

Coral-released creatinine is a chemical cue for spawning in reef-building corals

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Abstract

Successful sexual reproduction in broadcast-spawning corals relies on colonies releasing gametes within a narrow timeframe. Environmental cycles regulate reproductive timing, but the processes coordinating gamete release among neighbouring colonies remain unknown. Here, we show that coral-derived creatinine acts as a chemical cue for spawning. Paired metabolomic and volatilomic profiling revealed that creatinine and/or creatine were approximately 400-fold more abundant in gravid *Acropora spathulata* immediately before spawning relative to non-gravid conspecifics. Targeted liquid chromatography-tandem mass spectrometry subsequently distinguished these metabolites and showed that creatinine in seawater increased approximately 10-fold during the 40 minutes preceding spawning. Creatinine was undetectable three months later, whereas creatine occurred during both periods and was more abundant outside spawning. Experimental exposure to creatinine advanced spawning in gravid *A. spathulata* by up to eight days, with responses detected at concentrations as low as 10 μM , and also accelerated spawning in *Montipora aequituberculata*. The enrichment of creatinine in gravid coral tissues, its progressive release into seawater immediately before spawning, and its capacity to induce gamete release across coral genera identify creatinine as a spawning cue. This chemical signalling mechanism provides a new explanation for fine-scale reproductive synchrony in reef-building corals and a candidate tool for mitigating spawning desynchronisation under climate change.

Significance statement

Mass coral spawning is one of the most synchronized biological events, yet the molecular triggers coordinating gamete release remain unknown. This work identified creatinine produced in high amounts by corals before spawning, which when artificially supplied to the surrounding water, induced gamete release across coral genera. Creatinine concentrations significantly increased in natural seawater during spawning, suggesting this chemical may be a universal chemosignalling mechanism used by many species at the reef scale. Our results provide evidence of intraspecific chemical communication in adult cnidarians and identify the long-overlooked biochemical trigger of coral mass-spawning events.

Introduction

Coral mass spawning is a large-scale, highly synchronised event during which reef-building coral colonies release gametes within a very narrow time frame (i.e., minutes to hours; Baird et al. 2021). As adult corals are benthic and immobile, the synchronicity of this event across large spatial scales is critical for high levels of fertilisation and genetic recombination, representing the sole annual opportunity for sexual reproduction in ~80% of coral species (Harrison 2011). Several environmental factors regulate gamete development and the timing of spawning for many coral species, with seawater temperature (Lin and Nozawa 2023), the lunar phase (Komoto et al. 2023) and diel light dynamics (Boch et al. 2011) determining the month during which corals spawn. However, the final cues that determine the precise time (day, hour) of synchronous gamete release are currently unknown.

In other marine species, such as polychaetes, echinoderms and other marine invertebrates (Watson et al. 2003), the synchronised release of gametes is often biochemically driven. Yet, to our knowledge, there is no clear evidence of the role of specific molecules in driving the highly synchronous release of gametes among neighbouring, reproductively compatible coral colonies. More generally, direct evidence for intraspecific chemical communication among adult cnidarians remains limited (Table S1). Previous studies investigating the role of chemical cues in coral spawning have focused primarily on hormones. Sex steroids, such as oestrogens (notably oestradiol), peak immediately prior to spawning in *Sinularia polycactyla* (Slattery et al. 1999). In contrast, estradiol dosing to gravid *Montipora capitata* caused a reduction in sperm–egg bundles (Tarrant et al. 2004). Few studies have also tested the effects on corals of compounds that induce spawning in other marine invertebrates (Flint and Than 2016), but those tested are often detrimental to survival (e.g., hydrogen peroxide) or are indistinguishable from controls (e.g., 5-hydroxytryptophan; Flint and Than 2016). Other studies have shown that some compounds are not only ineffective at inducing coral spawning but also actively inhibitory, such as dopamine that inhibits spawning in *Acropora tenuis* (Isomura et al. 2013). As such, we currently do not know if biochemical cues are involved in gamete release during mass coral spawning.

Spawning asynchrony is exacerbated by abnormal environmental conditions caused by anthropogenic climate change, particularly ocean warming and the increased frequency of marine heat waves, which drive temperature anomalies that disrupt gametogenic timing and the alignment of environmental cues required for synchronous spawning (Shlesinger and Loya 2019). Consequently, as reproductively compatible adults spawn over increasingly disparate times, both the number and genetic diversity of viable offspring diminish (Shlesinger and Loya 2019). If specific compounds are involved in spawning synchronisation, their identification will offer a promising new avenue for intervention strategies that rely on spawning synchrony to bolster offspring populations and genetic diversity (Baums et al. 2022; Banaszak et al. 2023).

We adopted an iterative approach commonly employed in drug discovery to elucidate the biochemical cues that induce coral spawning. Specifically, we measured the compounds produced and emitted by gravid *Acropora spathulata* directly prior to spawning using metabolomic and volatilomic approaches (Bartels et al. 2024) and identified compounds that were significantly enriched in pre-spawning gravid colonies compared with their non-gravid conspecifics. We then tested whether these compounds could induce early spawning in *A. spathulata* and subsequently in other coral genera, and whether natural seawater concentrations demonstrate similar peaks in target compounds during spawning. Our results provide evidence of intraspecific chemosignalling between adult cnidarians and identify specific compounds that can trigger gamete release in corals, opening the door to novel strategies to mitigate the desynchronisation of coral spawning under climate change.

Results and Discussion

The search for chemical cues involved in coral spawning

We assessed metabolites in pre-spawning and non-gravid *A. spathulata* from the Great Barrier Reef (Australia), more specifically those present in the tissue, mucus, and the emitted volatiles. Metabolite profiles of the tissue and volatiles emitted into the water were significantly different between gravid and non-gravid corals (PERMANOVA, $p < 0.01$; Fig. 1, Table S2). Sixty compounds were more abundant in gravid corals directly prior to spawning onset (t-tests, $p < 0.05$; Fig. 1, Table S3), with 33 of these compounds increasing by more than 5-fold. Two tissue-derived metabolites, creatine and/or creatinine (their matching retention indices and fragmentation patterns make them indistinguishable via the gas chromatography-mass spectrometry (GC-MS) methods used here), increased 400-fold directly prior to the release of gametes. Creatine is commonly found in vertebrates and is involved in ATP recycling, but it may also function as a neurotransmitter (Mallik and Frank 2023). Creatine is synthesised through the methylation of guanidinoacetate, catalysed by guanidinoacetate *N*-methyltransferase (GAMT), and is reversibly phosphorylated to phosphocreatine by creatine kinase. Creatine-based phosphagen provide spatial and temporal buffering of ATP in cells with high and rapidly changing energetic demands, and are present in cnidarians. In marine invertebrate reproductive tissues and gametes, for example, a creatine kinase-dependant phosphocreatine shuttle transports high-energy phosphate along the flagellum of sea urchin sperm (Wothe et al. 1990). Both creatine and phosphocreatine can undergo spontaneous, nonenzymatic cyclisation to form creatinine (Kashani et al. 2020). We identified putative genes encoding GAMT and creatine kinase in the genomes of several *Acropora* species, indicating that corals possess the genetic capacity for creatine-phosphocreatine metabolism (Table S4). Creatinine released immediately before spawning could therefore originate from creatine-associated energy metabolism in gravid tissue or gametes. Importantly, metabolic by-products can acquire signalling functions when their abundance reliably reflects physiological state: in the polychaete *Platynereis dumerilii*, the nitrogenous metabolic end product uric acid is released with eggs and induces sperm release at submicromolar concentrations (Zeeck et al. 1998). We therefore hypothesise that creatine and/or creatinine may act as a short-range final-release cue or amplifier, reinforcing synchrony among colonies already primed to spawn by environmental and physiological signals.

A total of 24 volatile metabolites also increased by more than 5-fold, with the largest increases observed in compound classes with high volatility, such as aromatic hydrocarbons (primarily benzenoids) and esters with aromatic substituents, including methyl methacrylate and acetic acid derivatives. Seven mucus-derived metabolites increased by more than 5-fold in gravid corals, including gamma-aminobutyric acid (GABA) which has previously been shown to induce spawning in the sea urchin *Strongylocentrotus intermedius* upon injection into the body cavity (Takahashi 1986).

Spawning-associated concentrations of creatine and creatinine in seawater

For a metabolite to act as a waterborne inter-colony cue, it must be released into seawater and remain available long enough to reach nearby colonies at a biologically active concentration. Both creatine and creatinine are small, relatively stable polar compounds (18) that can diffuse quickly in seawater. In addition, compounds involved in, or produced during, the physiological transition to spawning are likely to become enriched in the surrounding seawater. Because the unresolved creatine/creatinine signal was

initially detected only in coral tissue, we used LC-MS/MS to discriminate and quantify both compounds in seawater collected 10-40 min before spawning and in comparative system-water samples collected three months later.

Creatinine was detected in seawater immediately before spawning but was not detected in any of the seawater samples collected three months later (Fig. 2; Table S7). Furthermore, the concentration of creatinine increased 10-fold in the minutes preceding spawning, from 0.01 μM 40 minutes before to 0.1 μM 10 minutes before spawning (Table S7). In contrast, creatine was detected during both sampling periods and occurred at substantially higher concentrations in post-spawning aquarium water than in aquarium water collected immediately before spawning. This broader temporal occurrence suggests that creatine is unlikely to function as a temporally specific natural inter-colony cue for coral mass spawning.

Creatinine induces spawning in *Acropora spathulata*

The spawning-associated occurrence of creatinine and the broader occurrence of creatine identify creatinine as the more plausible candidate inter-colony spawning cue. Alongside creatinine, all compounds that increased by more than 8-fold (3 compounds), together with those involved in reproduction and/or chemical communication (in corals or other organisms) were selected for further testing (Fig. 1d, Table S6): 1-acetonaphthone, phenyldodecane, decanal, GABA, methyl methacrylate, naphthalene and tridecane. The effects of these compounds on gravid *A. spathulata* were measured to determine their potential roles as chemical triggers of spawning.

In an initial trial, the addition of creatinine (1 mM nominal concentration) dosed individually to the water surrounding gravid corals induced spawning in 67% (4/6) of replicate colonies 8 nights prior to the peak spawning night of control fragments. None of the other 7 chemical compounds advanced spawning under the conditions tested, including the neurotransmitter GABA, a known inducer of spawning in sea urchins (Takahashi 1986). Despite the elevated abundance of GABA in the mucus layer of gravid *A. spathulata* (Fig. 1d), exposure to 1 mM GABA did not induce spawning in *A. spathulata* (1 mM). Extracellular GABA alone was therefore insufficient to trigger gamete release in *A. spathulata* under the conditions tested, although its enrichment may reflect an internal or localised role in the spawning process but not as an inter-colony cue. Furthermore, although our multiplatform analysis captured substantial chemical diversity across coral tissues, mucus and emitted volatiles, it represents only a subset of naturally occurring metabolites, and additional spawning-associated compounds may remain undetected. Nevertheless, creatinine was the only compound examined here to advance spawning, indicating that the response was not a nonspecific consequence of chemical addition (Table S6).

During the same spawning event, we conducted a concentration-range experiment using 10, 100 μM and 1 mM creatinine, extending the tested range two orders of magnitude below the initial 1 mM treatment. These concentrations remained higher than the approximately 0.1 μM detected in bulk seawater and were therefore selected to test whether creatinine could advance spawning under controlled conditions, rather than to reproduce measured environmental concentrations directly. Importantly, the concentration

detected in bulk seawater represents the net residual concentration after localised release, dilution, dispersion, uptake and potential transformation. It therefore does not necessarily represent either the initial amount released or the transient concentration encountered at the biologically relevant site of action. Short-lived concentration gradients within the coral diffusive boundary layer could be substantially higher than those measured in the surrounding bulk seawater, although their magnitude has not yet been quantified. Furthermore, the approximately 400-fold enrichment of creatinine in gravid coral tissues indicates a substantial organism-associated pool that is not represented by bulk-seawater measurements. Accordingly, this experiment was designed as a proof-of-biological-response and initial concentration-ranging assessment.

This subsequent trial began seven nights before the predicted peak spawning night. At 10 μM creatinine, two of the three fragments that spawned did so following the first night of dosing, 6 days earlier than the mean spawning time of the control fragments; the third fragment spawned at approximately the same time as the controls. Thus, early spawning occurred in 67% of fragments that spawned ($n = 2/3$), or 50% of all gravid fragments exposed to this treatment ($n = 2/4$). At 100 μM , all three fragments spawned, with one spawning 1 day earlier than the average control spawning time and the remaining two spawning at approximately the same time as the controls. Dosing with 1 mM creatinine significantly accelerated spawning in 100% of *A. spathulata* fragments. These fragments spawned an observed average of 3.5 days earlier than the spawning controls (range: 3–4 days), while the fitted model estimated a 4.26-day reduction in time to spawning (logistic survival model: coefficient = -4.255 , SE = 1.132 , $z = -3.760$, $p < 0.001$; Fig. 3a; Tables S7–S8). The ability of creatinine to accelerate spawning when added directly to seawater suggests that corals can sense or take up these compounds, which may then influence reproductive processes. If released by a gravid colony, these compounds may stimulate spawning in neighbouring corals, either by promoting gamete maturation, triggering gamete release or via secondary chemosignalling pathways. Further experiments incorporating lower concentrations and measurements closer to the tissue–water interface are required to establish the response threshold and determine its environmental relevance.

Species-specific or genera-general?

Creatinine is a common metabolite that has previously been identified in other coral genera (e.g., *Pocillopora* and *Seriatopora* (Pei et al. 2023), both of family Pocilloporidae) but not specifically during spawning. Given the universality of this compound, its success in inducing spawning in *A. spathulata* and the potential implications for addressing spawning desynchronisation, we next sought to determine whether its effect is specific to *A. spathulata* or potentially common across genera with similar reproductive modes. We therefore repeated the experiment by exposing *Montipora aequituberculata* (another genus of family Acroporidae), to 1 mM creatinine.

The exposure of *M. aequituberculata* to 1 mM creatinine resulted in spawning 1 night earlier than in the controls (AFT modelling, time ratio = 0.478 , $p = 0.001$; Fig. 3b, Table S7). However, inference for this species is constrained by reduced sample size resulting from heterogenous gravidity of fragments and

by inherent variation in natural spawning timing among controls ($n = 2$). Notably, the synchronicity of gamete release was more tightly conserved in *M. aequituberculata* dosed with 1 mM creatinine, with spawning occurring over 2 consecutive nights, whereas control corals spawned across 4 nights. Taken together, these results suggest that creatinine can induce earlier and more synchronous gamete release in different broadcast spawning genera.

Conclusion

Coral spawning is a spectacular natural phenomenon that has been extensively studied since its discovery four decades ago (Harrison et al. 1984), yet the chemical processes coordinating the final release of gametes have remained elusive. Here, we provide direct experimental evidence that creatinine can trigger gamete release in gravid *A. spathulata* and *M. aequituberculata*, together with evidence that creatinine is strongly enriched in gravid coral tissues and increases in seawater immediately before spawning. These convergent findings establish creatinine as a bioactive, spawning-associated molecule and support a role for small-molecule cues in coral spawning synchronisation. Whether creatinine acts directly on the gamete-release machinery, promotes final gamete maturation or has additional physiological or ecological functions remains to be determined. Chemical signalling between conspecifics plays well-recognised roles in sexual reproduction (Watson et al. 2003), aggregation (Ng et al. 2013) and threat signalling (Kamio et al. 2022) across marine taxa, yet its diversity and functionality in adult cnidarians remains largely unexplored. Our findings therefore provide a foundation for investigating how these early-diverging animal use chemical cues within and among species, and how such communication shapes their ecology.

Understanding and manipulating the biochemistry of coral spawning also provides a novel opportunity to enhance the scalability of restoration efforts based on coral sexual reproduction, including larval seeding and selective breeding (Banaszak et al. 2023). A chemical trigger could enable more reproductively compatible colonies to release their gametes within the same spawning window, expanding the pool of simultaneously available gametes and potentially increasing fertilisation success, offspring production and the representation of parental genetic diversity. Thus, direct dosing of creatinine to gravid corals could prove a useful and urgently needed tool to address the rising issue of spawning desynchronisation due to climate change (Shlesinger and Loya 2019).

Methods

Twelve partial colonies of *Acropora spathulata* (minimum surface area ~ 100 cm²) were collected from Davies Reef (18°49'48" S, 147°37'48" E; GBRMPA permit G21/38062.1) on 8 December 2022, one day after the full moon. Colonies were maintained at the National Sea Simulator (SeaSim, AIMS, Australia) in a 285 L semi-closed aquarium with filtered seawater at 28.5°C, undergoing three complete system turnovers daily. Aquaria were exposed to natural solar and lunar light cycles, allowing corals to perceive ambient full-moon cues while under controlled temperature conditions. The collection comprised six gravid colonies (confirmed through visualisation of mature egg bundles in nubbin cross sections) and six

non-gravid colonies that had spawned at AIMS one month earlier and been tagged for re-collection. Samples were collected for headspace volatile profiling and solvent-extracted metabolite profiling from gravid corals over three nights; colonies were sampled if they showed signs of “setting”, defined here as visible egg bundles within polyp mouths. To account for variability in spawning timing, only measurements taken directly before spawning (irrespective of the specific night for individual colonies) were compared with those from non-gravid colonies.

Measuring emitted volatile compounds

On sampling nights, corals were transferred ~30 min after sunset to airtight 3 L glass chambers (IKEA, Netherlands) and gently aerated with instrument grade air (BOC, Australia) via air stones at a flow rate of ~400 mL/min measured at the outflow valve (Fig. S1). Samples were taken via methods adapted from previous volatilomics studies (Lawson et al. 2021; Olander et al. 2023). Briefly, chambers were pre-purged for a minimum of 15 min to remove potential volatile contaminants from the water before attaching thermal desorption (TD) tubes (200 mg Tenax TA; Markes International Ltd., Llantrisant, UK). Chambers were purged for an additional 15 min, beginning ~30 min before spawning was expected, with the outlets collected onto TD tubes. Tubes were immediately capped following sampling and stored at 4°C until analysis (within 1 month of collection).

TD tubes were analysed as described by Bartels et al. (Bartels et al. 2024). 0.2 µL of chlorobenzene-D5 was added to each tube as an internal standard prior to desorption. TD tubes were desorbed at 300°C, concentrated at -30°C, flash heated to 300°C for injection and analysis via GC–MS (Agilent Technologies Inc., California, USA) with a mass-selective detector (Model 5975C; Agilent Technologies Inc., California, USA). The data were pre-processed to remove contaminating ions (73, 84, 147, 149, 207, 221 *m/z*) via OpenChrom Lablicate Edition (version 1.5.0, Lablicate GmbH, Hamburg, Germany) and the metaMS package (Wehrens et al. 2014) through the Galaxy Project platform (The Galaxy Community 2022). Compounds were assigned to peaks via the NIST mass spectral search program (NIST MS Search 2.2; NIST, Maryland, USA). All values were normalised to the surface area of the coral as estimated via photogrammetry (see below).

Tissue metabolite extractions

Small fragments (~2–3 cm branches from each colony) were taken for metabolite extractions from coral tissues and solid-phase extractions (SPE) of coral mucus from the same samples and were immediately snap frozen. Coral fragments were rinsed in 50 mL of 4°C ultrapure Milli-Q water to remove mucus and any remaining seawater, and the rinse water was collected into sterile tubes and frozen at -80°C for subsequent metabolite extraction via SPE. The tissue was removed from the coral skeleton by airbrushing into 10 mL of chilled (4°C) Milli-Q water as per Matthews et al. (2023). The resulting tissue slurry was lyophilised overnight in an Alpha 2-4LD freeze dryer (CHRIST, Lower Saxony, Germany). Metabolites were extracted from dried tissues as described by Bartels et al. (2024). 400 µL of 100% LC–MS-grade methanol containing the internal standard D-Sorbitol-¹³C6 (20 µg/mL final concentration) was added to the samples before bead-milling at 50 Hz for 3 min. An additional 600 µL of LC–MS-grade

methanol was added to samples before incubating them at 4°C on a rotisserie shaker for 30 min. Following centrifugation at 3000 × g for 30 min at 4°C, the supernatant was transferred to a new tube, and the remaining pellet was washed with 1000 µL of 50% methanol (at 4°C), vortexed, and centrifuged again (as above). The resulting supernatant was pooled with the first extracts and centrifuged at 16,100 rpm for 15 min to remove precipitates, and 250 µL of each sample was dried in 50 µL increments in glass inserts using an Eppendorf Vacufuge 5301 Concentrator Centrifuge (Eppendorf, Hamburg, Germany). The pellet was maintained at -80°C for subsequent protein content analysis.

Mucus metabolite extractions

Mucus samples were acidified with 20% hydrochloric acid to a pH of 2. Oasis hydrophilic–lipophilic balanced sorbent SPE cartridges (6 cc, 200 mg of universal polymeric reversed-phase sorbent; Waters Corporation, Massachusetts, USA) were attached to a vacuum manifold chamber and primed by passing 6 mL of 100% liquid chromatography–mass spectrometry (LC–MS)-grade methanol through the column, followed by the addition of 6 mL of Milli–Q water. The acidified samples were passed through the columns, followed by 6 mL of 0.01N HCl to remove salts from the column. Columns were then dried under vacuum at ~10 Hg for 20 min. Once dry, the metabolites were eluted from the column with 2 mL of 80% LC–MS grade methanol, and then, 1.5 mL of the extract was dried in 500 µL increments using an Eppendorf Vacufuge 5301 Concentrator Centrifuge (Eppendorf, Hamburg, Germany).

Both the tissue and mucus layer metabolite samples were processed and derivatised online via GC–MS by Metabolomics Australia using a gas chromatograph and triple-quadrupole mass spectrometer according to the methods described in Matthews et al (2023). Samples were reconstituted in 25 µL of methoxyamine hydrochloride (30 mg/mL in pyridine) and then shaken at 37°C for 2 h before 25 µL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, Thermo Scientific) was added to each sample, which were then incubated at 37°C for 1 h. The samples were incubated with $^{13}\text{C}_5$, $^{15}\text{N}_1$ valine and $^{13}\text{C}_6$ sorbitol, added as internal standards. The sample was left for 1 h before 1 µL was injected onto the GC column using a hot needle technique. Split (1:10) injections were done for each sample. The GC-MS system used consisted of an AOC6000 autosampler, a 2030 Shimadzu gas chromatograph and a TQ8040 quadrupole mass spectrometer (Shimadzu, Japan). The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). GC-MS was performed on a 30 m Agilent DB-5 column with 1 µm film thickness and 0.25 mm internal diameter column. The injection temperature (Inlet) was set at 280°C, the MS transfer line at 280°C and the ion source adjusted to 200°C. Helium was used as the carrier gas at a flow rate of 1 mL/min and Argon gas was used as the collision cell gas to generate the MRM product ion. The analysis of the derivatised samples was performed under the following temperature program; start at injection 100°C, a hold for 4 min, followed by a 10°C min⁻¹ oven temperature ramp to 320°C following final hold off for 11 min. Approximately 520 quantifying multiple reaction monitoring (MRM) targets were collected using Shimadzu Smart Database along with a qualifier for each target which covers about 350 endogenous metabolites and multiple ^{13}C labelled internal standards. Chromatograms and multiple reaction monitoring product ions were assessed in the Shimadzu GC-MS browser and

LabSolutions Insight software. Samples in both datasets were normalised to their respective internal standards. The tissue metabolite data were further normalised to the protein content of the extracted pellet, which was calculated via the Bradford colorimetric method (Bradford 1976). Metabolite data obtained from the mucus layer were normalised to the surface area of the coral fragment.

Surface area estimation

Following metabolite and volatile sampling, structure-from-motion photogrammetry was used to determine coral surface area. The corals were mounted on a white stage with a reference grid of known dimensions and photographed (~60 – 110 images, depending on colony size and complexity) while the stage was rotated to cover all possible angles. Images were taken using an iPhone 13 Pro (resolution = 4032 × 3024 pixels, ISO sensitivity = 40, focal length = 26 mm, f-stop = 1.5, shutter speed = 1/100 s; Apple, California, USA). These images were then used to estimate the surface area of each colony as per Olander et al. (Olander et al. 2023); 3D models were constructed in Meshroom (v. 2021.1.0m AliceVision; Griwodz et al. 2021) and scaled using the stage reference grid in Blender (v. 2.93.0, Blender Foundation).

Compound data processing and statistical analysis

Given the exploratory nature of this initial experiment and the inherent variability that may occur when grouping colonies that spawned over different nights, we applied more lenient quality controls to our data than those typically used in volatilomic or metabolomic work to avoid the exclusion of potentially important compounds. Volatile compounds were excluded from the analysis if they appeared in less than three replicates for either gravid or non-gravid colonies (original $n = 6$). Metabolites from both the tissue and mucus layer datasets were not filtered. Different criteria for the exclusion of data were applied because volatile data are inherently more variable, and without such thresholding, differences in compounds were masked in the analysis. All datasets were $\log_2$ -transformed and mean-centred prior to all analyses. Normality and homogeneity of variance were assessed, and non-parametric alternatives used were necessary.

Principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA), a permutation based upper tail test, were performed using Euclidean distances with 999 permutations with the Vegan package (version 2.6.4) in R (version 4.2.1; R Core Team, 2022). These analyses were conducted to determine if the full suite of metabolites found in the coral tissue, mucus layer, and volatilome varied between gravid and non-gravid corals.

Moderated T-tests (Limma) were used to compare differences in the abundance of compounds between samples taken directly prior to spawning versus non-gravid colonies. Significance was assessed following Benjamini-Hochberg FDR correction with a significance threshold of $FDR < 0.05$. Only compounds that were significantly more abundant in gravid colonies were investigated, as those less abundant are unlikely to serve as inter-colony chemical cues for spawning. Compounds that increased more than 5-fold in abundance in colonies just prior to spawning were further investigated to determine their functions both in corals and other organisms. If these compounds were identified to be involved in

reproduction in some way (e.g., as internal cues, pheromones that are highly abundant in other fertile organisms, etc.), they were shortlisted as potential spawning cues. Any compounds that increased more than 8-fold that did not meet these criteria but were still either commercially available or readily synthesised were also shortlisted. In total, 9 compounds met these criteria and were selected to be dosed to *A. spathulata* to determine if they were indeed a trigger for the release of gametes: 1-acetonaphthone, 6-phenyldodecane, creatine, creatinine, decanal, gamma aminobutyric acid, methyl methacrylate, naphthalene and tridecane.

Seawater analysis of creatine and creatinine.

To place experimental dosing concentrations in an environmental context, additional seawater samples were collected from the SeaSim experimental system (described above) in the minutes immediately preceding spawning and three months after spawning. These measurements reflect bulk seawater concentrations within the experimental system and provide a reference against which the dosing concentrations can be interpreted (Table S7). Replicate 1 L of seawater samples were collected from the same system on night 1 at 25 and 10 mins ($n = 3$ per timepoint) prior to spawning, and on night 2 at 40 and 15 mins ($n = 6$ per timepoint) prior to spawning, and postspawning ($n = 3$). Seawater measurements represent distinct samples collected from the same aquarium system at each stated time point; they were used to characterise temporal changes and were not treated as independent replicate systems.

Water samples were independently processed using solid phase extractions using 500 mL of each water sample (as described above). Creatine and creatinine concentrations in extracts were quantified using a Shimadzu LCMS-8060 triple-quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. Chromatographic separation was achieved on a Waters CORTECS HILIC column (2.7 μm , 50 mm $\times$ 2.1 mm) maintained at 30 °C. A 6-minute isocratic elution was performed using 80% acetonitrile containing 4 mM ammonium formate at a flow rate of 0.25 mL min⁻¹. The injection volume was 1 μL . Calibration curves were constructed using the peak area ratio of analyte to the internal standard, with concentrations extending to 100 ppb (μM) for both creatine and creatinine. The lowest non-zero calibration standards were 0.5 ppb (0.0038 μM) for creatine and 0.2 ppb (0.0018 μM) for creatinine. D₃-creatine was added to all calibration standards and samples at a concentration of 10 ppb. The first MRM transition for each compound was used for quantification, and the remaining transitions used as qualifiers for confirmation. The monitored MRM transitions (collision energy, CE; retention time, RT) were as follows: D₃-creatine, m/z 135.1 $\rightarrow$ 93 (−15 eV), 135.1 $\rightarrow$ 47.2 (−15 eV), and 135.1 $\rightarrow$ 43.1 (−20 eV), RT 3.71 min; creatine, m/z 132.1 $\rightarrow$ 90.2 (−15 eV) and 132.1 $\rightarrow$ 44.1 (−15 eV), RT 3.71 min; and creatinine, m/z 114.0 $\rightarrow$ 44.3 (−15 eV) and 114.0 $\rightarrow$ 86.0 (−15 eV), RT 2.36 min. Creatine and creatinine concentrations were converted from ppb to μM using molecular masses of 131.13 and 113.12 g mol⁻¹, respectively. Seawater data were summarised descriptively in R (version 4.2.1; R Core Team, 2022), and the figure was constructed using the “ggplot2” package. Arithmetic means $\pm$ SE were calculated from untransformed concentrations among samples with a numerical signal, including values extrapolated below the calibrated range. Samples in which no signal was detected were excluded from these

calculations. No formal inferential comparisons were performed because the samples represented two spawning nights and a single post-spawning sampling occasion.

Inducing spawning in *Acropora spathulata*

During the next summer spawning season (November–December 2023), six colonies of *Acropora spathulata* were collected from Davies Reef (permit number G21/38062.1) one day after the full moon (28th November 2023) and held at SeaSim (as described previously). Each colony ($n = 6$) was immediately broken into 11 partial colony fragments and allocated across 8 treatment and 3 control groups (Table S6). Each fragment was measured once. Fragments originating from the same parent colony were distributed across treatments, such that treatment groups were matched by colony identity; parent colony was therefore included as a blocking factor in the analysis. The fragments were allowed to recover for one day before experimentation began.

Chemical dosing began 2 nights post full moon (~6 nights prior to expected spawning, although source colonies ultimately spawned 9 – 10 nights after dosing began). The corals were subjected to their respective treatments in 550 mL glass jars that contained 300 mL of fresh 10 μm filtered seawater at 27°C at sunset. Care was taken to minimise air exposure while also minimising the amount of holding tank water entering the treatment jars. Jars were continually aerated via gentle bubbling to prevent deoxygenation and held in water baths to maintain a temperature of $27 \pm 0.5^\circ\text{C}$. Dosing commenced 30 min after sunset (*A. spathulata* typically begins spawning at ~9 pm), with compounds added to yield final nominal concentrations as per Table S6. These concentrations were determined by identifying those used in previous studies on other organisms or any biologically significant values. All concentrations were below the documented ED50 values in safety trials per each chemical's safety data sheet (Table S6). As some compounds required dissolution in ethanol (final concentration added to corals = <1%) and/or Milli-Q water, ethanol and Milli-Q water controls were also established.

Following dosing, corals were monitored closely for signs of setting as per the previous year. Once the fragments began spawning, aeration to the jar was immediately paused, and the timing of spawning recorded. Spawning was observed in four out of six replicates for creatinine after 2 nights of dosing only (Table S6). We then randomly allocated gravid fragments to creatinine at final nominal concentrations of 1 mM, 100 μM and 10 μM ($n=4$). Untreated and Milli-Q water controls were also run alongside the treatments.

Additional solid-phase extractions were performed to estimate the concentrations of creatinine recovered following dosing and to assess analytical recovery relative to the nominal dosing concentrations (Table S5). Extracts exceeding the calibration range were diluted before analysis and back-calculated using the corresponding dilution factor (2000-fold for the 1 mM samples, 200-fold for the 0.1 mM and 20-fold for the 0.01mM dilutions). Creatinine was recovered at all concentrations tested, with 1 mM, 100 μM and 10 μM back-calculated creatinine concentrations of 1.33, 409 μM and 68 μM respectively. Recovery therefore did not scale consistently with nominal concentration, with concentrations increasingly overestimated at the lower dosing levels. The back-calculated

concentrations (from ppb to μM) are provided in Table S5 but should be interpreted as estimates of analytical recovery rather than precise concentrations available to the corals.

Accelerated failure time modelling

Any fragments that had not spawned by the end of the experiment were cracked open using bone cutters and checked for the presence of visible eggs. Fragments containing eggs were classified as right-censored (gravid but not observed to spawn within the experimental window), whereas non-gravid fragments were excluded from analysis. Time-to-spawning was analysed using accelerated failure time (AFT) models implemented in the “survival” (version 3.5-7) and “survminer” (version 0.4.9) packages in R (version 4.2.1; R Core Team, 2022) to determine the efficacy of each compound and concentration in inducing spawning. AFT models directly estimate multiplicative shifts in spawning timing while retaining censored individuals in the analysis, providing a robust and biologically interpretable framework. Several parametric distributions were evaluated, and the distribution that provided the best fit with the least complexity was ultimately selected (Tables S9 & S10).

Determining the specificity or universality of spawning cues

To determine whether chemical cues inducing spawning were specific to *A. spathulata* or potentially universal across corals with similar reproductive modes, we subsequently repeated the experiment using *M. aequituberculata* during “out-of-season” spawning in May 2024. Corals were raised from larvae to adulthood at SeaSim in 1200 L semi-closed aquaria and have been successfully manipulated to spawn ~6 months earlier than their wild-reef counterparts using offset environmental profiles (Koukoumaftsis et al. 2024).

Six genetically distinct colonies were fragmented and allocated across treatments so that one fragment from each colony appeared in every treatment; control corals “dosed” with Milli-Q water and treatments with creatinine at a final concentration of 1 mM. Dosing commenced 30 min after sunset, after which corals were monitored closely for signs of setting and spawning. If spawning occurred, aeration to the jar was immediately ceased, and the timing of spawning recorded. AFT modelling was applied as per the methods described for *A. spathulata*, with the same censorship criteria applied to account for any fragments that were gravid and did not spawn and non-gravid fragments. This step proved to be essential for *M. aequituberculata*, which can exhibit non-homogeneous spread of egg bundles across the colony surface, ultimately resulting in the exclusion of a number of non-gravid fragments from the analysis.

Declarations

Data Availability

All raw metabolomics and volatilomics data files are available on the MassIVE mass spectrometry data repository; prior to publication, files are available via a temporary link

(<https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?accession=MSV000096894>) with password "Creatine". Following publication, the link will be made public and permanent (<ftp://massive.ucsd.edu/v09/MSV000096894/>). Processed data for statistical analysis are provided in the supplementary file.

Code Availability

All R scripts are available on GitHub (<https://github.com/NatashaBartels/coral-spawning-chemical-cues>).

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Author contributions

N.B, J.-B.R., J.L.M, D.J.S, C.A.L and M.P conceived of and designed this study. N.B, N.J.D, A.O, L.M.L, H.K and J.L.M collected the data with assistance from Z.J.C, D.J.H and L.K, and N.B, J.L.M, J.-B.R. and S.W. analysed the data. N.B. wrote the first draft of the manuscript, and all authors edited and approved subsequent versions.

Competing interests

The authors declare no competing interests.

Additional Information

Supplementary Information is available for this paper. Correspondence and requests for materials should be addressed to Jennifer.Matthews@uts.edu.au. Reprints and permissions information is available at www.nature.com/reprints.

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Figures

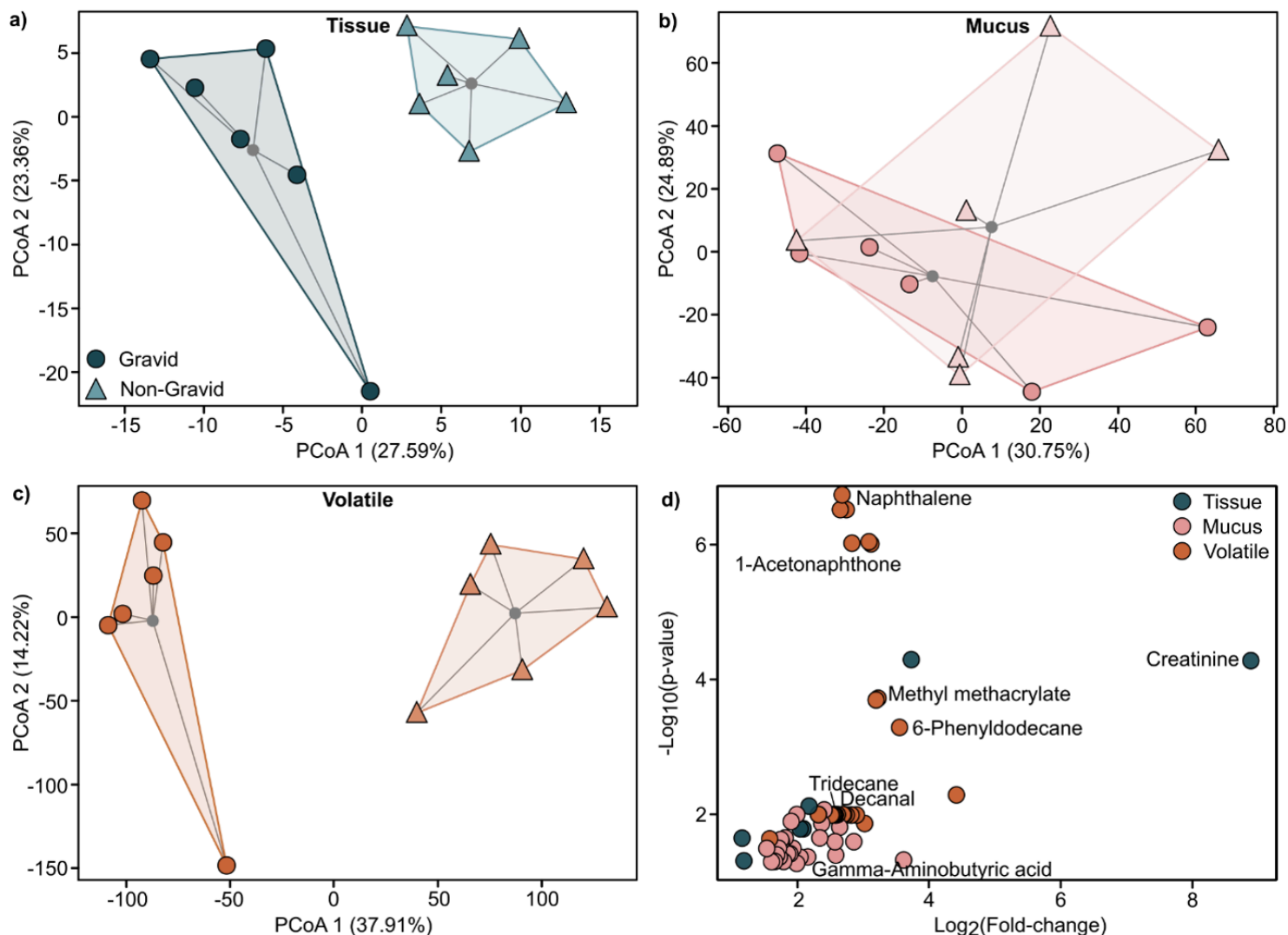


Figure 1

Principal coordinate analysis (PCoA) of the metabolite profiles of (a) coral tissues, (b) the mucus layer and (c) the volatilome of *Acropora spathulata* in pre-spawning (gravid) versus non-gravid colonies. (d) The 33 compounds that were more than 5-fold higher in pre-spawning gravid *A. spathulata* compared to non-gravid conspecifics. Only compounds that were ultimately selected for dosing are labelled.

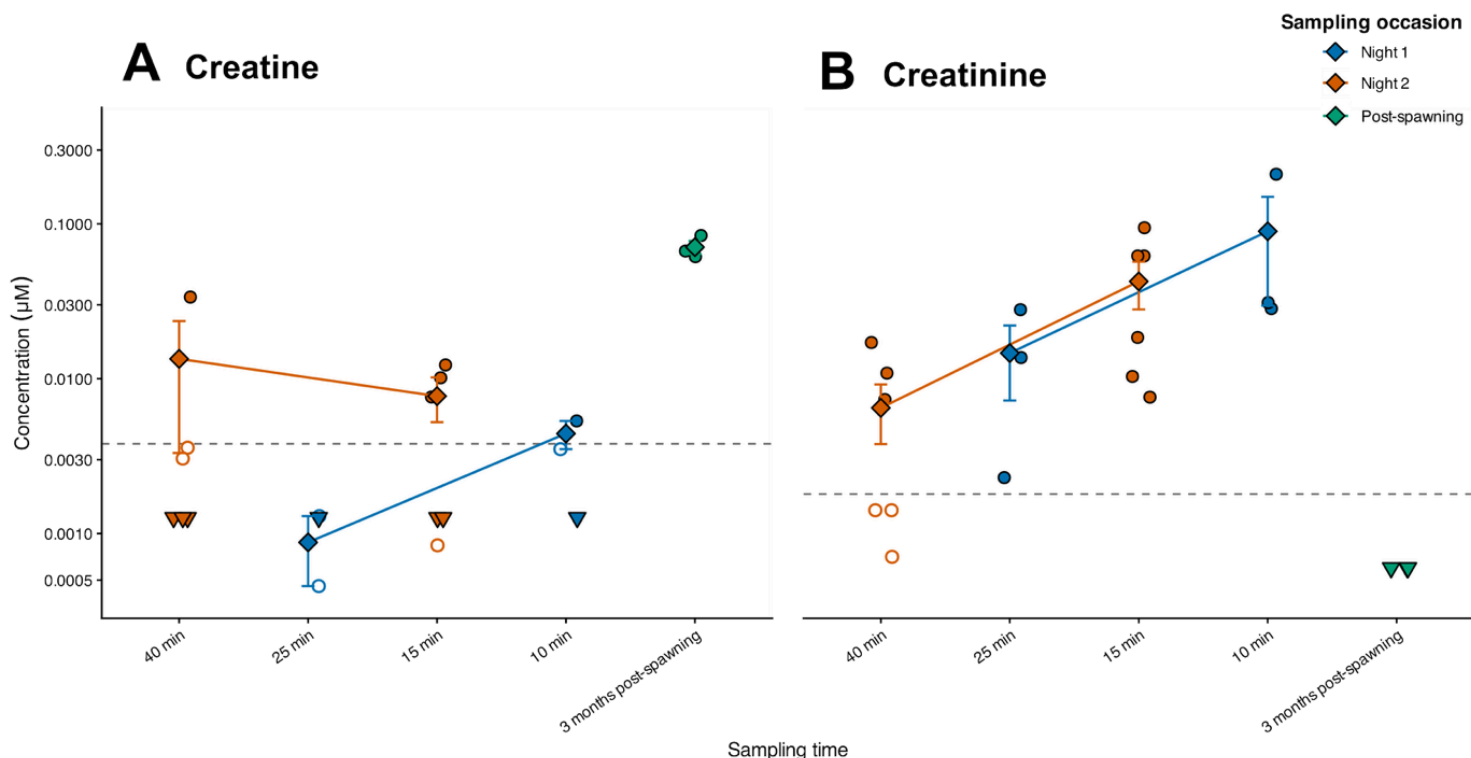


Figure 2

Creatine and creatinine concentrations in experimental system water immediately before coral spawning and three months after spawning. Concentrations are displayed on a logarithmic scale, with sampling occasion indicated by colour. Circles represent individual samples, and diamonds and error bars represent arithmetic means $\pm$ SE calculated from untransformed concentrations among samples with a numerical signal (Table S7). Dashed horizontal lines indicate the lowest non-zero calibration standards: 0.0038 μM for creatine and 0.0018 μM for creatinine. Values extrapolated below these concentrations were retained in the summary calculations and are displayed as hollow circles. Samples in which no signal was detected were not assigned a concentration or included in the summary calculations; for visualisation only, these samples are displayed as downward-pointing triangles at one-third of the corresponding lowest calibration concentration. Lines connect mean concentrations between sampling times within the same spawning night only. Only system water samples were included (40 and 15 mins $n = 6$ per treatment, 25 and 10 mins $n = 3$; postspawning $n = 3$).

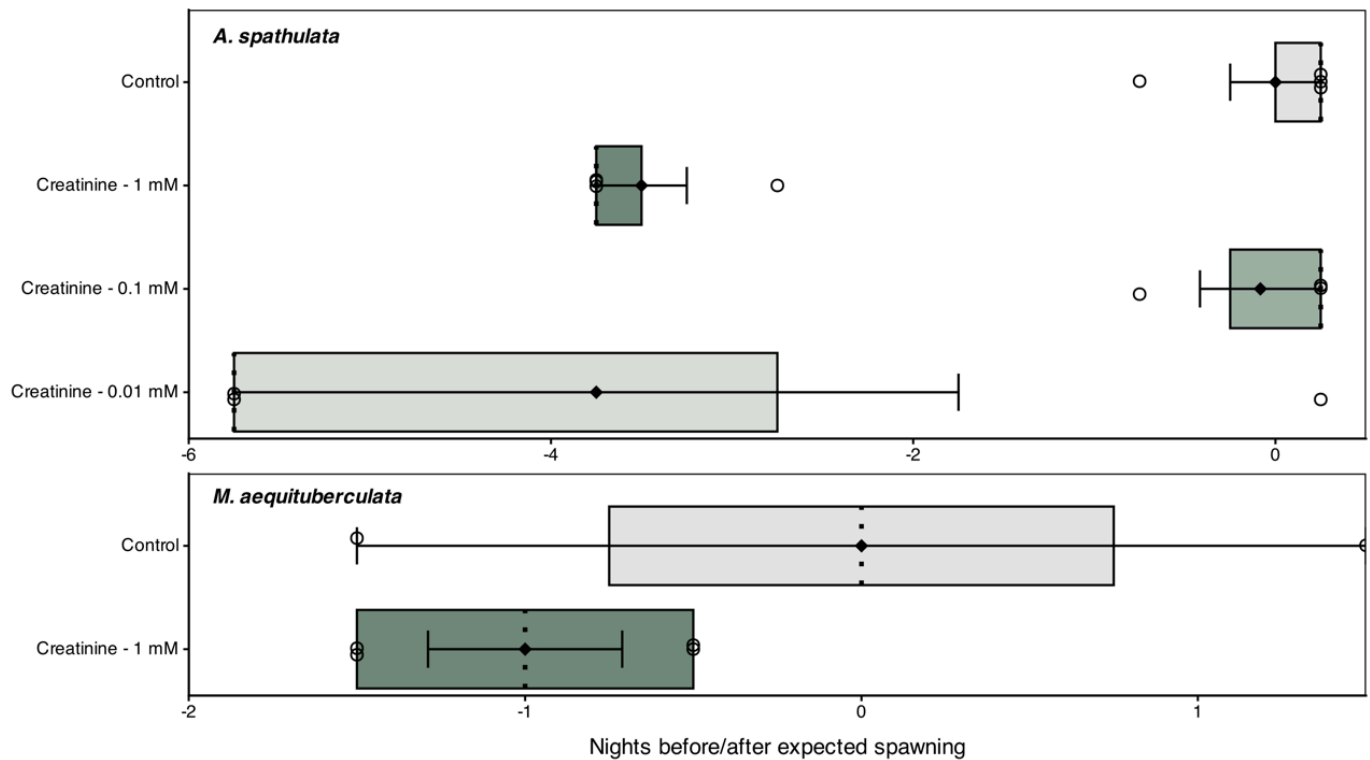


Figure 3

Timing of spawning for (a) *Acropora spathulata* and (b) *Montipora aequituberculata* fragments exposed to different concentrations of creatinine. The data are normalised to the average number of nights on which the control corals spawned. The dotted lines represent the median, diamonds represent the mean, and error bars represent the standard error of the mean. A significant difference was identified in the timing of spawning between control and creatinine 0.01 mM only (AFT modelling, $p < 0.05$).

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