



Anthropogenic low-frequency sound effects on resting metabolism and energy pathways in two marine benthic crustaceans

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Abstract

Anthropogenic sound caused by ship traffic as well as the construction and operation of offshore windfarms have increased exponentially in the last decades. While its impact on marine life is relatively well studied for mammals and fish, the implications of anthropogenic sound on benthic invertebrates are poorly understood. Here, we tested for potential stress responses of common marine invertebrates using two widespread mesograzing crustaceans: the isopod *Idotea balthica* and the amphipod *Gammarus locusta*. All experimental animals were gathered from laboratory cultures in the facilities of the Alfred Wegener Institute in Bremerhaven, Germany, in spring 2023. Oxygen consumption rates and the activities of four key mitochondrial enzymes (cytochrome *c* oxidase, electron transport system complex I and III, citrate synthase and lactate dehydrogenase) were examined under the influence of added low-frequency sound (+25 dB SPL_{RMS} re 1 µPa at 90 Hz, above background soundscape) to assess how basal energy demands and supplies were affected. The isopod *I. balthica* seemed to be robust against added sound exposure over 72 h as neither oxygen consumption rates nor enzyme activities were significantly altered. The amphipod *G. locusta*, however, displayed significantly lower oxygen consumption rates in response to both short-term (1–4 h; 39% reduction) and longer-term (68–72 h; 35% reduction) added sound exposure, although enzymatic activities were not significantly affected. This study underlines the need to address the potential impact of sound on the energy available for the growth and reproduction of small invertebrates. Overlooked vulnerabilities to noise pollution in key taxa could have far reaching implications for marine food webs, nutrient cycles and ecosystem functioning.

Keywords Anthropogenic sound · Isopoda · *Idotea balthica* · Amphipoda · *Gammarus locusta* · Mitochondria

Introduction

Sound in the oceans includes not only natural sounds such as wave motion, seismic activity and animal communication, but also anthropogenic noise such as ship traffic, industry and construction (Duarte et al. 2021). Anthropogenic low-frequency sounds (characterised by frequencies between 10 to 500 Hz) have increased constantly over the last decades and radiate over great distances, resulting in its omnipresence throughout the oceans (Duarte et al. 2021; Hildebrand 2009; Jalkanen et al. 2022; Kyhn et al. 2014). Ship traffic and wind turbine operational noise currently dominate the underwater anthropogenic low-frequency soundscape (Duarte et al. 2021; Hildebrand 2009; Tougaard et al. 2020), with 90% of added sound attributable to the former (Mala-koff 2010). Sounds from ship traffic can reach spatial distances of up to 10,000 km and therefore surpass all other continuous sound sources. Offshore wind farms produce

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low-frequency noise during construction dredging, profiling and shipping (Nedwell & Howell 2004) and during operation mainly via gearbox vibrations (Pangerc et al. 2016).

Anthropogenic changes in acoustics can negatively impact the behaviour of organisms (Frisk 2012; Kunc et al. 2016; Leiva et al. 2021; Purser et al. 2016; Slabbekoorn 2010; Wang et al. 2022). Several studies showed that anthropogenic sounds can affect marine vertebrates (Maciel et al. 2023; Payne et al. 2015; Purser et al. 2016; Stone & Tasker 2006). For example, anthropogenic sound exposure can lead to disrupted feeding behaviour in grey seals (+17 dB SPL_{RMS} re 1 µPa at 250 Hz to 20 kHz; Hastie et al. 2021) and bottlenose whales (+64 dB SPL_{RMS} re 1 µPa at 1 to 4 kHz; Miller et al. 2022), reduced male courtship and female spawning in gobies (+24–31 dB SPL_{RMS} re 1 µPa at 100 Hz; de Jong et al. 2018) and reduced time for resting and nursing in pilot and humpback whales (+3–15 dB SPL_{RMS} re 1 µPa at 0.02 to 120 kHz; Arranz et al. 2021; +24–48 dB SPL_{RMS} re 1 µPa at 0.02 to 20 kHz; Sprogis et al. 2020). While highly mobile animal groups such as marine mammals and fish can avoid or minimise their exposure to anthropogenic sound by swimming away from the source, most benthic invertebrates cannot relocate to other sites on a short-term scale (Solan et al. 2016). Accordingly, different crustacean species were shown to react to anthropogenic low-frequency sounds by means of decreased antipredator behaviour and foraging (*Carcinus maenas*; +10–22.5 dB SPL_{RMS} re 1 µPa at 0 to 3000 Hz; Hubert et al. 2018; +20–44 dB SPL_{RMS} re 1 µPa at 0.2 to 3 kHz; Wale et al. 2013a), reduced burial depth and bioturbation (*Corophium volutator*; +10 dB SPL_{RMS} re 1 µPa at 100 Hz; Wang et al. 2022) as well as lower attention and disrupted decision-making processes (*Homarus gammarus*; +20 dB SPL_{RMS} re 1 µPa at 100 to 200 Hz; Leiva et al. 2021).

The integrity of cell machinery can also be sensitive to low-frequency sound exposure. Electron microscope scans of the inner statocyst structure of cephalopods revealed damaged mitochondria after 48 h of sound exposure (André et al. 2011; Solé et al. 2013). This damage can be irreversible and negatively impact sensory perception, reflexes and balance (Solé et al. 2023). Reductions in metabolism and alterations in gene expression were also observed in the bivalve *Tegillarca granosa* (Linnaeus, 1758) under anthropogenic sound exposure (Shi et al. 2019). The sea cucumber *Apostichopus japonicus* (Selenka, 1867) showed suppressed cellular apoptosis and protein synthesis as well as heightened oxidative damage after exposure to low-frequency noise simulating offshore wind turbines (Cheng et al. 2024). Similar results were seen in the blue mussel *Mytilus edulis* Linnaeus, 1758 with reduced oxygen consumption and filtration rate as well as significantly increased oxidative damage when exposed to shipping noise (Wale et al. 2019).

The mitochondrion is a compartment of the cell that plays a fundamental role in cellular aerobic metabolism. In aerobic organisms, the main way to produce energy in the form of ATP is via the electron respiratory chain within mitochondria. This involves five enzyme complexes associated with the inner mitochondrial membrane. The double membrane separates the cytosol from the matrix. Electrons are passed down from complex to complex and release energy along the way, enabling complexes I, III, and IV to pump protons from the matrix into the intermembrane space. This forms a membrane potential, impelling complex V to transport the protons back into the matrix which then powers its conversion of ADP to ATP. Complex II is also part of another mitochondrial metabolic pathway: the citric acid cycle. This cycle is initiated by citrate synthase (CS) and oxidates carbohydrates, proteins and lipids to energy and essential intermediates for the synthesis of glucose and amino acids. Finally, the anaerobic pathway is important in hypoxic and anoxic environments and therefore particularly relevant to organisms inhabiting tidal environments. When the maintenance costs increase and more ATP is needed, the anaerobic metabolic rate is enhanced, which is sometimes essential under stressful conditions (Agnew & Taylor 1985; Clarke & Fraser 2004). Lactate dehydrogenase (LDH) plays an important role in this pathway for gluconeogenesis and lactic fermentation (Krebs et al. 1967).

We hypothesised that the documented damages to mitochondria after 48 h of low-frequency sound exposure (e.g. André et al. 2011; Solé et al. 2013) likely mean irreversible effects on mitochondrial outputs and thus on energy demands under this stressor. A method to quantify energy trade-offs is measuring the oxygen consumption rates in individuals (Clarke & Fraser 2004; Hervant et al. 1997; Norin & Clark 2016). The resting metabolism, which indicates the minimum costs for maintenance, can be determined using respirometry (Svendsen et al. 2016). Therefore, oxygen consumption is a good indicator of the physiological condition of individuals, especially when combined with the metabolic enzyme activities in the mitochondria (Dahlhoff 2004).

The current study investigated the sex-specific short-term (1–4 h) and longer-term (68–72 h) effects of continuous low-frequency sound exposure on the resting metabolism and main enzymatic mitochondrial outputs of the isopod *Idotea balthica* (Pallas, 1772) and the amphipod *Gammarus locusta* (Linnaeus, 1758) via the activities of four different enzymes. Both species, widespread in the European seas, are common components of marine benthic communities and thus essential for ecosystem functioning (Covich et al. 1999; Leidenberger et al. 2012; Moksnes et al. 2008; Väinölä et al. 2008). The two species are characterised by high phenotypic plasticity regarding their habitat

and feeding behaviour (Hallberg et al. 1980; Leidenberger et al. 2012; Poore & Bruce 2012; Väinölä et al. 2008) and are considered key species in the lower intertidal and subtidal zone as well as on floating algae where they dwell as effective grazers of macrophytes and epiphytes. They are also important prey for various fish species (Andersson et al. 2009; Beermann et al. 2018; Gutow et al. 2006, 2015; Leidenberger et al. 2012).

Material and methods

Animal maintenance

The isopod culture was maintained at 10 °C and fed with thalli of the brown alga *Fucus vesiculosus* Linnaeus, 1753. The amphipod culture was maintained at 18 °C and fed with a mixture of *Fucus vesiculosus*, carrots and sugar beets. The individuals selected for the experiment were separated by sex and were of similar body mass (*I. balthica*: 96 mg±23 mg; *G. locusta*: 30 mg±11 mg). The sound level in the culture rooms was not recorded, but the rooms were quieter than the experimental room in which sound levels were recorded during the experiment.

Experimental setup

Individuals were not fed after their transfer to the experimental setup. Four experimental units (12 L buckets made of polypropylene) were filled with natural filtered seawater (salinity: 35 PSU; 5 µm polypropylene yarn filter, Graver Technologies Europe GmbH, Germany; Fig. 1). The

experimental room was set to a constant light regime (L:D 12:12) and temperature of 13 °C. So-called “noise eggs”, or waterproof diving eggs (ovoid dry boxes used to safe-keep valuables while diving) with a vibration motor inside and powered by two rechargeable batteries, were used to generate low-frequency sound (de Jong et al. 2017) with a frequency of 90 Hz in the noise treatment level. It is important to note that while the dominant frequency produced by the noise eggs fall within the frequency range of ships and wind farms (0.01 to 20 kHz; Duarte et al. 2021), it does not simulate them, nor does it reach comparable sound pressure levels. The produced soundscape is not directly comparable to natural environments, partly due to the sound reflections from the walls of the bucket. Thus, this method of sound production is mainly for detecting basic cause-effect relationships of continuous, tonal noise on marine life. The batteries of the noise eggs were connected in parallel to extend capacity, while maintaining the same 1.2 V voltage and powered the motor for 72 h continuously without being recharged or replaced. They were only replaced with fresh batteries at the start of a new trial, which began with short-term measurements. The experimental units were supported by foam rubber mats, which helped to reduce the transmission of added low-frequency sound between the buckets. Each experimental unit contained a single noise egg, an aeration stone and a respiration chamber (60 mm length×20 mm diameter) connected to an OXY-4 (PreSens GmbH, Germany) to measure oxygen consumption over time. The OXY-4 was connected to a laptop to run the program OXY4v2_11, which visualised the oxygen concentration and recorded the data (Fig. 1). The oxygen sensors (SP-PS3-SA, PreSens GmbH, Germany) remained attached to the experimental

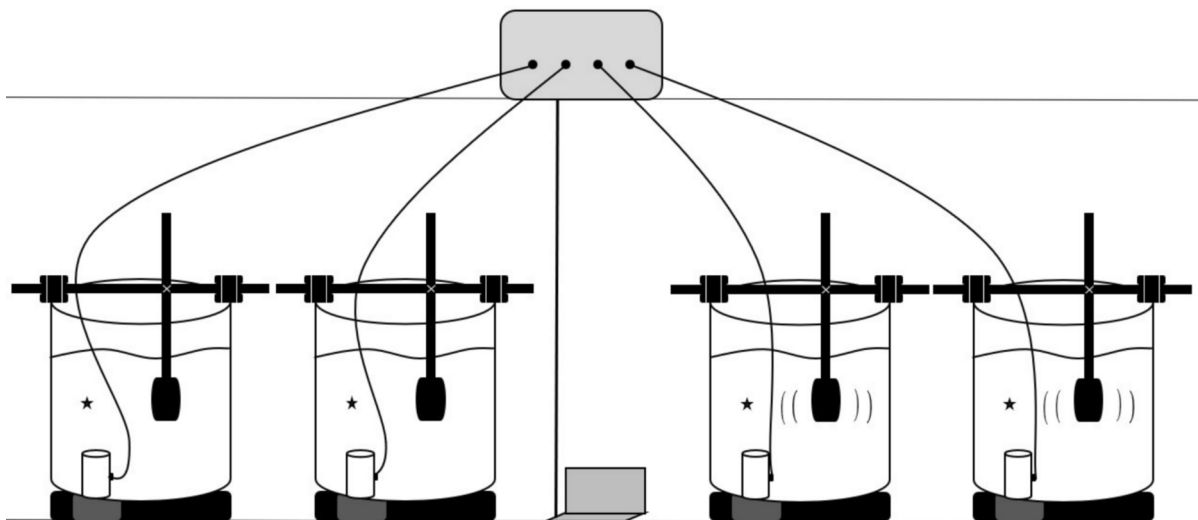


Fig. 1 Visualisation of the experimental setup. The OXY-4 (top) is connected to all four respiration chambers inside each bucket as well as the laptop (bottom, middle) for data acquisition. Both buckets on the left were without added noise and both buckets on the right

were treated with added noise. Aeration stones are not depicted to improve figure readability. The stars show hydrophone positions during recordings

units from each of the two treatment levels (without added noise; added noise). The setups for added noise and without added noise were identical, with unpowered noise eggs in the experimental units that belonged to the group that was not exposed to added noise. The noise eggs were fastened to the end of PVC pipes (410 mm length $\times$ 32 mm diameter) which were fixed with transversal stick segments (450 mm length). Sandbags were placed underneath the transversal sticks to mitigate the transmission of vibrations from the noise egg to the bucket walls (see Wang et al. 2022; Fig. 1).

In the noise treatment level, individuals were exposed to low-frequency sound for 72 h. Respiration measurements were made for 4 h at the beginning (hours 1–4; henceforth “short-term”) and at the end (hours 68–72; henceforth “longer-term”) of the experiment to evaluate short-term and longer-term effects. One trial was defined as one complete set of short- and longer-term measurements (72 h) with four animals being measured simultaneously (each in a separate respiration chamber). After the final respiration measurement, individuals were snap frozen with liquid nitrogen and stored at -80°C for the analysis of mitochondrial enzymatic activities; this marked the end of the trial. After every trial, all water content from the buckets and respiration chambers were exchanged. In total, 28 isopods and 28 amphipods were used in the experiments (14 females and 14 males each; $n=7$ for each combination of treatment “Noise” [without added noise; added noise] and “Sex” [female and male]; some replicates were discarded due to failed measurements). Sixteen trials were performed using 56 individuals (4 per trial) and 8 blanks without individuals to account for microbial oxygen consumption. Thus, 128 single measurements were made in total (64 short-term and 64 longer-term).

Sound levels were recorded for 30 s during each respiration measurement (short- and longer-term). For this purpose, a hydrophone (Aquarian Scientific AS-1, USA) was connected to a preamplifier (Aquarian Scientific PA-4, USA), then attached to an audio interface (Zoom UAC-2, Japan). The interface was connected to a laptop and the ambient noise was recorded using the free software Audacity (version 3.2.5; www.audacityteam.org). Data were analysed with R (version 4.4.2, R core team, 2024) using the PAMGuide code (Merchant et al. 2015). The power spectral density of the recorded sounds showed a higher sound pressure level under added noise at 90 Hz compared to without added noise (approximately 115 dB and 90 dB, respectively; Fig. 2). As the climate room was not fully silent due to cooling systems and machinery, the term “noise” will henceforth be used as a synonym for “added low-frequency noise”. The peaks at 50 Hz and its harmonic frequencies (e.g., 100, 200 Hz) were from ground loop noise in the recording and thus not actually present in the soundscape of either treatment level at the sound pressure levels depicted (Fig. 2).

Respirometry

Oxygen saturation was measured in four chambers simultaneously (two without added noise and two with added noise). At the bottom of each chamber was a magnetic stirring bar for homogenising the oxygen concentration within the chamber water column. Each bar was enclosed by a short PVC ring (5 mm height $\times$ 20 mm diameter) with a 500 μm mesh glued on top to prevent individuals from coming in contact with the rotating bar. The buckets containing the chambers were then placed on magnetic stirrers (IKA®-Werke GmbH & CO. KG, Germany). Every chamber contained a 500 μm mesh sheet (20 mm $\times$ 5 mm) to provide a substrate for the isopods to cling on, mitigating stress unrelated to noise. For the amphipods, a black 1 mm mesh sheet (40 mm $\times$ 40 mm) was placed inside the chambers as shelter for their negative phototactic behaviour. Compressed air and sodium sulfite (Na_2SO_3) were used to calibrate the 100% and 0% O_2 references, respectively, while considering temperature, salinity and atmospheric pressure. To standardise, all results were calculated per gram wet weight of the tested individuals.

To minimise stress responses, individuals were transferred to the respiration chambers and acclimatised for 2 h before the added sound exposure and measurements began. A 1 mm mesh lid (25 mm diameter) prevented escape during this period. Then, during the respiration measurements, a closed lid (25 mm diameter) and closed outlets ensured an airtight system. After the first (short-term) oxygen consumption measurements, the outlets were opened to allow water circulation and the closed lid was replaced again by the mesh lid to keep the individual in the chamber during the sound exposure while minimising handling. For the end point (longer-term) oxygen consumption measurements, the mesh lid was replaced once more with the closed lid.

Enzymatic activity analyses

A homogenisation buffer (20 mM Tris(hydroxymethyl)aminomethane, 1 mM EDTA, 0.1% Tween 20, pH 7.4) was added to the snap frozen samples in a ratio of 1:4 (w/v) for isopods and 1:8 (w/v) for amphipods (Hardewig et al. 1999). Each used animal was weighed individually. The samples were homogenised with a Precellys homogeniser (Bertin Technologies SAS, France) for two 15 s cycles at 5,000 rpm with a 15 s break. The temperature was set to 4°C using a Cryolys cooler (Bertin Technologies SAS, France) filled with liquid nitrogen (Yusseppone et al. 2018). Following the protocol of Hébert-Chatelain et al. (2008), the samples were centrifuged for 5 min at $500\times g$ at 4°C (Eppendorf Centrifuge 5417R, Germany). All enzyme activities were measured in supernatant. The remaining supernatant was

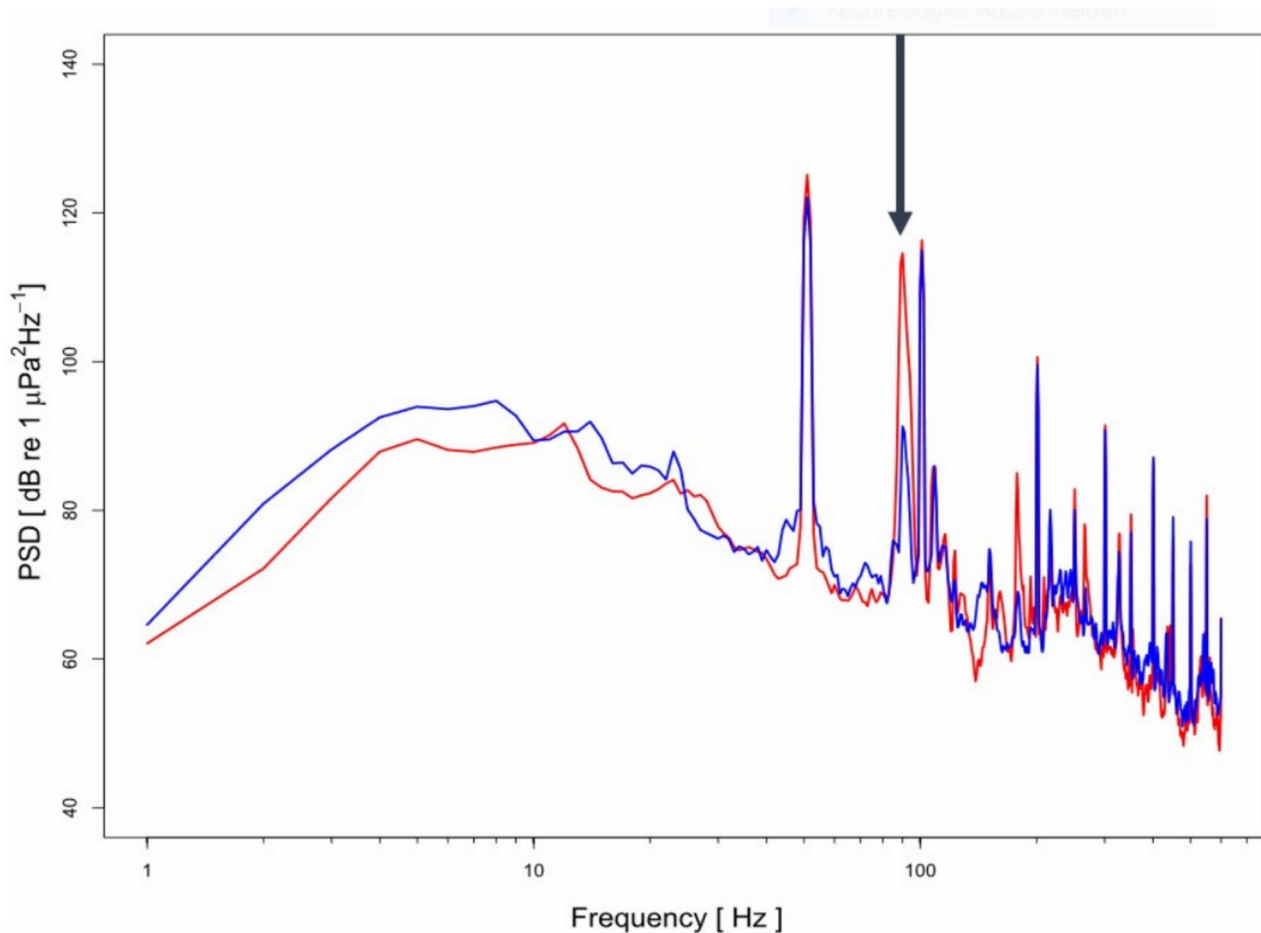


Fig. 2 Power spectral density (PSD) of without added noise (blue) and added noise (red) treatment levels. The dB scale is logarithmic. Therefore, an increase of 10 dB implies a tenfold power increase. Among treatment levels, there was a difference of about 25 dB at 90 Hz, which means the added noise at that frequency was over 100 times louder in

the noise treatment level (arrow). The peaks at 50 Hz and its harmonic frequencies (e.g., 100, 200 Hz) were from ground loop noise in the recording and were not actually present in the soundscape of either treatment level at the sound pressure levels depicted

frozen at -20°C for soluble protein measurements as per Bradford (1976) to standardise enzyme activities per mg protein ($\text{U}\cdot\text{mg}_{\text{protein}}^{-1}$; $\text{U}=\mu\text{mol substrate}\cdot\text{min}^{-1}$). The activities of four enzymes were measured in triplicates at room temperature using a microplate photometer (TriStar LB941, Berthold Technologies, France) following this order: cytochrome c oxidase representing complex IV (COX; Moyes et al. 1997), electron transport system complex I and III (ETS; Lannig et al. 2003), citrate synthase representing complex II (CS; Srere et al. 1963) and lactate dehydrogenase (LDH; Greenway & Storey 1999; Thibault et al. 1997) as a marker of the lactate anaerobic pathway.

Data analysis

All analyses were performed in R (version 4.4.2, R core team, 2024) while the boxplots with Tukey whiskers were prepared with the software GraphPad Prism (version 8.0.2).

Data were analysed through linear mixed-effects models using the package lmerTest (Kuznetsova et al. 2017). The full model included the experimental repetition (trial) as a random factor, while added noise and sex were considered fixed factors (full factorial design). For the analyses of oxygen consumption, time since start of the experiment (short- and longer-term exposure) was also considered as a fixed factor. Here, the main effects and two-way interactions of all fixed factors were tested but not three-way interactions (Tables S1 and S3). Backward model selection for linear mixed models was carried out using Akaike information criterion (AIC) scores (Tables S1, S2, S3 and S4). Several models were fitted to the data: a) the most complex (all factors, all interactions), b) the additive model (all factors, no interactions) and c) the simplest (with factors „Noise “ and „Sex “, no interaction). Models were then ranked according to their AIC score. If the simplest model had the lowest AIC, that model was chosen. If the difference between the

lowest AIC score and the second lowest score (ΔAIC) was greater than 3, the model with the lower AIC was selected regardless of differences in complexity. Hypothesis testing (likelihood ratio tests) was applied only when ΔAIC was less than 3. When models differed significantly ($p < 0.05$), the one with lower AIC was selected; in the opposite situation, the principle of parsimony was applied and the model with lower number of parameters was selected. Table S4 contains the AIC values for all models and indicates which models were ultimately selected. The package performance (Lüdecke et al. 2021) was used to check that residuals were approximately normally distributed with homogeneity of variance without data transformation.

Results

Idotea balthica

Oxygen consumption decreased from short- to longer-term exposure ($F_{(1, 40.633)} = 22.648$, $p \leq 0.000$; Fig. 3a, b; Table S5) reflecting an acclimation to the experimental setup. Neither treatment, sex, nor the interaction among the fixed terms had an effect on respiration rate. Males without added noise had an oxygen consumption rate of $388.05 \pm 152.22 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ (mean $\pm$ standard deviation), while males under added noise had an oxygen consumption rate of $442.73 \pm 389.17 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ (Fig. 3a). Females exhibited an oxygen consumption rate of $307.47 \pm 106.26 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ without added noise and $251.58 \pm 88.81 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ with added noise (Fig. 3a).

Fig. 3 Resting metabolism and enzymatic activities measured in *Idotea balthica* without added noise (light grey) and added noise (dark grey). Oxygen consumption of males and females ($n = 7, 6, 6, 7$) after (a) short-term (1–4 h) and (b) longer-term (68–72 h) exposure. Enzymatic activities of (c) cytochrome c oxidase, (d) electron transport system, (e) citrate synthase and (f) lactate dehydrogenase in males and females ($n = 7, 6, 7, 7$) after 72 h of exposure

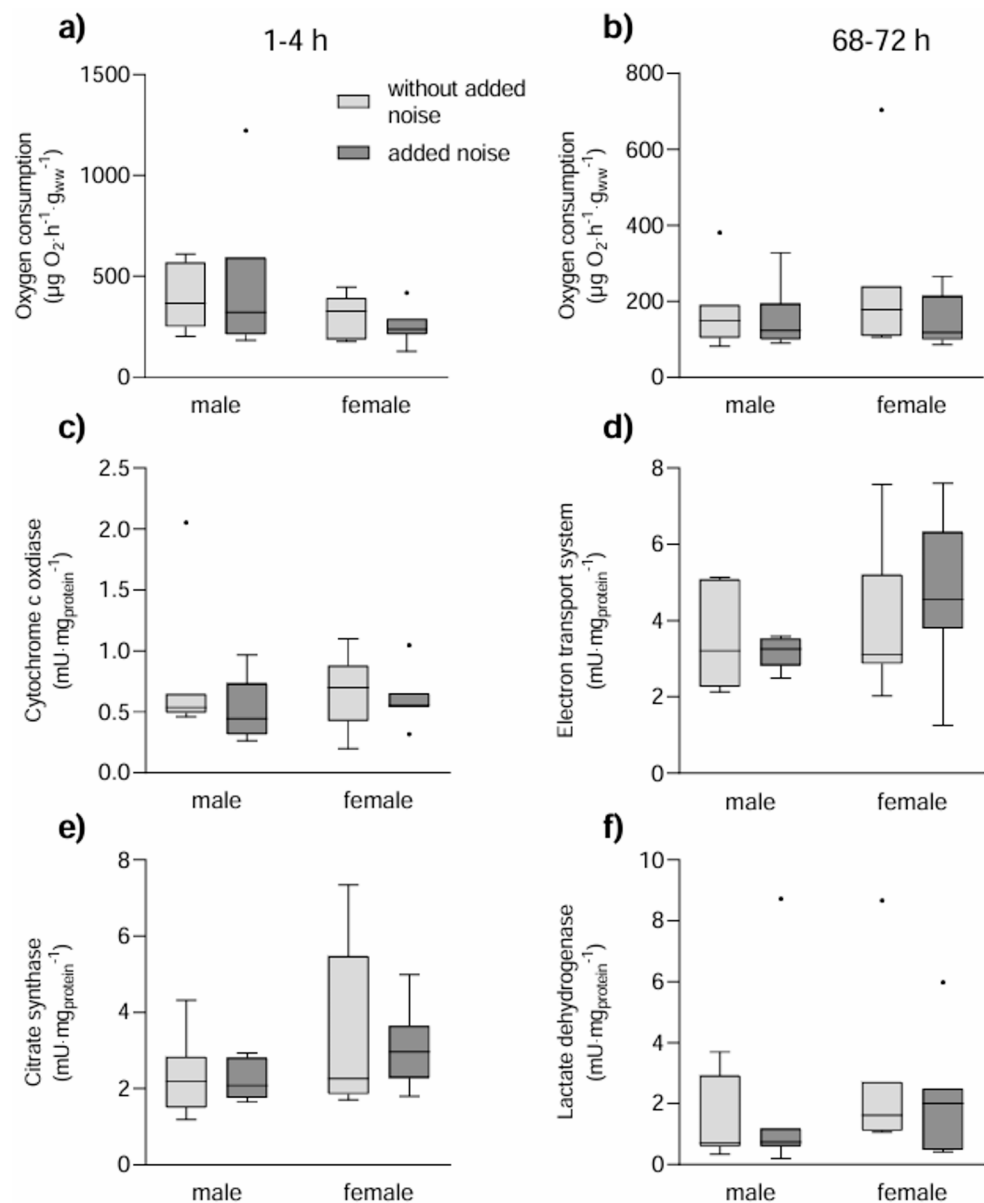
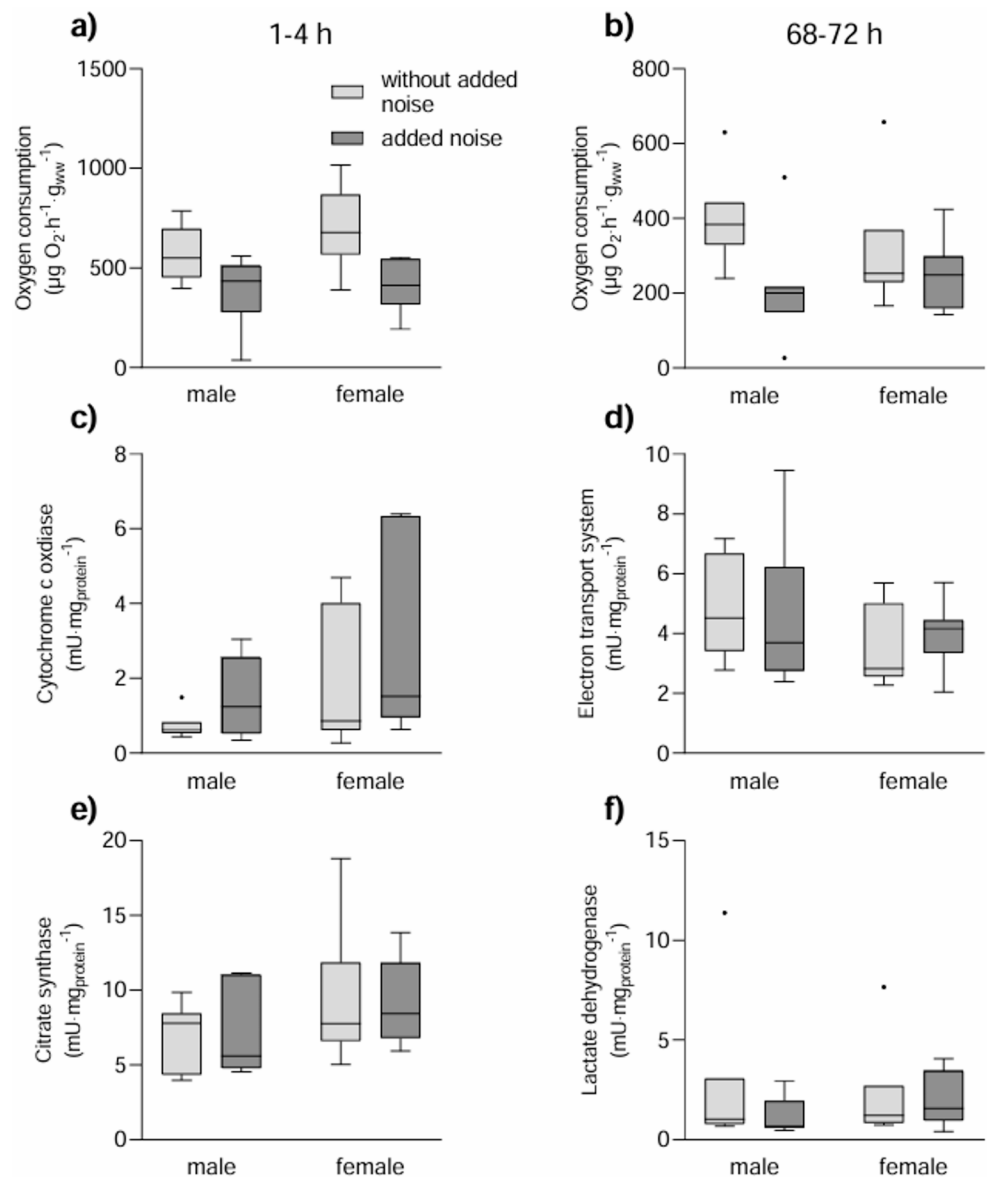


Fig. 4 Resting metabolism and enzymatic activities measured in *Gammarus locusta* without added noise (light grey) and added noise (dark grey). Oxygen consumption of males and females ($n=7, 7, 7, 7$) after (a) short-term (1–4 h) and (b) longer-term (68–72 h) exposure. Enzymatic activities of (c) cytochrome c oxidase, (d) electron transport system, (e) citrate synthase and (f) lactate dehydrogenase in males and females ($n=7, 7, 7, 7$) after 72 h of exposure



Similar to the respiration data, sex-specific responses to noise could not be detected in any of the measured enzymes. Correspondingly, enzyme activities did not vary significantly between individuals without added noise and added noise (Fig. 3c–e).

Gammarus locusta

Oxygen consumption decreased from short- to longer-term exposure ($F_{(1, 44.187)} = 32.497$, $p \leq 0.000$; Fig. 4a, b; Table S6), reflecting, like in the isopods, an acclimation to the respiration chambers. However, contrary to the isopods and disregarding the overall decrease along measurement time points, oxygen consumption was consistently lower under added noise compared to oxygen consumption without added noise ($F_{(1, 44.187)} = 23.598$, $p \leq 0.000$; Fig. 4b; Table S6). In

the first 4 h, males without added noise exhibited an oxygen consumption rate of $574.44 \pm 147.49 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ compared to $374.26 \pm 184.56 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ with added noise, while females exhibited an oxygen consumption rate of $695.86 \pm 203.81 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ without added noise and $402.17 \pm 127.42 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ under added noise (Fig. 4a). Similarly, after 72 h, males without added noise had an oxygen consumption rate of $398.09 \pm 121.81 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ while those under added noise had an oxygen consumption rate of $210.41 \pm 146.68 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$. Females, after 72 h of exposure, had an oxygen consumption of $313.55 \pm 163.49 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ without added noise and $248.26 \pm 94.89 \mu\text{g O}_2 \cdot \text{h}^{-1} \cdot \text{g}_{\text{ww}}^{-1}$ under added noise (Fig. 4b). There was no sex effect nor interaction among fixed terms for the oxygen consumption of the amphipods.

The interaction of added noise and sex was not significant for all enzymes measured in the amphipods. The activities of the enzymes CS ($F_{(1, 24)}=4.276$, $p=0.050$; Table S6) and COX ($F_{(1, 19)}=5.2860$, $p=0.033$; Table S6) varied significantly between sex with higher values in females (Fig. 4c–f).

Discussion

High tolerance of anthropogenic low-frequency sound in *Idotea balthica*

Anthropogenic low-frequency sound did not affect the oxygen consumption rates of *Idotea balthica*, regardless of exposure duration (1–4 h; 68–72 h), and enzymatic activities were also unaffected after 72 h of exposure. The metabolic rates of isopods are largely linked to their moult cycles. The oxygen consumption rates of *I. balthica*, for example, can increase two- to threefold when individuals are close to moulting (Bulnheim 1974). Therefore, the observed outliers in the current study were most likely due to differences in individual moult cycles (Fig. 3a, b). Given that ETS activity and oxygen consumption are closely linked (Verslycke et al. 2004), it follows that no statistically significant differences were detected in the responses of either parameter to the noise exposure.

The results did not highlight important sex-specific differences in metabolic strategies, although oxygen consumption in females was generally lower. This pattern was already observed in other studies (Strong & Daborn 1979) where sexual differences regarding behaviour in *I. balthica* were investigated, especially during breeding season. Females are much less active and prefer hiding under stones and shells, while males are more active and prefer exposed habitats (Jormalainen & Tuomi 1989; Strong & Daborn 1979; Vesakoski et al. 2008). The predator avoidance behaviour of females ensures their reproductive success (Jormalainen et al. 2001; Merilaita & Jormalainen 1997). In contrast, the higher activity in males is linked to their search of potential mates (Jormalainen & Tuomi 1989). A study using the isopod *Dynamene bidentata* (Adams, 1800) observed a difference in energy storage use: males utilised lipids for energy, while ovigerous females used lipoproteins or proteins. Females also ceased feeding during breeding and utilised all food reserves for yolk production, whereas males replenished their food reserves after their final moult (Hölldich 1971). Furthermore, males and females of *I. balthica* have different nutritional needs for growth and reproduction which manifest as different feeding preferences (Jormalainen et al. 2001). In general, higher activity entails an increase in metabolic rates to produce the required energy,

which would explain higher oxygen consumption of males in the current study (Bulnheim 1974).

Research about the impact of anthropogenic low-frequency sound on invertebrates is scarce. To our knowledge, no other research has been done on the effect of low-frequency sound on enzymatic parameters of isopods thus far. *Idotea balthica* has been used as a model organism in a number of experiments dealing with different forms of environmental stress. A steady increase in temperature, for example, linearly increased the oxygen consumption rates of *I. balthica* (Bulnheim 1974; Gutow et al. 2016; Salomon & Buchholz 2000). However, temperature stress did not affect CS activity, suggesting that increased respiration was due to a behavioural response to an elevated temperature range (escape response) rather than a metabolic adaptation process (Salomon & Buchholz 2000). Furthermore, in experiments with rapid temperature and salinity changes, *I. balthica* adjusted its oxygen consumption and acclimated within only a few hours (Bulnheim 1974). Therefore, this species appears to be rather flexible and can acclimate to chronic stressors. This may also be the case with low-frequency sound as indicated by the results of the current study. Some isopod species use acoustics for communication (Nakamachi et al. 2015) and should therefore depend on functioning acoustics. However, their tolerance to vibrations of various intensities is currently unknown. As *I. balthica* mostly lives in coastal habitats (Gutow et al. 2006), this species is subjected to natural sounds such as those caused by the tidal rhythm, which can reach a sound pressure level between 72 and 108 dB re 1 $\mu\text{Pa}_{\text{RMS}}$ that occur at frequencies of up to 100 Hz (Willis et al. 2013). Therefore, at least *I. balthica* may be characterised by a certain degree of adaptation to noisy environments, which may explain why the isopods were unaffected by added low-frequency sound in the current study.

Sensitivity to anthropogenic low-frequency sound in *Gammarus locusta*

In contrast to the isopods, the amphipod *Gammarus locusta* exhibited lower oxygen consumption rates when exposed to added low-frequency sound. This was observed during both short-term (39% reduction) and longer-term (35% reduction) exposure to the low-frequency sound. The enzymatic activities, however, seemed to be unaffected after 72 h. Gammarids exhibit specific predator avoidance behaviours such as hiding and reducing activity (Alexander et al. 2013; Beermann et al. 2018). The added sound in the current experiment could have triggered such antipredator behaviour in *G. locusta*, i.e., decreased activity to avoid detection by a potential predator, along with the suppression of

metabolism to reduce energy expenses and the direction of energy to basic maintenance.

Several studies have reported metabolism-suppressing effects of low-frequency sound in intertidal organisms. For example, larvae of the shrimp *Crangon crangon* (Linnaeus, 1758) exhibited lower oxygen consumption rates along with higher excretion rates with exposure to low-frequency sound (+30–35 dB SPL_{RMS} re 1 µPa at 31.5 to 100 Hz; Regnault & Lagardere 1983). The subtidal rock shrimp *Rhynchocinetes typus* H. Milne Edwards, 1837 showed significantly lower oxygen consumption rates while exposed to motorboat sound (+25 dB SPL_{RMS} re 1 µPa at 0.020–50 kHz; Ruiz-Ruiz et al. 2019). In contrast, the shore crab *Carcinus maenas* exhibited significantly higher oxygen consumption rates when exposed to ship noise (+37–47 dB SPL_{RMS} re 1 µPa; Wale et al. 2013b). This contradiction likely reflects different behavioural/physiological strategies to handle stressful situations — a reduction in metabolism and lower excretion reduces the chances of being detected by predators, whereas an increase in metabolism and therefore more energy may facilitate escape reactions (Ruiz-Ruiz et al. 2019). Differential responses to sound were also observed in the blue mussel *Mytilus edulis* and the giant sea scallop *Placopecten magellanicus* (Gmelin, 1791). Exposure to shipping noise resulted in an increased valve gape with reductions in metabolism-related rates such as oxygen consumption and filtration in *Mytilus edulis* (Wale et al. 2019), while *Placopecten magellanicus* exposed to pile-driving noise reduced their valve gap, had lower mantle water oxygen levels and significantly increased their metabolic rates (Cones et al. 2024). Similar to *I. balthica*, metabolic rates of *G. locusta* are also closely linked to moult cycles with massive increases in oxygen consumption during the moult (Bulnheim 1972). Therefore, the observed outliers in this part of the study most likely also reflect differently timed individual moult cycles.

The higher CS and COX activity in females could be explained by higher mitochondrial capacity to swim faster (Cherry et al. 2020), invest more energy into gametes than males (Sornom et al. 2012) and moult more in order to reach sexual maturity (Sutcliffe 1992; Hyne 2011). Citrate synthase initiates the citric acid cycle and its activity is often used as a marker for mitochondrial density while the COX enzyme, or complex IV of the electron transport chain, converts oxygen molecules into water. The released energy is then invested into pumping protons across the membrane (Chinnery & Schon 2003). Excess COX activity can mean uncontrolled electron flow, heightening the production of reduced ETS intermediates and hence higher reactive oxygen species (ROS) production. Although the organisms in the present study consumed less oxygen under added noise, the environment in the chamber was never hypoxic. Multiple studies demonstrated that in hypoxia, COX activity

and gene expression decreased along with respiration rate as an adaptive strategy for survival (Chandel et al. 1996; Vijayasathya et al. 2003). These studies and others showed the close relationship between dissolved oxygen concentration and COX activity (Singtripop et al. 2007). Thus, in the current study, the decreased oxygen consumption rates in *G. locusta* of both sexes and the high levels of CS and COX activities observed in females align with transient antipredator behaviour events as cell machinery was not suppressed by the significantly lower respiration rate.

This is further supported by the observed unaltered levels of LDH activity, a marker of anaerobic metabolism. An experiment investigating the effect of declining oxygen saturation on the amphipod species *Marinogammarus pirloti* (Sexton & Spooner, 1940) and *Marinogammarus obtusatus* (Dahl, 1938) yielded contrasting results: in hypoxia, both species compensated by increasing their oxygen consumption. Furthermore, the organisms accumulated lactate in anoxia, which represented high LDH activity levels in anaerobic metabolism (Agnew & Taylor 1985).

How amphipods generally respond to other stressors could help make sense of the responses observed in this study. For instance, exposure of the amphipods *Gammarus oceanicus* Segerstråle, 1947 and *Gammarus duebeni* Liljeborg, 1852 to pollutants resulted in lower oxygen consumption rates and higher nutrient excretion (Tedengren et al. 1988). Long starvation periods can also lead to significantly lower oxygen consumption rates and lower ETS activity in *Gammarus fossarum* Koch, 1836 and *Corophium volutator* (Cammen et al. 1990; Mezek et al. 2010). Due to synthesis and degradation, changes in enzyme activities may take longer to appear than respiration responses, which are comparatively much more promptly evident (Cammen et al. 1990). The duration of the added low-frequency sound could have been too short to observe clear responses of the four enzymes studied. Thus, enzymes may be more effective as metabolic markers for long-term experiments (Smith & Chong 1982). Similar to *I. balthica*, *G. locusta* inhabits mostly coastal waters and is exposed to natural soundscapes (Andersson et al. 2009; Gutow et al. 2015). However, for some amphipod taxa such as *Gammarus*, a lateral line organ was described which may allow the sensing of vibrations (Enright 1962; Platvoet et al. 2007). For this reason, amphipods could be more sensitive to anthropogenic low-frequency sound than isopods, as corroborated by the resting metabolism results of the current study.

Marine fish and some invertebrates sense and respond to acoustic stimuli via particle motion instead of sound pressure levels (Hawkins & Popper 2017; Nedelec et al. 2016; Popper & Hawkins 2018). The cuttlefish *Sepia officinalis* Linnaeus, 1758 and the hermit crab *Pagurus bernhardus* (Linnaeus, 1758), for example, were shown to largely

respond to particle motion (André et al. 2016; Roberts et al. 2016). However, we were unable to measure particle motion in this current study due to the lack of technical equipment. It therefore remains to be elucidated how the added low-frequency sound created in the current study might translate into particle motion and thereby affect the animals. The experimental design of the current study cannot be directly compared to conditions in the natural environment as the purpose was to simply test for causal connections between anthropogenic noise and marine invertebrates. Therefore, any detected cause-effect relationships deserve further investigation to assess their implications for sensitivities under natural environmental conditions.

In conclusion, oxygen consumption measurements successfully provided basic information on the status of mitochondria over a short-term and longer-term period of noise exposure. This method is simple and easily implemented alongside standard measurements. In contrast, enzyme measurements are complex and likely more effective in long-term experiments with noise. The information generated, however, is much more detailed and accurate. We believe that the two methods are complementary and should be implemented in future studies aiming to identify physiological effects of anthropogenic low-frequency sound on benthic invertebrates. As such sounds led to a reduction in the metabolism of the amphipods in this study, it is plausible that this stressor also affects the energy budgets of other invertebrate species. Amphipods could thus be good model organisms for monitoring the effects of underwater anthropogenic noise pollution on aquatic life. Short-term compensation via metabolic suppression may temporarily suffice, but the species may struggle to maintain homeostasis if noise persists. With the continuous increase in ship traffic and thus exposure to anthropogenic low-frequency sounds, particularly sensitive invertebrate species may be threatened.

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Author contribution Anna Terschek: Methodology, data curation, formal analysis, investigation, writing—original draft, writing—review & editing. Nelly Tremblay: Conceptualisation, methodology, data curation, writing—review & editing. Sheng V. Wang: Methodology, writing—review & editing. Jan Beermann: Conceptualisation, resources, writing—review & editing, supervision.

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Data availability The datasets are available from the corresponding author under request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

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