevated uncoupling and that the cadmium-induced impairment of mitochondria is strongly exacerbated during acute warming (48). At control temperature (20°C), effects of Cd exposure on intrinsic rates of mitochondrial oxidation were negligible, whereas at 28°C, a rapid and pronounced decrease of mitochondrial oxidative capacity was found in Cd-exposed oysters (14). Cadmium exposure also resulted in a decline in mitochondrial abundance in oyster tissues (13), causing a decrease in tissue aerobic capacities. Therefore, one may expect Cd-induced impairment in the function of active aerobic tissues (such as heart), which would result in a reduced scope for cardiac performance.

On the other hand, decreased temperature-dependent oxygen saturation of body fluids via ventilatory performance and/or oxygen carrying capacities may contribute to the observed hypoxemia at high temperatures. Since oysters lack respiratory pigments such as hemoglobin or hemocyanin, the observed Cd-induced effect on acid-base status and ion regulation by affecting the activity of carbonic anhydrase (28, 56), does not imply an immediate effect on oxygen supply. Oxygen-carrying characteristics of oyster hemolymph depend on physical solution alone, and thus, the Cd-induced hypoxemia at high temperatures seems to be linked to decreased oxygen uptake capacity at the gills, combined with the capacity limitation of cardiac circulation. Gills are expected to be particularly sensitive to exposure to waterborne metals because the fact that they have a large surface area to facilitate gas and ion exchange and represent a major site of metal uptake in aquatic animals. Moreover, the tissue-specific variation in cellular protection capacities may contribute to the gills’ sensitivity to metals (5). In this context, a recent study on C. virginica indicates that gills are slower in producing protective proteins, metallothioneins, in response to cadmium exposure compared with other tissues (such as hepatopancreas) and that cadmium exposure results in damage to the cellular proteins of gill cells, as indicated by an early upregulation of heat shock proteins already after 4 h of cadmium exposure (29). Most importantly, respiratory stress in trace metal-exposed animals due to structural gill damage, cell necrosis, and enhanced mucus production has been described in numerous earlier studies (2, 34, 50, 51, and references therein). In conjunction with cadmium-induced impairment of mitochondrial capacities, this is likely to make the gills highly susceptible to metal-induced injury, which goes hand in hand with reduced ventilatory performance, especially at elevated temperatures when mitochondrial damage is strong, oxygen demand is high and oxygen solubility in the water is reduced.

To conclude, the synergistic effects of high temperature and cadmium on aerobic metabolism of oyster caused hypoxemia as a first sign of thermal stress thereby narrowing the thermal tolerance window of the animals. According to the concept of oxygen- and capacity-limited thermal tolerance in marine ectotherms (see 40, 42), which in invertebrates is linked to ventilatory and circulatory limitation (24, 41), pollution exacerbates the deleterious impact of increasing temperature on aerobic metabolism by impairing oxygen uptake and distribution and concomitantly increasing energy demand. The associated downward shift of the upper critical temperature indicates that climate change will expose animals in contaminated areas to a higher risk than their conspecifics in unpolluted areas.

**Perspectives and Significance**

In the context of imminent global climate change, the investigation of interactive effects of temperature and other environmental stressors on animal bioenergetics represents an important issue in current and future ecophysiological research. The synergism between stressors similar to that described in this study is not specific to metals and can be extrapolated to other pollutants or factors that affect respiratory gas exchange and/or inhibit cellular energy production. As a result, stress tolerance to environmental parameters such as hypoxia or high temperatures will be reduced in animals inhabiting polluted areas of intertidal and coastal regions. In the future, studies of such interactive effects should take into account the genetic background of the organisms, as well as differences in sensitivity between developmental stages and animals with different physiological (e.g., reproductive or disease) status. Understanding of the physiological mechanisms of aerobic limitation in response to combined temperature and pollution stress could provide a framework for improved toxicokinetic models of the effects of these stressors, assist in determining factors that set limits to species distribution in polluted environments, and ultimately lead to better strategies of environmental risk assessment in the face of global climate change.
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