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RESEARCH ARTICLE

Cardiac mitochondrial energetics of the Australasian red spiny lobster, *Jasus edwardsii*, when exposed to isoeugenol within the commercial anaesthetic AQUI-S

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ABSTRACT

The anaesthetic isoeugenol has been used as metabolic suppressant for commercial transport of live lobsters in order to decrease energy expenditure and improve survival. Given the central role of mitochondria in metabolism and structural similarities of isoeugenol to the mitochondrial electron carrier coenzyme Q, we explored the influence on mitochondrial function of isoeugenol. Mitochondrial function was measured using high-resolution respirometry and saponin-permeabilised heart fibres from the Australasian red spiny lobster, *Jasus edwardsii*. Relative to vehicle (polysorbate), isoeugenol inhibited respiration supported by complex I (CI) and cytochrome *c* oxidase (CCO). While complex II (CII), which also reduces coenzyme Q, was largely unaffected by isoeugenol, respiration supported by CII when uncoupled was depressed. Titration of isoeugenol indicates that respiration through CI has a half-maximal inhibitory concentration (IC_{50}) of 2.4±0.1 µmol l^{-1}, and a full-maximal inhibitory concentration (IC_{100}) of approximately 6.3 µmol l^{-1}. These concentrations are consistent with those used for transport and euthanasia of *J. edwardsii* and indicate that CI is a possible target of isoeugenol, like many other anaesthetics with quinone-like structures.

KEY WORDS: Crustacean, Coenzyme Q, Quinone, Mitochondria

INTRODUCTION

Air transport is the standard method for long-distance transport for sub-tidal crustaceans. While crustaceans often show high rates of survival despite extended periods of emersion from water (Spanoghe and Bourne, 1997; Taylor and Waldron, 1997; Morris and Oliver, 1999), emersion and the handling processes involved in the preparation and transportation of live crustacean species negatively impact post-transport survival and welfare, which impacts the quality and welfare of live lobsters such as the Australasian spiny red lobster, *Jasus edwardsii* (Lorenzon et al., 2007; Sladkova and Kholodkevich, 2011).

Generally, wild lobsters captured from temperate waters are prepared for live transport by chilling through immersion in near-freezing water, which lowers the metabolic rate (Group, 1999). Maintaining cool temperatures over extended periods during freighting is problematical, and as holding temperatures rise, so do the metabolic rates of lobsters and their mortality (Forgan et al., 2014). Therefore, suppression of metabolism could extend lobster survival, and some animals suppress metabolism through hibernation, aestivation or diapause and therefore they can effectively 'place life on hold' to protect metabolic reserves (Pinder et al., 1992; Michaelidis et al., 2008). While this slows the production of deleterious metabolic end products, the general strategy of metabolic suppression appears to be mediated through decreasing ATP consumption, and ATP synthesis by mitochondria (Pinder et al., 1992; Reilly et al., 2014). The reversible suppression of mitochondrial function may therefore present a tool to suppress metabolism and extend survival during transportation of lobsters to market.

While there is debate as to how many anaesthetics function, most are assumed to do so through neurotransmission blockade, but they may also suppress metabolism through membrane destabilisation and mitochondrial suppression (Footitt et al., 2008; Pavel et al., 2020; Voss and Sleigh, 2020). Where mitochondria have been implicated in anaesthesia, they often relate to the interactions of respiratory complex I (CI). Drugs such as amobarbital, ketamine and propofol disrupt the mitochondrial electron transport systems (Stewart et al., 2009; Venâncio et al., 2015; Finsterer and Frank, 2016). All have phenolic group structures that mirror the structure of the electron transporter ubiquinone/ubiquinol (Q), also known as coenzyme Q.

Two food-safe phytochemicals eugenol (methoxy-4-phenol) and isoeugenol (2-methoxy-4-propenylphenol) also share structural similarities to Q. Both molecules lack the isoprenoid group of Q, yet both share short allyl groups. Eugenol is found in clove oil and acts as an anaesthetic and analgesic. Isoeugenol is the active ingredient of the commercial anaesthetic AQUI-S®, and is used to anaesthetise and improve the resilience and condition of transported aquatic animals (Javahery et al., 2012). Both compounds have been found to inhibit rat liver mitochondrial respiration (Cotmore et al., 1979). However, the mode of action within mitochondria of invertebrates, such as lobster, remains unclear.

Here, we aimed to explore the direct effects of isoeugenol on cardiac mitochondria. We tested lobster cardiac mitochondrial function of the red spiny rock lobster, *Jasus edwardsii*, as the heart has an abundance of mitochondria, and considerable control over metabolism and survival. Mitochondrial respiration can be readily measured *in situ*, i.e. within permeabilised muscles of the crustacean heart, with minimal amounts of tissues (Iftikar et al., 2010). We used two approaches to explore the impact of isoeugenol on mitochondrial function using high-resolution respirometry and a structured substrate–inhibitor–uncoupler titration protocol. Given that Q is reduced from ubiquinone to ubiquinol, at respiratory complexes I and II (CI and CII), and oxidised at complex III (CIII; Fig. 1), and is lipid soluble, we could test where isoeugenol interacts by titration of different substrates and inhibitors in different order to

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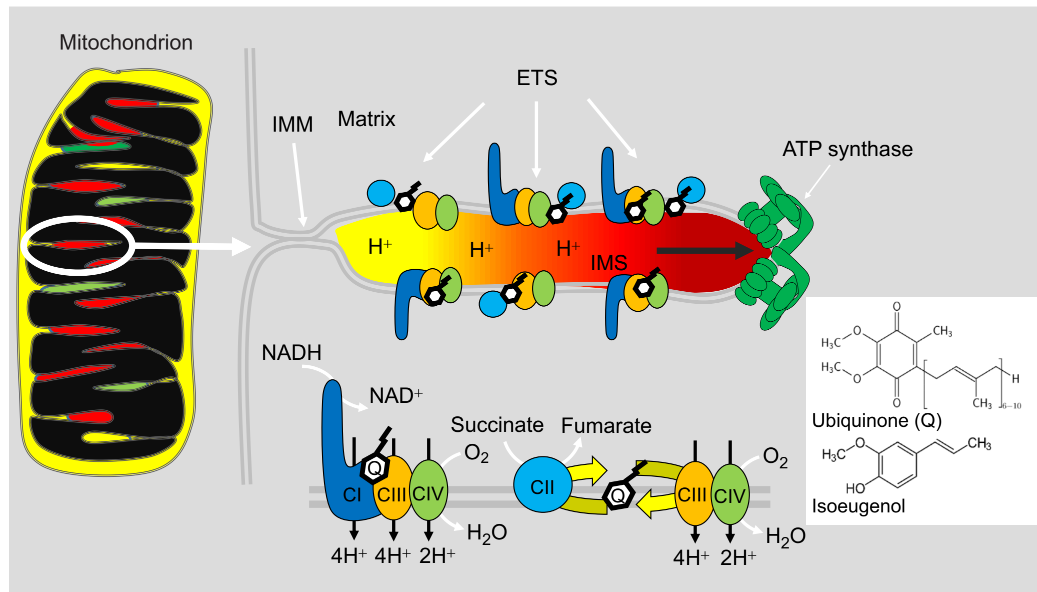

Fig. 1. Mitochondrial function. The current model of mitochondrial function is that each cristae (circled) has an independent membrane potential with electron transport systems (ETS), which are found on the flat surfaces of cristae, transferring protons across the inner mitochondrial membrane (IMM) into the intermembrane space (IMS). A proton gradient is formed through electrons motivating proton translocation. Protons are then focused on the ATP synthases. The electron transport systems vary in composition, with respiratory complexes forming different chain types: complex I (CI, which oxidises NADH) and complex II (CII, which oxidises succinate) form separate systems and transfer a different net number of protons, i.e. CI and CII systems move 10 and 6 protons, respectively. Coenzyme Q (Q) accepts electrons from either CI or CII, and these are transferred to complex III and IV (CIII, CIV) and oxygen. The plant-derived compound isoeugenol (inset) has a similar structure to Q and therefore should interact with the ETS.

assess its effects on respiratory complexes and the phosphorylation system.

MATERIALS AND METHODS

Jasus edwardsii (Hutton 1875) (~400–1800 g which represents legal harvest size), within the intermoult phase were obtained from the inner Hauraki Gulf, New Zealand, using traps and by hand capture by divers. Lobsters were transported to the University of Auckland's Leigh Laboratory, where they were held in a 200 l holding tank supplied with flow-through aerated seawater (~15°C) with a 12 h photoperiod and fed twice a week for at least 2 weeks on a diet of green-lipped mussels (*Perna canaliculus*) and urchins (*Evechinus chloroticus*). Lobsters were starved 72 h prior to experimentation. All experiments for this study were approved by the University of Auckland's Animal Ethics Committee (AEC 001242).

Heart permeabilised muscle fibre preparation

Specimens of *J. edwardsii* were determined to be in the intermoult phase using the pleopod method as per Musgrove (2000) (*N*=4 in all experiments, mean±s.e.m. mass 586.67±76.46 g), then chilled on ice and euthanised by pithing. The heart was removed by opening the carapace of the cephalothorax with a small Dremel saw. The heart was immediately dissected and immersed in 2 ml of modified relaxing buffer (in mmol l^{-1}: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 5.77 Na_2ATP, 6.56 $MgCl_2$, 20 taurine, 20 imidazole, 0.5 dithiothreitol, 50 MES and 40 KCl, pH 7.1; Iftikar et al., 2010) at 0°C. This buffer was modified from that used for mammalian muscle, with omission of creatine phosphate and addition of 400 mmol l^{-1} KCl to increase the osmolarity to 1000 mOsm. The heart was dissected into muscle fibre bundles and placed into 1 ml respiration media (RM) with 50 µg ml^{-1} of saponin and incubated on ice for 30 min, before transfer into mitochondrial respiratory medium formulated for marine crustaceans (in mmol l^{-1}: 0.5 EGTA, 3 $MgCl_2$, 100 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 200 sucrose and 250 KCl, with 1 g l^{-1} BSA, essentially free fatty acid, pH 7.24) at 20°C for 10 min (Iftikar et al., 2010). Fibres were then rinsed in 2 ml of RM, blotted dry on filter paper and weighed as bundles of approximately 30±1 mg, and used immediately. All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Mitochondrial respiration experiments

Heart muscle fibres were added to 2 ml chambers containing RM at 17°C in an Oroboros Oxygraph-2k™ respirometer (Oroboros Instruments, Innsbruck, Austria). Media oxygenation was maintained above 200 nmol ml^{-1}. Respiration was measured as mass-specific oxygen flux (pmol O_2 s^{-1} mg^{-1} wet mass), and respiratory flux (J_{O_2}) was calculated in real time as the negative time derivative of the oxygen concentration using Oroboros DatLab Software V (Oroboros Instruments). To assess mitochondrial function in the context of the anaesthetic isoeugenol, two substrate–uncoupler–inhibitor titration (SUIT) protocols were used to measure substrate and coupling control of the mitochondria in permeabilised fibres (adapted from methods used for crab: Iftikar et al., 2010; Fig. 1); in addition, isoeugenol was compared with vehicle (PEG/control).

The two SUIT protocols both involved the addition of CI and CII substrates and specific inhibitors but differed according to when isoeugenol was added, and aimed to determine the effects of isoeugenol on coupling of oxidative phosphorylation (OXPHOS). Both titration protocols involved the addition of the CI-supporting substrates glutamate (10 mmol l^{-1}) and malate (3.8 mmol l^{-1}) to measure the LEAK state respiration through CI in the absence of ADP (LEAK-CI). ADP (2.5 mmol l^{-1}) was then added to stimulate oxidative phosphorylation (OXPHOS-CI). In the first protocol, isoeugenol (6 µmol l^{-1}) or vehicle (control) was added prior to ADP. In the second protocol, isoeugenol or vehicle was added after ADP. This permitted us to test the effects of isoeugenol on CI and OXPHOS

coupling while respiration was supported by CI, and therefore whether isoeugenol uncouples or inhibits respiration at CI. The remainder of the two SUIT protocols was identical and involved poisoning of CI with rotenone (1 µmol l^{-1}) and the initiation of CII-mediated oxygen flux with succinate (10 mmol l^{-1}). This allowed us to test electron transport through CII and provided a relative index of inhibition of CI. All experiments were conducted in duplicate.

LEAK state respiration in CII substrates was then determined (LEAK-CII) following addition of carboxyatractyloside (12.5 µmol l^{-1}) an inhibitor of the adenonucleotide translocase. Carbonyl cyanide-*p*-(trifluromethyl) phenylhydrazone (FCCP; 1.5 µmol l^{-1}) was then added to uncouple mitochondrial respiration to test for limitations by the phosphorylation system and maximise flux of the electron transport system (ETS) at least supported by CII. CIII was then inhibited by the addition of antimycin-a (5 µmol l^{-1}), to test the activity of cytochrome *c* oxidase (CCO/CIV) by addition of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD; 0.5 mmol l^{-1}) and ascorbate (1.2 mmol l^{-1}), and the background and auto-oxidation of these compounds were determined following addition of sodium azide (40 mmol l^{-1}). Oxygen was maintained above 200 nmol ml^{-1} by the direct addition of oxygen gas to ensure the saturation of cardiac fibres.

Table 1. Effect of isoeugenol on mitochondrial respiration

	Vehicle	Isoeugenol
Protocol 1		
LEAK ratio CI	0.229±0.025	0.465±0.037**
LEAK ratio CII	0.618±0.038	0.649±0.069
UCR	2.115±0.064	1.045±0.123***
CI/CII	0.846±0.032	0.358±0.035***
Protocol 2		
LEAK ratio CI	0.089±0.014	0.175±0.024
LEAK ratio CII	0.332±0.056	0.542±0.056*
UCR	2.498±0.236	1.234±0.209**
CI/CII	1.321±0.153	0.314±0.048***

Vehicle and isoeugenol were either added pre-ADP (pre-oxidative phosphorylation; SUIT protocol 1) or post-ADP (post-oxidative phosphorylation; SUIT protocol 2). The LEAK ratios for complexes I (CI) and II (CII) represent the fraction of oxygen consumed attributed to non-phosphorylating respiration relative to that in phosphorylating states with either CI or CII supporting substrates. The uncoupling control ratio (UCR) represents the fractional increase in flux following uncoupling of respiration with FCCP relative to phosphorylating flux supported by CII. CI/CII represents the ratio of CI to CII flux in phosphorylating states and therefore represents the contributions of each complex. Data are means±s.e.m., *n*=4 animals. **P*<0.05, ***P*<0.005, ****P*<0.001.

Dose–response

To assess mitochondrial function in relation to increasing concentrations of isoeugenol, a titration protocol was used consisting of the CI-supporting substrates glutamate (10 mmol l^{-1}) and malate (2 mmol l^{-1}) in the presence of FCCP (1.5 µmol l^{-1}) to maximise ETS flux (Fig. 1). Isoeugenol was titrated at 0, 0.3, 1.5, 3 and 6 µmol l^{-1}, with 0.5 µl of each concentration added successively to the chamber.

Calculations and statistical analysis

LEAK ratios were used as a comparative measure of OXPHOS capacity before and after the administration of isoeugenol in OXPHOS-CI and LEAK-CI states. An additional measure of membrane integrity with CII was determined from the OXPHOS-CII phosphorylation flux relative to respiration after atractyloside inhibition (LEAK-CII). These provide a simple proxy to measure the integrity of the mitochondrial inner membrane when phosphorylated or not phosphorylated with the inner mitochondrial membrane (IMM) under a high electrochemical gradient (Iftikar et al., 2010). Limitation or control of the phosphorylation system was determined from the comparison of uncoupled respiration in the ETS relative to phosphorylating respiration of CII (OXPHOS-CII). This can be useful as it reflects loss of ETS capacity (Iftikar et al., 2010). The ratio of phosphorylation at CI to total phosphorylation (CI/CII) was determined by comparison of OXPHOS-CII/OXPHOS-CI.

Where repeated dosage measurements were made on the same heart from a single lobster, replicates involved a heart from four different lobsters. Differences between treatments were evaluated using a repeated measures Student's paired *t*-test or ANOVA in the case of multiple mean comparisons, with the level of significance set at *P*<0.05. In dose–response assays, hyperbolic curves were fitted to determine the half-maximal inhibitory concentration (IC_{50}) and full-maximal inhibitory concentration (IC_{100}) using GraphPad Prism™.

RESULTS

The effects of AQUI-S on mitochondrial respiration

The addition of isoeugenol prior to ADP doubled the LEAK ratio with CI substrates (Table 1). However, there was minimal increase in respiration when isoeugenol was added in LEAK-CI (state 2 respiration) prior to ADP, suggesting that the increase in CI LEAK ratio (LEAK ratios for CI, Table 1) was not from an increase in LEAK respiration itself, but due to a limited increase in phosphorylating respiration on the addition of ADP (Fig. 2A). Moreover, addition of isoeugenol did not appear to alter the non-phosphorylating oxygen flux of mitochondria. Following ADP addition, the titration of isoeugenol depressed OXPHOS by 62% relative to the control. Protocol 2, where isoeugenol was added after initiating OXPHOS, also resulted in the depression of oxygen flux by approximately 67% of the control (*P*<0.005, Fig. 2B). As the vehicle had no apparent effect, this indicates isoeugenol inhibits CI-supported electron transfer.

Oxygen flux did not differ for OXPHOS-CII between treatments under the pre- or post-phosphorylation additions of isoeugenol (Fig. 2A,B), indicating that CII was largely intact and not inhibited by isoeugenol. The depression of CI was further reflected by decreased flux ratios of CI/CII in both treatments (*P*<0.0001; Table 1). With the addition of carboxyatractyloside, LEAK-CII flux rates differed slightly between protocols. In protocol 2, there was a small increase in the LEAK-CII ratio (*P*<0.04; Table 1), while in protocol 1 there was no apparent difference in LEAK ratio relative to controls. This provides little evidence that isoeugenol acts as an uncoupler.

The induction of the uncoupled respiration state (ETS-CII) following FCCP addition showed differences from control for both protocols, as isoeugenol addition suppressed the uncoupled oxygen flux by approximately 50%, while control flux rates were higher in both phosphorylation states compared with those in the presence of anaesthetic (*P*<0.005). This suppression was reflected in the uncoupling ratios (UCR-CII; Table 1) for both treatments being depressed by 50% (*P*<0.0005). Isoeugenol also suppressed CCO (CIV) flux when added pre- (*P*=0.05) or post-ADP (*P*<0.01) states.

Dose–response assay

Respiration supported by CI substrates was inhibited in a dose-dependent manner by isoeugenol. A hyperbolic curve was fitted to this response (R^2=0.99, ANOVA, $F_{4,15}$=3.81, *P*<0.05; Fig. 3) and a mean theoretical inhibitory concentration, where 50% inhibition

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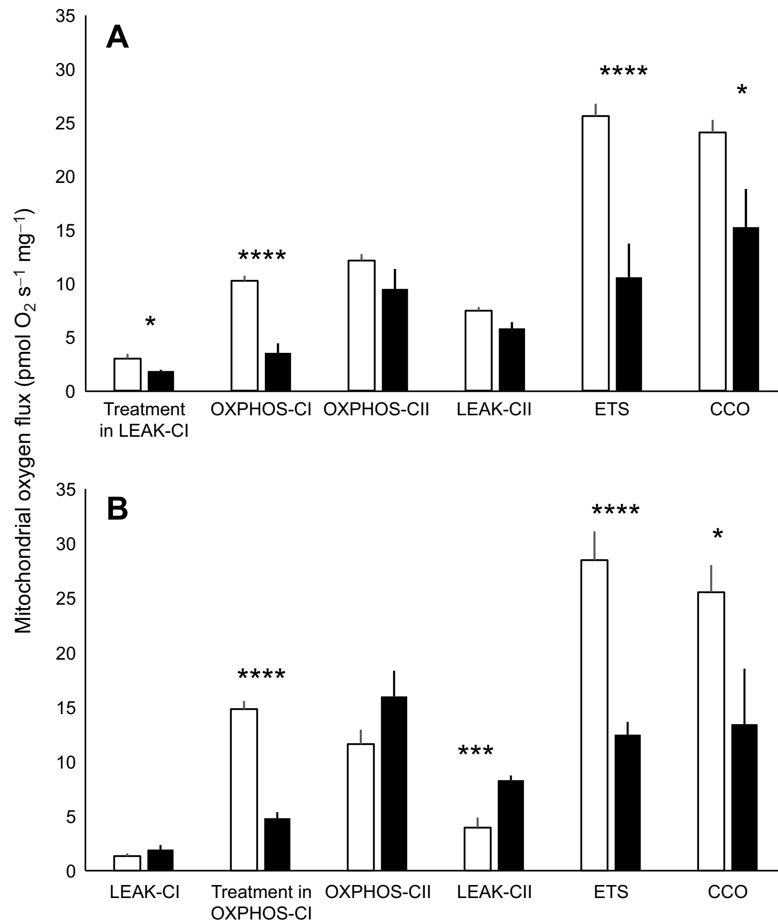

Fig. 2. Measurement of substrate and coupling control of mitochondria in permeabilized cardiac fibres. Two different substrate–uncoupler–inhibitor titration (SUIT) protocols were used to measure mitochondrial oxygen flux, and involved the titration of mitochondrial substrates, inhibitors and uncouplers to test different components and respiration states in permeabilised cardiac muscle. Bars indicate values in the presence of isoeugenol (black) or vehicle (white). CI-supporting substrates (glutamate and malate) were added to initiate LEAK (non-phosphorylating, LEAK-CI) respiration. Protocols differed in the timing of treatment with isoeugenol or vehicle (control), with addition prior to (A) or after (B) ADP (OXPHOS-CI). Rotenone was then added to test CII (OXPHOS-CII) in both protocols, and this was followed by addition of carboxyatractyloside to inhibit ADP–ATP exchange and initiate LEAK-CII. The chemical uncoupler FCCP was added to maximise ETS flux. Antimycin-a was then added to inhibit CIII, and TMPD-ascorbate was added to support cytochrome *c* oxidase (CCO) flux. Asterisks denote a significant difference between vehicle and isoeugenol treatments: *$P \leq 0.05$, ***$P \leq 0.005$ and ****$P \leq 0.001$. Error bars represent s.e.m., n=4 animals.

occurs (IC_{50}) was calculated as 2.43±1.03 μmol l^{-1} and 100 inhibition (IC_{100}) as 6.3 μmol l^{-1}.

DISCUSSION

As with many anaesthetics and analgesics, the mechanism by which isoeugenol works has yet to be fully resolved. Here, we show that isoeugenol depresses mitochondrial respiration in crustacean heart. While isoeugenol may depress respiration at numerous steps of the ETS, the most obvious effect was mediated through CI-supported respiration, with potential effects elsewhere within the ETS. Isoeugenol also depressed respiration following chemical uncoupling (ETS) following addition of FCCP, indicating the compound is active elsewhere in succinate-fuelled electron transport.

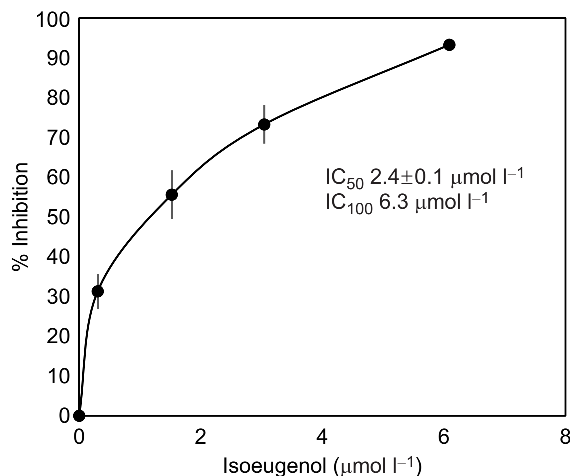

Fig. 3. Progressive inhibition of cardiac muscle respiration supported by CI substrates by addition of increasing concentrations of isoeugenol. Data are presented as percentage oxygen flux relative to vehicle controls. A hyperbolic curve was fitted to determine the IC_{50} and IC_{100}, which approximated 80 and 200 ppm, respectively. Error bars represent s.e.m., n=4 animals.

Since ancient times, oils extracted from cinnamon and cloves with their active agents eugenol and isoeugenol, have been used as antimicrobial/antiseptic (Gill and Holley, 2004) and antispasmodic agents (Rossignol, 2012), and these are found in household products, fragrances, skin care products, flavourings, and dental and pharmaceutical products. Both compounds contain phenolic/quinone structures that resemble the electron carrier ubiquinone (Q) of the ETS. These quinone structures also have putative antioxidant properties as found in other plant-based phenolics (e.g. vanillin and curcumin), and have similar reduction potentials to Q (∼400 mV) (Sağlam et al., 2016). While this indicates that they could accept electrons within a similar range to Q molecules, or fit within active sites of ETS enzymes (Fig. 1), they lack carbonyl groups to which protons can bind, which are required for electron transport.

The molecular structure of isoeugenol (Fig. 1) may in part explain its inhibitory effects on mitochondrial function, and therefore suppress metabolism. The most significant inhibition occurred with a 62–65% depression in O_2 oxygen flux derived from substrates that support CI oxygen flux. This CI inhibition occurred regardless of when ADP was added, suggesting that CI activity is inhibited more than the remainder of the ETS. Isoeugenol was also found to inhibit

rat liver mitochondria at CI (Cotmore et al., 1979). As it is lipophilic it is less likely to interact with matrix dehydrogenases and more likely to target CI or the inner mitochondrial membranes. The addition of succinate also restored O_2 flux, indicating isoeugenol does not appreciably inhibit CII or CIII in phosphorylating states, and therefore some ETS function is maintained.

Like CI, CII also binds and reduces Q, so why is it not also inhibited? The mechanisms of electron transport within CI and CII differ considerably. Rotenone inhibits the electron transport from Fe-S complexes to Q in a specific Q binding pocket in CI but not CII (Fendel et al., 2008). The same site is also reversibly targeted by amytal (Stewart et al., 2009), which also shares similar cyclic structures to isoeugenol and also does not inhibit CII. Note that respiration was largely restored to similar levels by the addition of succinate in test and control protocols. However, CII does not directly transfer protons and thus contributes less to the membrane potential and is therefore less efficient at forming ATP (Fig. 1).

We used two tests to better understand whether isoeugenol altered coupling of the ETS to the oxidative phosphorylation system. In the first test, isoeugenol was added either before or after initiating phosphorylation (addition of ADP). The second test involved the addition of atractyloside, an inhibitor of the adenine nucleotide transporter, which inhibits ADP and ATP exchange. Given that isoeugenol inhibited CI flux, we could not determine whether LEAK-CI respiration was enhanced by isoeugenol in either protocol. Therefore, it was less clear whether isoeugenol also acts as an uncoupling agent. Inhibition of CII-supported phosphorylating respiration was apparent in control tests using carboxyatractyloside in the presence of isoeugenol, which indicates it has limited to no activity as an uncoupling agent.

Eugenol has been reported to interact with lipid membranes and to decrease mitochondrial membrane potential, and to elevate reactive oxygen species (ROS) (Jaganathan et al., 2011). In the present study, the proton ionophore FCCP, dissipates the proton gradient and accelerates ETS flux. This effect was apparent in control experiments, yet it did not occur in the presence of isoeugenol. This suggests some inhibition of the ETS occurs in addition to that at CI, and ETS flux was depressed by approximately 50%. In both experiments, the final step of the ETS, CCO, was also significantly depressed. As this step was tested specifically, it is likely that this is an additional site of action for isoeugenol, or that isoeugenol disrupted the mitochondrial inner membrane and overall ETS function. CCO is also the oxygen binding site so will impact oxygen gradients and diffusion into cells.

Like most anaesthetics, isoeugenol induces sedation in a dose–dependent manner in a variety of vertebrate and invertebrate species (Wagner et al., 2003; Coyle et al., 2005; Iversen and Eliassen, 2009; Neiffer and Stamper, 2009; Barrento et al., 2011; Javahery et al., 2012; Kiessling et al., 2009). This indicates a common mechanism of action that may transcend receptor inhibition. Mitochondrial function is largely conserved across eukaryotes, and, as expected, the mitochondrial respiration rate was inhibited with increasing isoeugenol concentration (Fig. 3). A maximal inhibition concentration is likely to be above 6 µmol l^{-1}, with an IC_{50} of 2.4 µmol l^{-1} (Fig. 3). These values are consistent with the data from the commercial supplier AQUI-S®, which suggest 30 ppm (approximately 1 µmol l^{-1}) for sedation of whole crustaceans, and concentrations exceeding 200 ppm isoeugenol for euthanasia (listed at 250 ppm for 2 h or 7.6 µmol l^{-1}, https://www.aqui-s.com/applications/euthanasia). The effect of eugenol is dose dependent, with sedation in male rats at 1.5 µmol l^{-1} (50–60 mg kg^{-1}) i.v. and has a half-life in plasma or blood of 14 and 18 h, respectively (Mohammadi Nejad et al., 2017). For finfish, isoeugenol is considerably more potent, as sedation is apparent at 0.15–0.30 µmol l^{-1} (5–10 ppm) for trout. Rotenone is also well known for its potency in finfish, and while reversible, isoeugenol has similar inhibitory effects to rotenone on mitochondrial respiration (IC_{50} ∼2 µmol l^{-1}).

Eugenol and isoeugenol have also been proposed to have antioxidant capacities, yet both may elicit pro-oxidant activities, as they deplete intracellular levels of reduced glutathione (GSH) in cultured human submandibular cells (Atsumi et al., 2005). In addition, isoeugenol had an order of magnitude greater cell cytotoxic response (CC_{50} 50 µmol l^{-1}) than eugenol, with greater elevations in ROS production (Atsumi et al., 2005). Investigations of ROS production and antioxidant defences are warranted for isoeugenol treatment of lobsters. These may provide further information of the mechanism and effects of isoeugenol as an anaesthetic.

The inhibition of mitochondria by isoeugenol may be considered a negative property and detrimental for animal survival. However, the mechanisms of common anaesthetics remain unclear even in medical settings. Numerous common anaesthetics reversibly inhibit mitochondria in addition to their blockade of neurotransmitter receptors, as oxygen concentrations often increase in tissues prior to suppression of neuronal activation (Voss and Sleigh, 2020). Moreover, mutation of GABA receptors in mice oblates electrophysiological responses of propofol, yet the hypnotic response remains (Lee et al., 2015). Moreover, inhibition of CI may be beneficial. CI is commonly depressed in reperfusion injury, and amobarbitone reversibly inhibits CI, yet following drug washout is protective of CI activity in tissue reperfusion settings (Chen et al., 2018). Further work could explore mitochondrial function and stability following emersion with and without isoeugenol.

Our data show that isoeugenol inhibits mitochondrial respiration, and its most potent effect is at CI. However, isoeugenol may further inhibit other respiratory complexes within the ETS such as CCO. While these processes should decrease mitochondrial ATP synthesis and enhance anaerobic metabolism, isoeugenol and eugenol have been found to decrease lactate accumulation in animals exposed to hypoxic environments (Wie et al., 1997; Iversen and Eliassen, 2009; Small, 2004), and this indicates that higher order processes may be active with additional sites of action. Notably, metabolic suppression in hibernation also occurs with suppression of ATP turnover and mitochondrial ATP production, and this also coincides with depressed lactate formation (Chazarin et al., 2019; Fedotcheva et al., 2010). If cardiac mitochondrial respiration is a reliable indicator of whole-animal metabolic activity, isoeugenol clearly reversibly decreases mitochondrial function in lobster heart and may therefore provide mechanisms for metabolic suppression.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.R., A.J., A.J.R.H.; Methodology: J.R., C.P.H., A.J.R.H.; Validation: J.R., C.P.H., A.J.R.H.; Formal analysis: J.R., A.J.R.H.; Investigation: J.R., C.P.H., A.J.R.H.; Resources: A.J., A.J.R.H.; Data curation: A.J.R.H.; Writing - original draft: J.R., A.J.R.H.; Writing - review & editing: A.J., A.J.R.H.; Supervision: A.J., C.P.H., A.J.R.H.; Project administration: A.J.; Funding acquisition: A.J.

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