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Short Communication

An examination of relationships between air-time survival, dehydration rate and morphology in bivalves: implications for climate changes and pollution

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ABSTRACT

Quick and cheap biomarkers to evaluate water quality are urgently needed to accommodate remote and low-budget laboratories. Recently, three simple and cheap biomarkers were proposed for water quality assessments using two morphological biomarkers (condition factor-CF and growth index-GI) and a tolerance test to air emersion. A multiple regression model was used to find predictors of air survival in wild bivalve populations. The data revealed that the air lethal time (LT) was negatively related with weight loss (WL) at the 7th day of exposure to air. This suggests that WL >25% at the 7th day predicts LT below the natural variation range. The relationship was significant with the CF, GI and air temperature indicating that larger animals with sustained growth and in warmer air temperature resist less time to air exposure. Moreover, the relationship was also significantly related to oxidative stress (lipid peroxidation) and decreased temperature dependence of mitochondria activity. For intertidal *Mya arenaria* clams, a temperature of 28.4 ºC could harm clams between tides (LT=0.5 day) which is in agreement to the reported maximal temperature (28.7 ºC) of the southern limit of clam distribution in North America coast. This relationship was statistically stronger at polluted sites than in pristine sites. In conclusion, this study permits to determine the critical range of air exposure time and temperature range during drought and strong temperature changes caused by global warming at contaminated sites.

KEYWORDS: air temperature, lethal time, dehydration rate, growth, metabolic activity, oxidative stress.

INTRODUCTION

Bivalves are important component of the benthic community and often considered as sentinel species to determine the anthropogenic impacts at local and regional scales of pollution and global warming [1, 2]. Indeed, these mollusks thrive at the water-sediment interface, a nexus for various reservoirs of contaminants, live for relatively-long periods (2 to hundreds of years as with the artic clam *Artica islandica*) and feed on the suspended matter [3]. It is not surprising that many species of this group of invertebrates are compromised leading to risk of extinctions [4]. Intense fishing of the resource as a food source could also bring about this problem for the other more abundant species. In addition, the sustainability of bivalve populations are compromised by global warming, exposing coastal populations to more extremes such as increased temperatures (high temperature peaks) and water quantity/quality (increased precipitations and release of untreated wastewaters) events [5, 6]. Ecotoxicological studies are usually on the most abundant species (not a risk of extinction) so that data produced on one species may not protect other more sensitive species [7]. The use of physiological or allometric descriptors to determine impacts across species would be of value. Hence, studies

dealing with the most abundant species are not guaranteed to protect the biodiversity of bivalves.

In a recent study, three simple biomarkers were proposed to assess water quality problems [1]. These biomarkers were significantly associated to biomarkers of toxicity at the molecular level such as xenobiotic biotransformation, lipid peroxidation (LPO), genotoxicity, altered mitochondrial respiration activity and inflammation. These biomarkers were two morphological (condition factor-CF: clam weight/shell length; growth index (GI): shell length/age) and functional (tolerance to air and loss of water in air) endpoints. These tests are simple, quick and inexpensive to perform, requiring only an analytical balance and a ruler. The air tolerance test determines the air lethal time (LT) and dehydration rates or weight loss (WL) in time. This test is so-called the stress on stress (SOS) response and has been proven a sensitive and simple means to determine the general health status in both freshwater and marine bivalves under various environmental stress such as pollution, loss of habitat from water level changes and heat waves. For example, SOS response has identified problems from marina/harbors [8, 9], oil sand extraction sites [10] and miscellaneous contaminants [11, 12]. In the context of global warming, increased temperature, heavy rainfall events and rises in coastal water levels are already observed [5, 12, 13]. In the context of climate changes, the air temperatures will reach over maximal values more often and perhaps by longer periods thereby compromising the survival of organisms especially at the geographical limit of species distribution. On the one hand, drought could increase in scales with longer periods of high temperature, thereby exposing local bivalves for longer period of high temperatures depending on the river/lake hydrological properties [5]. On the other hand, the increase in CO_2 will contribute to acidification from CO_2 dissolution in water and increase air temperature leading to rises in water levels from polar snow melts and rainfalls. Moreover, higher water levels (coast) and intense precipitations could overwhelm water and wastewater management systems (sewers and wastewater treatment works) [13]. Indeed, the release of rainfall overflows is accompanied with the release of untreated raw wastewaters, which could increase the problem of pollution from various contaminants and microorganisms [14, 15]. This will bring about a highly complex interplay between increase in air temperature, droughts and inputs of pollutants in coastal systems where sessile endobenthic organisms thrive. The ability of bivalves to occupy a given habitat is dictated by both external factors (habitat, substrate and food availability) and intrinsic physiological properties such as metabolic activity and assimilation of energy from carbohydrates, lipids and proteins for biomass [7]. Allometric considerations linking mass or size with physiological traits would prove helpful to derive physiological and morphological traits that spans across the boundary of species [16]. Hence, there is need for cost-effective biomarkers that could integrate these interactions in respect to limitations for remote and/or low-budget laboratories.

The purpose of this study was therefore to determine predictors of the capacity of the clam *Mya arenaria* to survive air exposure such as initial weight loss by dehydration, condition factor, growth index and air temperature in the attempt to predict bivalve mortality during periods of drought taking into account pollution and air temperature in these times of global warming. The influence of pollution was taken into account by measuring oxidative damage (lipid peroxidation, age-related pigments) and temperature-dependent mitochondria energy expenses. These relationships were also examined across species based on previously published studies in the attempt to highlight physiological predictors that spans different bivalve species of both marine and freshwater origin.

METHODS

Mya arenaria clams were collected from three sites in the Saint-Lawrence Estuary at the confluence of the Saguenay fjord and one site upstream the fjord on the Eastern part of Canada (Québec, Canada). The sites Baie du Moulin à Baude (48° 09’ 15’’ N; 69° 39’ 52’’ W) at the estuary site and Baie Sainte-Marguerite (48° 15’ 15’’ N; 69° 58’ 14’’ W) in the Saguenay fjord were selected as the ‘reference’ sites since they are not direct source of pollution. This site is visited regularly by Beluga whales and feed on local bed clams. Although this site does not have any harbors, the site receives the Saint-Marguerite River and faces a seaway

passage for large commercial boat traffic. The sites of Tadoussac (48° 06' 30'' N; 69° 43' 39'' W) and Baie Sainte-Catherine (48° 06' 30'' N; 69° 43' 39'' W) were selected as the contaminated sites because they support harbor activities such as personal and commercial boating activities (including site-seeing and whale-watching activities). Clams (n=50) were collected during the morning's low tide during the last week of September (according to the schedule of tides) in 2018, 2019 and 2020. They were transported back in cooled containers to a local laboratory hub for morphological measurements and estimation of air time survival. A subsample (n=12) of clams was frozen first at -20 ºC for ease of transport (no longer than one week) followed by freezing at -85 ºC for biomarker analysis in the main laboratory.

Morphological analysis consisted in evaluation of wet weight, maximal shell length and age as previously described [1]. Age was estimated by the number of major grooves on shells. The condition factor (CF: total weight/shell length) and the growth index (GI: shell length/age) were determined. The capacity of bivalves to survive air exposure (lethal time: LT) was determined. Briefly, a total number of 15 organisms were placed in plastic containers and incubated at 12 ºC, 14 ºC, 15 ºC and 23 ºC (for the year 2018) under 80% humidity to determine the air LT at different temperatures. Bivalves were weighted each day to monitor the dehydration rates or the weight loss (WL). The WL was calculated by the following formula: (weight at t-initial weight)/(initial weight) for each individual. The lethal time (LT) was determined as the day of death as evidenced by opened shells and loss of shell closure upon stimulation. The data were expressed as the LT (days) for each individual.

Physiological markers of health and pollution were determined by measuring lipid peroxidation (LPO), temperature-dependent mitochondria electron transport activity (MET_T) and the accumulation of age pigments (AP) in soft tissues of clams and freshwater mussels as described by André *et al.* [7]. Briefly, *Mya arenaria* clams (n=12) were thawed on ice and dissected for the visceral mass (without the foot and gills). The tissues were weighed and immediately mixed in 250 mM sucrose containing 25 mM Hepes-NaOH, 1 mM dithiothreitol, 1 mM ethylenediamine tetracetate (EDTA) and 1 µg/mL apoprotinin at pH 7.4. The tissues were homogenized using a Teflon pestle tissue homogenizer (5 passes) and a 1 mL sample of the homogenate was centrifuged at 1500 × g for 15 min at 4 °C and the supernatant centrifuged at 9000 × g for 20 min at 2–4 °C. The supernatant was carefully removed from the pellet containing the crude mitochondria fraction and resuspended in 0.5 mL of the homogenization buffer. Total proteins were determined using the Coomassie blue dye as previously described using standard solutions of bovine serum albumin for calibration [17]. Mitochondrial electron transport (MET) activity was determined by spectrophotometry as previously described [18]. Briefly, the crude mitochondria fraction was mixed with 0.1% triton X-100, 5% polyvinylpyrrolidone, 1 mM NADH, 0.2 mM NADPH and 1 mM p-iodonitrotetrazolium and the absorbance read at 520 nm for each 2 min interval for 30 min. The rate of reduction of the dye was determined at 20 ºC and 4 ºC. In order to determine sensitivity to temperature changes, MET activity was determined at 4 °C and at 20 °C and the temperature-dependent MET (MET_T) was calculated by the following equation: MET_{20oC}-MET_{4oC} / 16 ºC. MET activity was expressed as the increase in absorbance (dye reduction)/min/mg proteins. The levels of LPO were determined in the homogenate fraction using the thiobarbituric acid reactants (TBARS) methodology using fluorescence at 540 nm excitation and 590 nm emission [19]. The data were expressed as µg TBARS/mg proteins using standards of malonaldehyde (tetramethoxypropane) for calibration. Another fraction of the homogenate was mixed with 2 volumes of ethanol and incubated at 4 ºC for 12 hours for the extraction of age-related pigments (AP) as previously described [20]. The mixture was centrifuged at 3000 x g for 5 min and the supernatant was diluted 1/10 with ethanol and fluorescence readings were taken at 360 nm excitation and 460 nm emission using a portable fluorometer (TBS-380, Turner Designs, CA, USA). The instrument was calibrated with an external standard of 10 µM quinine sulfate internal standard at 1000 fluorescent units.

The data were obtained from n=15 clams for morphometric analysis (CF and GI), air-time

survival (LT, WL) and physiological biomarkers (MET_T, LPO and AP) for each of the 4 sites for 3 three consecutive years (2017, 18, 19) making a data set of 180 points. For *Mya arenaria* clams, the regression model was produced using the best fit algorithm from the software package. The best fit relationships between LT and the WL at each day before time of death and the other endpoints were examined using multiple regression analysis to determine the best predictors of air LT (days). Outliers were determined by the Student's t-test for residuals and were removed for the regression model. Significance was set at $p<0.01$ using the Statsoft software package (version 13, USA).

RESULTS AND DISCUSSION

The normal (natural) range of the LT to air emersion was between 11-14 days (25-75% centiles) and was significantly related ($r=0.41$; $p<0.001$) to the weight loss at the 7th day (WLd7) (Table 1). The WL for the other days were not significantly related to LT ($p>0.05$). This relationship provides a useful predictor to determine whether molluscs become more sensitive to air exposure in polluted area or during prolonged exposure to air. For example, in clams from the reference site Baude, the lower limit of air LT was LT=11 days for the 25th centile, and the WLd7 was estimated at 26% loss of body weight. The WL was less significant at the 6th day ($r=0.25$; $p=0.03$). Hence, a WLd7 ≥ 26% would decrease the air LT outside the natural variation range for these populations (i.e. <11 days). Based on a previous study with *Mya arenaria* clams, the WL values varied between 10-34% at natural sites under no pollution pressures reaching 48% at polluted sites [21]. This indicates that the LT could decrease to 9.5 and 7.2 days, respectively in these more extreme cases.

The air LT was also significantly related to the CF and the GI (Table 1). In clams from the reference sites, these variables were more strongly related to LT than with the WLd7. The contribution of the WLd7 increased when polluted or stressed sites were included in the regression model. Taking into account the CF and GI in the regression model, the WL at LT≤11 was estimated at 0.35 with a CF and GI values of small clams and slowly growing clams (25th centile values). The WL was lowered at 0.22 with CF and GI values in larger and rapidly growing clams (75th centile values). This suggests that heavier and growing clams survive less time in air below the natural variation range when the WLd7 reaches 22%. At the more extreme case, i.e., considering the 95% centiles for CF, GI and WL (0.48), the LT could decrease to 6.9 days. In addition, the air temperature displayed the strongest correlation with the LT ($r=0.86$; $p<0.001$). The lower natural range of LT <11 (corresponding to the 25% centile) with a normal (mean) WL at d6 of 0.21 would give a critical air temperature of 12.8 ºC. This suggests that air temperatures above this value will reduce the air survival time below the natural range of LT in *Mya arenaria* clams. However, this has no

Table 1. Regression analysis models for the prediction of air LT and dehydration rates of clams.

LT vs	**r; p**	**Model**
Marine: *Mya arenaria*		
WLd7	0.35; p<0.001	LT(days)=15.2 -16.4(WLd7)
with GI	0.4; p=0.03	LT(days)=19.3 -13.4(WLd7)-0.55(GI)
with CF	0.4; p=0.01	LT(days)=18.2-13.7(WLd7) -6.5(CF)
with air temperature	0.86; p<0.001	LT(days)=19.6 -(4.3WLd6)-(0.6 air Temp)
Freshwater: *Elliptio* and *Pyganodon sp*		
Wld7	0.71; p=0.03	LT(days)15.5 – 21 (WLd7)
with GI	nd	nd
with CF	0.63; p=0.05	LT(days)=18.5-29.7(WLd7)-3.1(CF)

bearing to intertidal clams since they are usually exposed to no more than 0.5 day between tides. This could have some significance in situation when bivalves would be exposed to drought events spanning many days or weeks provided that these relationships hold for other species. For *Mya arenaria* clams, the estimated temperature required to reduce LT of 0.5 days with a WLd6=0.48 at the upper range (95% centile) [22] was estimated at 28.4 ºC according to a previous survey. This suggests that temperatures above this value could compromise the survival of clam populations. The measured air temperature changes for this sector (Tadoussac, Québec, Canda) ranged from -14 to +20.3 ºC (July) for the years 1991-2021 (https://fr.climate-data.org/amerique-du-nord/canada/quebec/tadoussac-206783/). In the context of global warming, this provides some degree of protection i.e., the maximal temperature is some 8 ºC below the air LT of 0.5 days for intertidal clam beds for this area. The geographical distribution of *Mya arenaria* clams on Northern hemisphere of the Atlantic starts from Virginia of USA (mid-USA) up north to NewFoundland/Labrador and the Saint-Lawrence Estuary of Canada (https://www.sealifebase.se/summary/Mya-arenaria.html). The reported temperature range of the southern range (Virginia, USA) spanned from 23.5 to 28.7 ºC during 1991-2019. This is consistent with the estimated critical temperature for LT of 0.5 days of 28.4 ºC where this clam species would not survive at sites south of Virginia where the maximum temperature would be higher.

We examined the relationship of the LT with the WLd7, CF and GI in freshwater species of similar size to *Mya arenaria* (5-9.5 cm shell length) and confirmed the significant relationship between the LT and WLd7 for *Elliptio* and *Pyganodon sp.* (Table 2). The same was found with smaller (1-3 cm) freshwater mussels (*Dreissena bugensis*). However, the significant relationship between LT and WL occurred at day 3 (WLd3) and day 5 (WLd5) corresponding to 42 and 71% of the mean LT for *Dreissena* sp. The relationships became more significant when the CF was included in the model suggesting that high CF (and mass) contributes to decreased LT. Allometry is the study of scaling relationships between body size or other morphological traits and functional mechanisms such as metabolic rate or production of reactive oxygen species during respiration [23]. An allometric relationship between log mass and the LT was also obtained by considering 6 different species of freshwater and marine bivalves (r=0.65), suggesting that mass (or CF) is an important endpoint. In general, metabolic rates (mitochondria or cellular respiration) are related to the log mass values spanning many orders of magnitudes ranging from yeasts to elephants and whales [23]. This suggests that rate of living or metabolism could also be associated to the air LT across the species horizon. The air LT was previously correlated not only with mass but with mitochondria LPO, shell length (size) and longevity as well [7]. In the present study, the LT was significantly correlated with WLd7 when LPO and MET_T were included in the regression model where high oxidative stress (from pollution) and reduced MET_T decrease the air LT, i.e. the capacity of clams to resist air exposure (Table 3). A LT drop below the natural variation range (<11days or the 25th centile), increased the

Table 2. Regression analysis with lower-sized mussels.

LT vs	r; p	Model
WLd3	0.61; p<0.001	LT=6.5 –(12.8WLd3)
with CF	0.64; <0,001	LT=8.4-(2.2CF)-(12.7WLd3)
WLd5	0.68; <0.001	LT=7.6-(8.7WLd5)
with CF	0.71; <0.001	LT=7.8-(0.14CF)-(9.2WLd5)
log mass[1]	0.65;<0.001	LT=2.7+(6.7 log mass)

[1]*Elliptio* (1: *complanata*), Pyganodon (2: *grandis*, *undetermined*), *Lasmigona* (*costata*), *Eurynia dilatata*, *Dresseina bugensis* (from André *et al*., 2019) and *Mya arenaria* (this study).

Table 3. Relationship of air LT with pollution biomarkers.

LT vs WLd7	r	Model
	0.51; 0.03	LT=15-(21*WLd7)
with LPO	0.63; 0.04	LT=19.2-(21*WLd7)- (57*LPO[1])
with MET	r=0.2; p>0.1	NS
with MET_T	0.65; 0.04	LT=17.5-(22.7*WLd7)-(63.2*LPO)+(6.4*METT).
with ARP	0.8; <0.001	LT=2.4-(3.4*CF)+(1.1*GI)+ (0.1AP)-(0.06*WLd7)

[1]Expressed as µg TBARS/mg proteins.

LPO levels at 0.053 µgTBARS/mg proteins compared to 0.03 in natural conditions (reference site) corresponding to 1.8 fold induction. Since LPO is increased by various pollutants such as metals, cyclic aliphatic and aromatic hydrocarbons, oxidative stress is one of the paths from which pollution and temperature contribute to reduced survival in bivalves [24, 25, 26]. The air LT was reduced to 5.6 days for more extreme situation i.e., if we consider the 95th centile for LPO and 5th centile for MET_T activity. Thus, high oxidative stress (LPO) and loss of temperature dependence in mitochondria activity both contribute to reduced resistance to air emersion below the natural variation range (< 25th centile). Given that most of the production of reactive oxygen species occurs in mitochondria [27], the inability to adapt to temperature changes could contribute to oxidative stress leading to LPO. It seems that MET activity remains higher at both low and high temperatures but lower temperature dependence of MET contributes to sustained oxidative damage and to the formation of AP or lipofuscin [20]. AP consist of oxidized protein/lipids amalgam that cells cannot eliminate and are considered biomarker of physiological age. In the present study, the air LT was positively related to AP and negatively with WLd7 ($r=0.75$; $p<0.001$) suggesting that physiologically younger clams and more susceptible to WL could reduce the air LT. This suggests that in normal conditions (mean) the levels of AP could decrease from 32 fluorescence units/mg proteins in normal conditions to 3 (10-fold) fluorescence units/mg proteins in more extreme case (i.e., 75% centile of CF, GI and WLd7) corresponding to 25th centile of LT=11 days based on a previous study [21]. This suggests that the presence of AP/lipofuscin is associated to clam's resilience towards air exposure; however, the contribution (slope) was lower than the CF and GI. Indeed, the partial correlation of AP was at the limit of significance at $p=0.05$. Clams with low GI and high CF are the main drivers of reduced air LT in relation to WLd7 and AP. A possible explanation for this resides in that fast growing organisms have increased energy demands leading to oxidative stress not reaching yet the accumulation step of AP [28]. Indeed, growth is associated to insulin receptor pathway involved in energy metabolism in clams where increased glucose intake is needed for growth and respiration. The accumulation of AP can be prevented initially by the activation of autophagic processes to remove damaged (oxidized) proteins in cells [29]. Another possible explanation is that in large individuals (CF), the accumulation of AP reduces metabolic activity and growth in clams which have more influence in lowering resistance to air exposure than AP and WLd7. These interactions will require more in-depth mechanistic investigations at the biochemical level in future studies to better predict impacts of climate changes and pollution to resident bivalve populations.

CONCLUSION

This study examined the relationships between bivalve size, growth, resistance to air emersion and air temperature as quick and cost-effective means to highlight the negative effects of pollution in the context of climate changes. The WL observed at the 7th day in *Mya arenaria* and other species of similar mass significantly predicted mortality and this could be used as a non-destructive means to predict mortality in local populations.

The effect of pollution and temperature was modeled by including oxidative stress (LPO) and temperature-dependent mitochondria activity. These tests are easy to implement by laboratories of different sizes and capacity at any area.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest of any nature.

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