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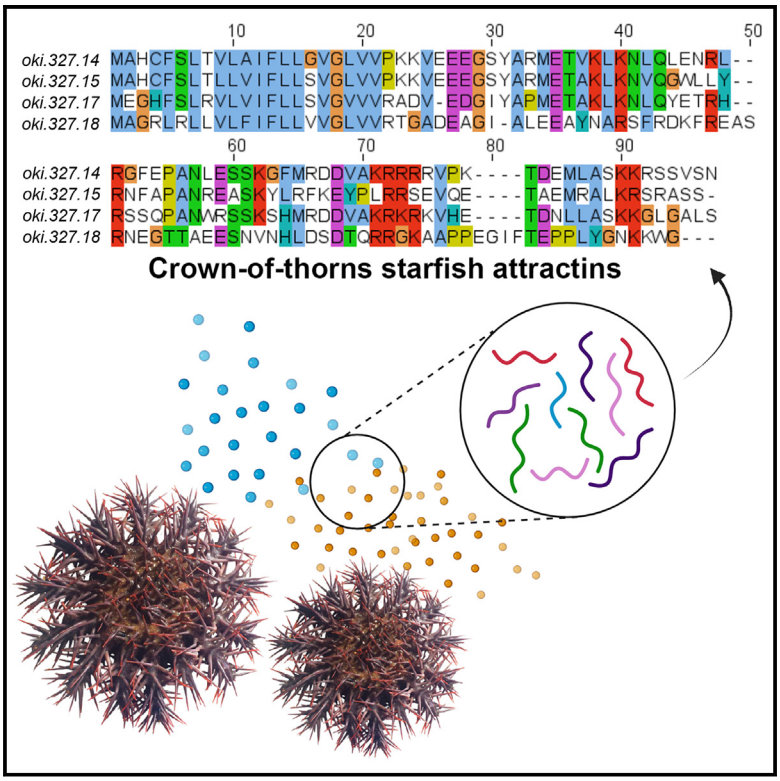
A family of crown-of-thorns starfish spine-secreted proteins modify adult conspecific behavior

Graphical abstract



Highlights

- Spine putative olfactory receptors were upregulated during reproductive season
- Characterization of a genus-specific family of secreted proteins (*Acanthaster* attractins)
- Synthetic peptides derived from *Achanthaster* attractins cause attraction behavior in adults
- *Achanthaster* attractins may be applied as semiochemical control methods

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In brief

Biochemistry; Evolutionary biology; Evolutionary ecology

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A family of crown-of-thorns starfish spine-secreted proteins modify adult conspecific behavior

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SUMMARY

With growing interest in utilizing semiochemicals to control pest species, recent investigations have begun to consider semiochemicals to control outbreaks of crown-of-thorns starfish (CoTS; *Acanthaster* cf. *solaris*), a corallivore contributing to coral reef degradation. In this study, differential gene expression analysis of adult CoTS spines led to the identification of (1) numerous G-protein coupled receptor genes enriched at the reproductive stage, possibly reflecting enhanced sensitivity to semiochemicals and (2) genes encoding secreted proteins at the non-reproductive stage. We also demonstrated that these proteins belong to an uncharacterized family of secreted proteins that are unique to *Acanthaster* spp., being released into the surrounding water. A synthetic peptide mixture derived from this protein family demonstrated no toxicity yet did modify conspecific adult behavior, eliciting attraction. Based on this evidence, we suggest a pheromonal role beyond reproduction. The discovery of these provides a tool for future innovative semiochemical biocontrol in CoTS management strategies.

INTRODUCTION

Olfactory communication is an effective and ancient form of information exchange among organisms, and its purpose is to influence or manipulate the physiology or behavior of the receiver.[1] These molecules are termed semiochemicals, and their purpose is to convey ecological information between organisms to elicit an innate response (behavioral or physiological) or to promote learning.[2–5] Semiochemicals can be subdivided into pheromones (intraspecific signals) and allelochemicals (interspecific signals).[5–7] The information that semiochemicals convey can be precise, such as the sex and age of an individual, what species they are, the location of the source, and altering physiological processes such as to induce reproductive processes, including spawning.[5,6,8] These chemicals are primarily detected by chemoreceptors, e.g., G-protein-coupled receptors (GPCR) and ionotropic glutamate receptors.[9,10] Yet, despite their importance, the chemical and physiological basis of semiochemical exchange among many species is poorly understood, particularly in aquatic organisms.

Due to their effectiveness in altering an organism's behavior, semiochemicals have found utility in pest control strategies, particularly for many insect species[11,12] and are showing promise in aquatic environments.[13–16] However, in contrast to land-based efforts, uptake in the aquatic context has been limited, largely due to a lack of current understanding of the semiochemicals themselves and their effects among aquatic species.[14] With growing interest in utilizing semiochemicals to control pest species, recent investigations have begun to consider semiochemicals to control crown-of-thorns starfish (CoTS; *Acanthaster* spp.,[17] omitting sister species *A. brevispinus*) population outbreaks.[18–20] Coral reef degradation via predation from CoTS has been recognized as a global ecological problem.[21,22] This overconsumption of coral by CoTS not only significantly reduces the numbers of healthy coral and thereby impacting the overall reef system health but also affects reef resilience by preventing coral from being able to adapt to their biggest threat, climate change. Due to the acute severity of coral cover degradation by feeding CoTS, manual culling strategies supported by governments are currently the only viable solution to control CoTS numbers; however, these remain labor and cost intensive efforts.[22]

Although CoTS are known to use visual phototaxis as a form of sensory input to navigate their surroundings,[23–26] olfaction has

been recognized as the primary sense[27,28] and is implicated in the mediation of a suite of CoTS behaviors across their life history.[20,27] For example, as a broadcast spawner, aggregation is of critical importance to ensure fertilization success.[29] Aggregation is also beneficial to avoid predators.[30] Yet, the molecular basis for these behaviors (e.g., olfactory receptors, semiochemical attractants) has only recently been revealed through multi-omics research,[31–36] initiated by interrogation of the CoTS genome.[19] These studies have identified an expanded family of GPCRs that are differentially expressed within the CoTS tube feet and sensory tentacles[31,32] and likely have an olfactory reception function based on their similarity to GPCRs from other species with confirmed function.[37] Proteomics analysis of CoTS-conditioned seawater, confirmed to elicit behavior changes in adults, has also provided some insights into candidate pheromone signaling molecules, whereby the presence of an expanded family of ependymin-related proteins has attracted most attention.[19,31,32] Additionally, studies into the molecular components that vary between wild and cultured CoTS, as well as between reproductive stage and sex, has implicated numerous other secreted proteins as key candidates for pheromone signaling.[36,38] For instance, more than 2,000 protein-coding genes change significantly between summer and winter.[36] Yet, the precise identity of CoTS semiochemicals and the tissues in which these are produced and released from has remained elusive.

The sharp calcareous ossicular spines, that give CoTS their name, are distributed on the aboral surface of the body, providing their infamous mechanical and chemical defense. The spines have been shown to release a multitude of molecules, the majority of which play a role in defense, and include secondary metabolites, small molecules (e.g., saponins)[39] and proteinaceous venom toxins (e.g., plancitoxin and phospholipase A_2s).[38,40,41] In addition, tissue-specific gene expression has implicated 288 genes as being upregulated in the spines compared to all other tissues (i.e., coelomocytes, papulae, radial nerve, skin, sensory tentacles, and tube feet), including 57 predicted to encode secreted proteins.[35] It is hypothesized that many of these uncharacterized molecules may have functions associated with conspecific communication,[18,19,35,38,42] the discovery of which might provide new semiochemicals for integrated pest management.

In recognition that CoTS spines have previously shown seasonal differences in gene expression[36] and are sources of excretory-secretory proteins,[38] we focused on these to elucidate candidate semiochemical components. First, a comparative spine RNA sequencing (RNA-seq) analysis was performed based on reproductive status and then subsequent sequence characterization was undertaken on genes significantly differentially expressed. Finally, a targeted biomolecular and behavioral investigation was then conducted on a novel secreted protein family that is abundant in CoTS spines and secreted into the water, which also appeared to be *Acanthaster* specific.

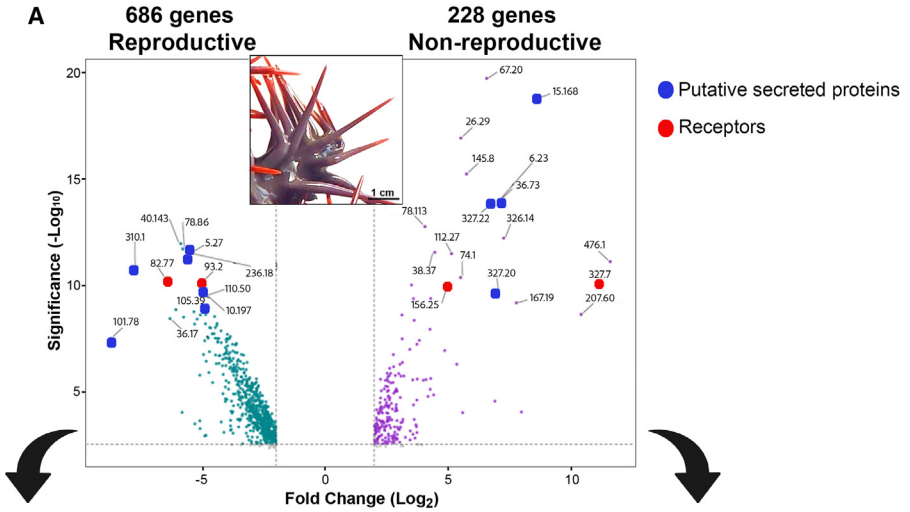
RESULTS AND DISCUSSION

CoTS spines are critical for semiochemical communication

To elucidate potential olfactory-related components present within CoTS spines, a comparative reproductive stage differential gene expression analysis was performed, demonstrating that 686 and 228 genes were significantly upregulated in reproductive versus non-reproductive spines, respectively (Figure 1A and S1A). Notably, at each stage, several genes encoding predicted secreted proteins, including a lectin, mucin, and a Na^+/Cl^--dependent amino acid transporter at the reproductive stage, and uncharacterized and unknown proteins at the non-reproductive stage, exhibited the highest level of differential expression. Overall, at the reproductive stage, genes associated with catalytic, hydrolyze, and transferase activity were prominent (Figure 1B), including genes encoding enzymes, defense proteins and those involved in signaling processes. At the non-reproductive stage, genes associated with transferase activity were most prominent (Figure 1C). Of note, only four identified differentially expressed genes encoded proteins that had previously been identified from COTs spine proteomics analysis (*oki.12.167*, *oki.42.47*, *oki.27.48*, and *oki.27.33*); none of which include recognized CoTS toxins such as plancitoxins or phospholipase A_2s.[38] These toxins are likely required irrespective of reproductive stage.

At the reproductive stage, of those involved in signaling processes, there was a notable enrichment of GPCR genes (Figure 2), some that may encode putative olfactory receptors (ORs), consistent with an elevated need to sense environmental semiochemicals.[43,44] In particular, glutamate receptor 2 (*oki.31.225*), previously implicated in CoTS sensory tentacle olfactory detection,[31,32] demonstrated the highest level of differential upregulation in reproductive stage spines. Enrichment of two other receptors, a metabotropic glutamate receptor 8 (*oki.163.34*) and an octopamine receptor (*oki.90.57*), has also previously been demonstrated in CoTS spine tissue with the former also showing upregulation in males compared to females.[35] Glutamatergic mitral and ruffed cells are the principal cells found in the olfactory bulb of fish, with members of the metabotropic glutamate receptor-like group shown to be responsible for olfaction,[45] lending support to the hypothesis that the glutamate receptors in CoTS spines may also have an olfactory function. Scanning electron microscopy has revealed the stereom of CoTS spines contains pore-like structures that allow for the secretion of molecules, notably defensive toxins,[38] and the presence of these pores, in combination with our discovery, suggests that CoTS spines might also act as a key interface for semiochemical detection. By analogy, the olfactory sensillum of insects also contains pore structures that house olfactory receptors.[46] The crustacean antennule, which have specific secretory glands and ionotropic receptors that support chemosensation, also have a multitude of functionality, e.g., locomotion, grabbing, mechanosensation and as defense spines,[47] further supporting a similar multifaceted role for CoTS spines.

At non-reproductive stage, a significant increase in spine gene expression was observed for two newly identified genes, *oki.327.20* and *oki.327.22*. Upon closer analysis, five additional genes having significant sequence identity were identified in close genome proximity: *oki.327.14*, *oki.327.15*, *oki.327.17*, *oki.327.18*, and *oki.327.21* (Figure 3A). Identity was particularly high within the N-terminal predicted signal peptide region, as well as several conserved residues, most notably E_{27}, G_{29}, A_{32}, E_{35}, R/K_{40}, R_{51}, D_{68}, R_{72}, T_{83}, and R/K_{91} based on *oki.327.14*. Of particular note



oki. Gene ID	Fold-change	BLAST match
105.39	3762.0	Alpha-N-acetylgalactosamine-specific lectin
236.18	1873.3	Mucin-19
110.50	1045.3	Na+- and Cl- -dependent amino acid transporter
40.143	562.8	L-ascorbate oxidase
310.1	415.4	Semaphorin-2A isoform
10.197	321.7	vWF domain-containing protein 2
82.77	242.0	Interleukin-17 receptor A-like
78.86	181.9	Neurogenic locus notch homolog protein 2
5.27	159.9	Protocadherin Fat 4
101.78	155.7	Hyalin-like isoform X1

oki. Gene ID	Fold-change	BLAST match
74.1	8372.9	Alpha-crystallin B chain
145.8	542.2	Deleted in malignant brain tumors 1 protein
15.168	359.1	Uncharacterized protein
327.22	332.8	Unknown
67.20	243.5	Flavin-containing monoamine oxidase A
26.29	219.8	Biomineralization proteins SpP19L long form
476.1	199.2	ATP-dependent RNA helicase DHX40
38.37	185.0	Uncharacterized protein
327.20	154.0	Unknown
78.113	109.9	ATP-dependent RNA helicase DHX40

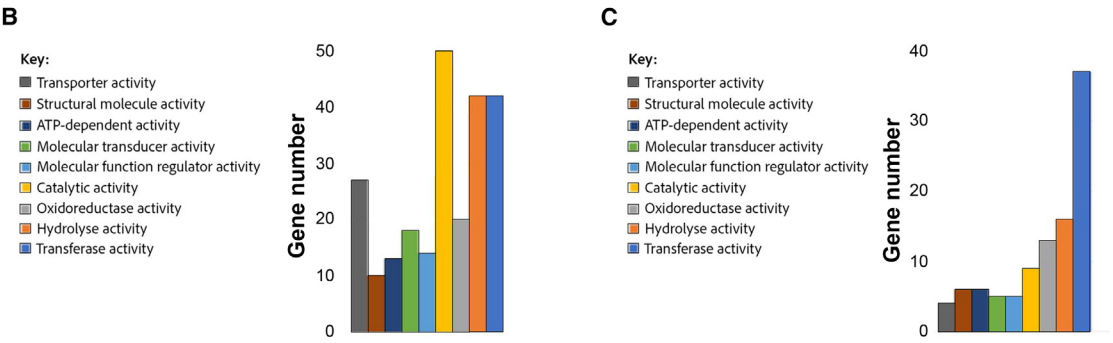


Figure 1. Differential gene expression of crown-of-thorns starfish (CoTS; *Acanthaster* spp. complex omitting *A. brevispinus*) spine tissue between reproductive and non-reproductive stages

(A) Volcano plot shows significant (sig. -Log_{10} > 2.2; Log_2 fold change (FC) between -1.8 and 1.8) differentially expressed genes, including table with top hits based on FC and significance labeled with gene identifier. All gene IDs are based on CoTS oki (Okinawa) genes. Tables show the top 10 DEGs based on highest FC.

(B) Graph showing biological function GO annotation of all significantly upregulated genes at reproductive stage.

(C) Graph showing biological function GO annotation of all significantly upregulated genes at non-reproductive stage.

is the presence of basic residues that predict cleavage by prohormone convertases prior to secretion. Although *oki.327.20* and *oki.327.22* genes in spines were differentially expressed between reproductive stages, these other five related genes were similarly highly expressed, irrespective of reproductive stage (Figure 3B). Further, all seven related genes were lowly expressed in, or absent from other CoTS tissues analyzed (Figure 3B). Spine tissue expression enrichment is supported by a previous study.[36] To explore the function of this group of seven genes, proteomic analysis was performed on conditioned water of non-reproductive CoTS. The presence of three of these proteins, oki.327.14, oki.327.15, and oki.327.18 (Figure S1) was revealed; only oki.327.14 had previously been identified from CoTS-conditioned water following exposure to its predator, the Giant Triton snail (*Charonia tritonis*).[19] NCBI and UniProt database searches revealed that these secreted proteins had no primary or tertiary sequence similarity with any known protein families, suggesting species-specificity. However, upon preparation of a multi-tissue transcriptome for the CoTS sister species, *Acanthaster brevispinus*, we confirmed sequence identity with two *A. brevispinus* proteins (Figure 3A), which indicated at least some level of genus conservation. *A. brevispinus* are found in coral reef ecosystems,

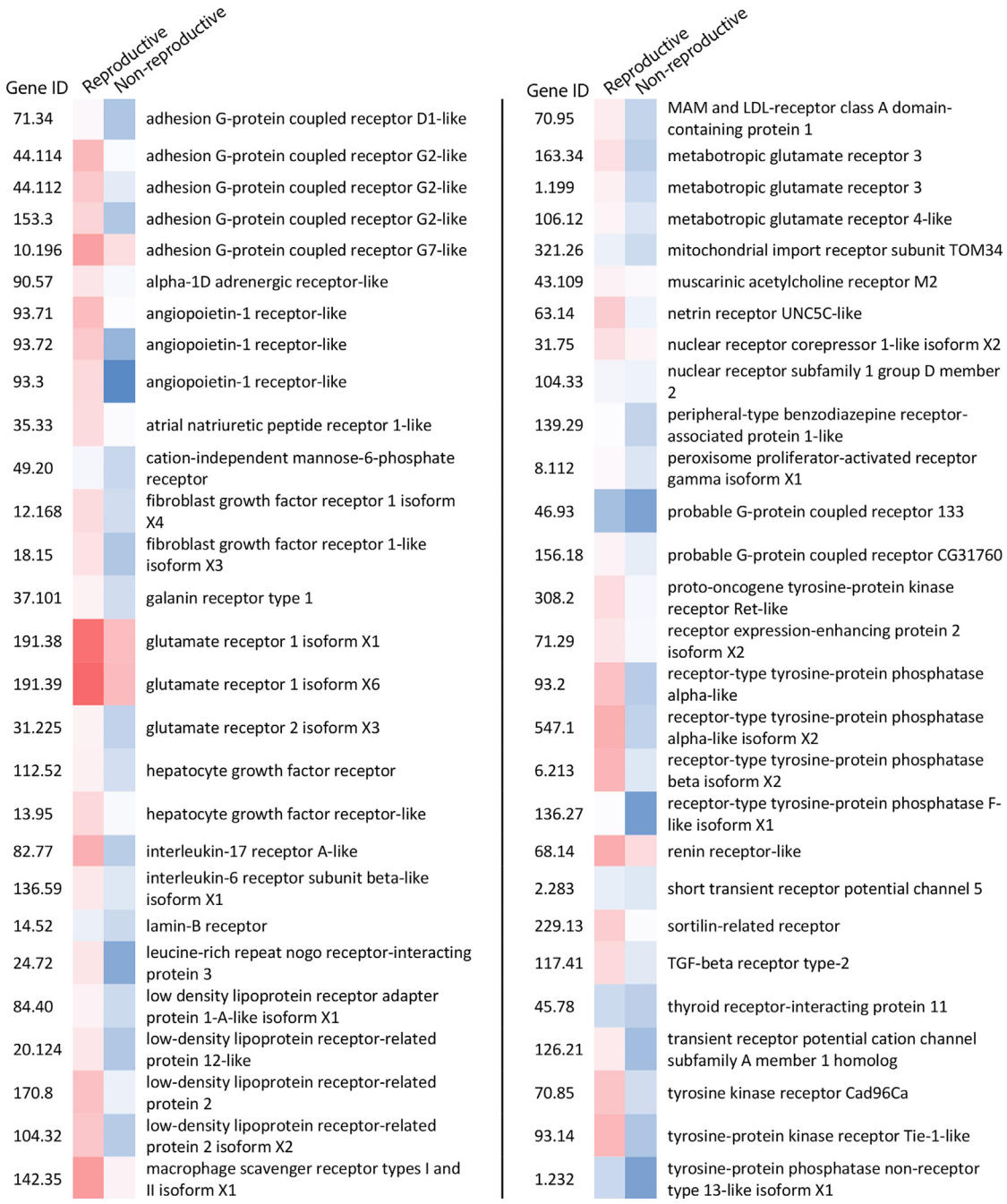


Figure 2. Annotation and expression (based on Log2 value) of significantly upregulated receptors within crown-of-thorns starfish (CoTS) spine tissue at the reproductive stage

The color scale represents the level of CoTS gene expression: high = red, medium = clear, and low = blue. For raw transcripts per million (TPM) gene expression values, see Table S1.

however, are not strict corallivores and prefer deeper water soft bottom habitats.[48]

Synthetic peptide assays confirm pheromone bioactivity

Based on predicted precursor cleavage sites (Figure 3A) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) of detected peptide fragments (Figure 1), synthetic peptide variants were prepared for the family of CoTS spine secretory proteins (Table 1). Initially, to determine whether they may play a role in defense as toxins, a synthetic peptide mixture (SPM) was prepared and exposed to brine shrimp at 100 pM, 10 μM, and 100 μM. This revealed no significant toxicity at

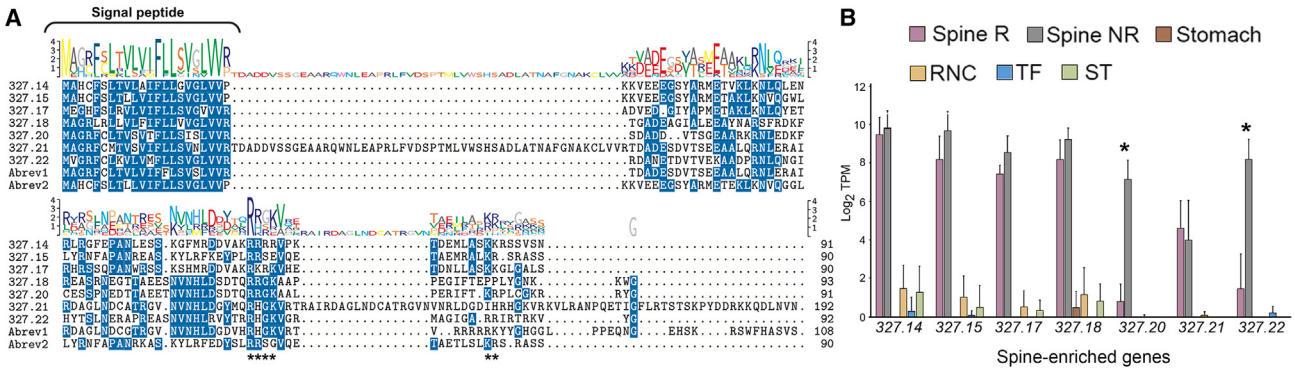


Figure 3. Comparative protein sequence and tissue expression

(A) Multiple sequence alignment of seven forms of the crown-of-thorns starfish (CoTS) spine-secreted proteins, as well as *Acanthaster brevispinus* homologs (Abrev1, 2). Asterisks indicates predicted cleavage sites.

(B) Graph comparing gene expression across various CoTS tissues: spine reproductive (R), spine non-reproductive (NR), stomach, radial nerve cord (RNC), tube foot (TF) and sensory tentacle (ST). Asterisks indicate significant difference ($p < 0.05$) between R and NR spines.

48 h post-exposure ($p < 0.05$) (Figure 4A). Thus, it was hypothesized that the CoTS spine secretory protein family may play a role in semiochemical communication.

Subsequently, the CoTS SPM was applied to assess its influence over CoTS behavior. Upon exposure to SPM at 1 nM in a two-current choice flume behavioral assay, adult CoTS showed a significant chemotaxis change (Figure 4B), reduced meandering (Figure 4C) and an increased total time spent in cue arm (Figure 4D). Meandering is a behavior defined as the change in direction of movement relative to the distance moved and is measured as direction of turning per unit distance. A high degree of meandering is indicative of searching or foraging behavior particularly when the target cannot infer the source of the cue, e.g., when there are multiple different or unknown chemical stimuli or a lack of stimuli altogether.[49–52] Conversely, relatively low meandering, such as that observed here in response to CoTS SPM, can indicate a preference for a specific directional stimulus, i.e., toward the CoTS SPM.

The CoTS SPM was introduced to the flume at a concentration of 1 nM, thereby establishing a potential exposure concentration at the picomolar (pM) to femtomolar (fM) range. The concentration for a semiochemical to initiate a response can vary; however, some have been shown to be effective at miniscule pM[53,54] and fM[55] concentrations. An important factor that impacts olfactory reception is the sensitivity of the receptors in the receiving organism. Mimicking natural concentrations is important in bioassays since high concentrations can cause spurious results due to non-pheromone stimulation of some components within a mixture or the overstimulation of receptors.[8] It may be that the further evolution of an olfactory system in the spines, in addition to the tube feet and sensory tentacles,[32] has allowed CoTS to develop a greater sensitivity to semiochemicals, which in turn might explain why they are so efficient at forming large feeding and spawning aggregations. Alternatively, the three different morphological regions used for olfaction may support the detection of different types of semiochemicals (e.g., pheromones versus allelochemicals). An in-depth comparison of receptor subtypes between the spines, sensory tentacles, and tube feet will allow for the testing of this hypothesis.

Based on the gene expression abundance and its secretion from the spines, lack of toxicity, conspecific attractiveness (Figure 4), as well as their sequence novelty (exclusive to *Acanthaster* spp. and no other seastars, e.g., *Asterias rubens*, *Asterias amurensis*, *Zoroaster* cf. *ophiactis*, *Plazaster borealis*, *Marthasterias glacialis*, *Archaster typicus*, *Echinaster* (*Othilia*) *brasiliensis*, *Pisaster brevispinus*, and *Luidia sarsi*), we have named the family of related proteins as *Acanthaster* attractins. We propose that the *Acanthaster* attractins family are a suite of conspecific pheromones that may work most effectively synergistically (a common phenomenon with pheromones[56]), with a possible purpose to stimulate conspecific aggregation, yet this function remains unverified. Pheromones and their associated behaviors are usually not mediated by a single molecule but rather a species-specific ratio of a combination of molecules.[8]

Table 1. List of crown-of-thorns starfish (CoTS) synthetic peptide sequences based on CoTS spine-secreted protein genes, including gene ID, amino acid sequence, and molecular weight (MW) in daltons (Da)

Originating CoTS gene ID	Amino acid sequence	MW (Da)
oki.327.14	KKVEEEGSYARMETVKLKNLQLENRLRGFEPANLESSKGFMRDDVA	5315.02
oki.327.14	VPKTDEMLASK	1218.43
oki.327.15	KKVEEEGSYARMETAKLKNVQGWLLYRNFAPANREASKYLRFKEYPL	5624.46
oki.327.15	SEVQETAEMRAL	1363.51
oki.327.17	DVEDGIYAPMETAKLKNLQYETRHRSSQPANWRSSKSHMRDDVA	5119.64
oki.327.18	DEAGIALEEAYNARSFRDKFREASRNEGTTAEESNVNHLDSDTQ	4945.13

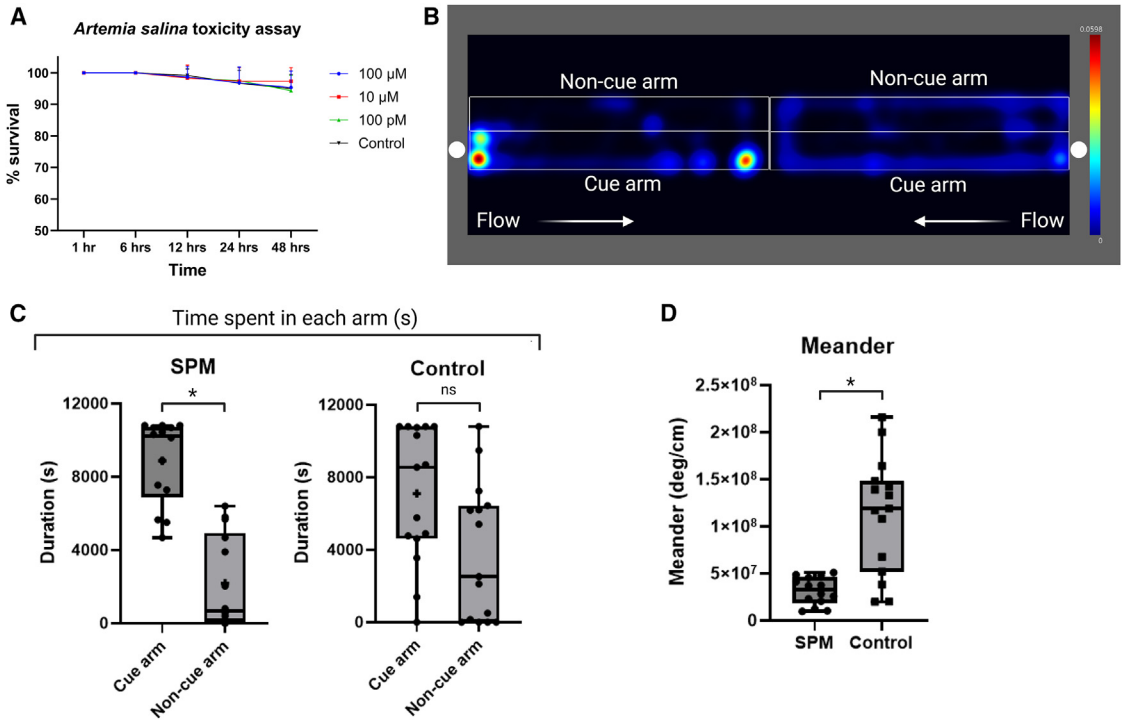


Figure 4. Toxicity and behavioral assays to assess the crown-of-thorns starfish (CoTS) synthetic peptide mixture (SPM)

(A) Brine shrimp (*Artemia salina*) lethality assay ($n = 14$ *A. salina*/well) to assess toxicity of the CoTS SPM ($n = 5$ replicates per concentration). Behavioral response of CoTS to SPM. A two-way ANOVA was used to test significance.

(B) Cumulative heatmaps from dual flumes showing attraction of CoTS to the SPM ($n = 14$; $n = 9$ left flume and $n = 5$ right flume—the white dots on either side of the cue arm indicate where the source of the SPM was fed into each respective cue arm.

(C) Combined meandering (deg/cm) when exposed to control ($n = 15$) and SPM at 1 nM ($n = 14$).

(D) Combined time spent in arm (s) when exposed to control ($n = 15$) and CoTS SPM at 1 nM ($n = 14$). An independent samples t test was used to test significance with a threshold of $p < 0.01$ indicated by an asterisk.

It is this ratio that triggers an innate or learned response from individuals. Therefore, further investigation of the ratio of *Acanthaster* attractins might aid in eliciting a stronger response.

The future of utilizing *Acanthaster* attractins for control management

Given the observed positive chemotaxis of non-reproductive CoTS to *Acanthaster* attractins, efforts to determine if they could be utilized in successful semiochemical control of CoTS to support and alleviate manual culling labor should be sought. With the growing successes of semiochemical pest control management, these results provide an exciting avenue to pursue similar control strategies with regards to CoTS. Future research needs to determine (1) the most effective ratio of the *Acanthaster* attractin molecules, (2) the efficiency of these molecules in attracting more than one CoTS individual, (3) the seasonal efficacy of these molecules in attracting CoTS, and (4) their effects on other co-habiting reef organisms, particularly closely related echinoderm species, thereby extending the practicality of semiochemical biocontrol to a reef environment.

Conclusions

This study has identified many differentially expressed genes within CoTS spines, at reproductive versus non-reproductive stages, including putative olfactory receptors at the reproductive stage, suggesting a heightened level of chemosensation. We additionally highlight the relative abundance of newly identified spine secretory proteins, supported by proteomics, which constitute an expanded family of proteins that are *Acanthaster* specific, the CoTS attractins. Using a SPM derived from these proteins, no evidence of toxicity was determined, however, exposure did stimulate significant behavior changes in non-reproductive adult CoTS. These data highly suggest that the spines play a key role in chemosensation, both in the emission of chemical cues and the detection through the presence of highly expressed receptors. Furthermore, these CoTS attractins might be candidates for use as semiochemical control agents to aid in manual culling efforts of CoTS population outbreaks.

Limitations of the study

Attractants might work differently in wild vs. aquaria-based experiments.[57] CoTS are made naive (are isolated from other conspecifics) and have limited food resources for up to 1 week prior to behavioral experiments, therefore the attractant might have a greater confounding effect on those individuals compared to CoTS that are regularly receiving chemical stimuli from conspecifics and the natural environment. In addition, individual

variation exists for wild derived specimens, and therefore we recognize the requirements for large sample sizes for experimental analysis. However, our research did integrate data from multi-omics analysis (transcriptomics and proteomics), which together provide strong support for our findings, and is supported by functional analysis.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Scott F. Cummins (scummins@usc.edu.au).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data: RNA-sequencing data have been deposited at NCBI as BioProject PRJNA901199 and PRJNA548418, and are publicly available as of the date of publication.
- Code: This paper does not report original code.
- Additional Information: Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

C.A.M., K.N., N.S., A.E., and S.F.C. conceptualized the project and secured funding; R.J.H., A.K.H., B.S.L., T.W., L.D.B., S.K., and S.S.-A. conducted experimental procedures; R.J.H. and A.K.H. wrote the first draft; R.J.H., C.A.M., A.E., and S.F.C. provided additional commentary on the first draft; all authors edited subsequent drafts.

DECLARATION OF INTERESTS

Authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2025.112161.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RNAlater	Invitrogen (ThermoFisher)	AM7020
TRI Reagent	Sigma-Aldrich (Merck)	T9424
RNase-Free Water	Sigma-Aldrich (Merck)	W4502
Trifluoroacetic acid	Sigma-Aldrich (Merck)	T6508
Formic acid	Sigma-Aldrich (Merck)	F0507
Trypsin	Promega Corporation, Madison, USA	VA9000
Sep-Pak C18 3 cc Vac. Cartridge (500 mg)	Waters Corporation (Milford Massachusetts, USA)	WAT020805
Aeris 3.6 μm PEPTIDE XB-C18 100 Å, LC Column 100 x 2.1 mm	Phenomenex, Sydney, Australia	00D-4506-AN
Experimental models: Organisms/strains		
Brine shrimp (*A. salina*)	Seaview Aquarium Centre	SKU:00199
Software and algorithms		
SignalP (V.5.0)	Department of Health Technology	https://services.healthtech.dtu.dk/services/SignalP-5.0/
TMHMM (V.2.0)	Department of Health Technology	https://services.healthtech.dtu.dk/services/TMHMM-2.0/
BLASTp	NCBI (NIH)	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
OmicsBox	BioBam	https://www.biobam.com/omicsbox/
I-TASSER	Zhang Lab	https://zhanggroup.org/I-TASSER/
PEAKS v7.0	Bioinformatics Solutions Inc. (BSI, Canada)	https://www.bioinfor.com/versions/
MS Data Converter (Beta 1.3)	SCIEX	http://sciex.com/software-downloads-x2110
ChinaPeptides Co., Ltd	Pudong New Area, Shanghai, China 201202	http://www.chinapeptides.com/english/index.aspx
SPSS Statistics V.24.0.0.	IBM (Armonk, New York 10504-1722 United States)	https://www.ibm.com/products/spss-statistics/resources
GraphPad (Prism 9 V.9.5.1.)	GraphPad Software Boston, MA 02110, United States	https://www.graphpad.com/
EthoVision XT v.17	Noldus (Wageningen, the Netherlands)	https://www.noldus.com/ethovision-xt
Deposited data		
RNA-sequencing data	NCBI	PRJNA901199
RNA-sequencing data	NCBI	PRJNA548418

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

Adult CoTS (15-30 cm) were collected during September (non-reproductive stage) and late November (reproductive stage) 2021 by GBRMPA divers (under permit G21/38062.1), working on the Great Barrier Reef (GBR), Cairns region, Northern Queensland (Australia). Prior to sampling, non-reproductive CoTS were maintained at the University of the Sunshine Coast (Sippy Downs, QLD) aquarium facility, where they were briefly kept in a 1500 L re-circulating protein skimmed and aerated saltwater system. Due to logistics, reproductive CoTS were transported to the Australian Institute of Marine Science (AIMS, Townsville, QLD), where they were maintained in a 10,000 L flow-through outdoor tank. Prior to sampling, their sex was determined by gonad biopsy,[58] then individuals were kept segregated for 7 days in 30 L aerated flow-through tanks during which time they were not fed. Isolation ensured

the CoTS became 'naïve' – eliminating any interference from exposure to cues from conspecifics or food sources. Non-reproductive adult CoTS (20-30 cm) were collected during the month of July 2024 by GBMPRA divers (under permit G17/38293.1) from the Townsville region, Northern Queensland (Australia).

A single adult female of *A. brevispinus* was collected by a commercial trawling vessel off the coast of Cairns in Queensland, Australia, and maintained at AIMS (Townsville) on a diet of beach clams until sampled. *A. brevispinus* is a short-spined sister species of CoTS that preys primarily on bivalves and which is not prone to population irruptions.[48]

METHOD DETAILS

CoTS spine tissue collection, RNA isolation and sequencing

Aboral spines were removed (cut 3 mm from the base) from 6 non-reproductive CoTS (September 2021; sex unable to be determined) and 3 reproductive male and female CoTS ($n = 6$ total; November 2021; sexed based on microscopic analysis of gonad), then immediately transferred to pre-cooled RNAlater (Thermo Fisher) and stored at -20^{0}C until processing.

The outer epithelial layer of the spine (referred to herein as spine tissue) was gently removed using a scalpel blade, then total RNA was extracted using TRI Reagent (Merck), as per the manufacturer's protocol. Total RNA was eluted in RNase-free distilled water (30 μL; Thermo Fisher Scientific), quantified using a Nanodrop 2000 (Thermo Scientific), and then stored at -80°C. RNA samples of high quality (based on RNA integrity number) were sent on dry ice to Novogene (Singapore) for library preparation and sequencing (3 Gb per sample) on an Illumina NovaSeq 6000 sequencing platform. Raw sequence data was obtained and deposited into the SRA NCBI GenBank database under PRJNA901199.

Differential gene expression analysis of CoTS spine tissues, and compared to other tissues

Trimmed CoTs spine RNA-seq data were annotated by alignment against the CoTS genome,[19] using the CLC Genomics Workbench (Ver. 21.0.3) with default parameters. RNA-seq quantification for each gene was determined by transcripts per kilobase million (TPM). Putative secreted proteins were predicted based on the presence of a signal peptide, absence of a transmembrane domain in the protein precursor sequences, and propeptide region, using SignalP (V.5.0), TMHMM (V.2.0), and ProP (V.1.0) webtools.[59–61] Expression of genes encoding identified putative secreted proteins was investigated in the spine tissues using the publicly available transcriptomes from the CoTS genome browser (http://marinegenomics.oist.jp). This comprised of CoTS tube foot, mouth, spine, sensory tentacle, radial nerve cords (male and female), testis, ovary, egg, and mid-gastrula tissues.[19,31] Gene expression in TPM values was transformed to z-scores before being visualized in a heatmap using the ClustVis webtool.[62] General functions of identified putative proteins were investigated based on the Gene Ontology (GO) annotation. In brief, full-length sequences of putative secreted proteins were annotated against a non-redundant database on the National Center for Biotechnology Information (NCBI) using a BLASTp search on the Omicsbox software (BioBam). GO annotation, limited to the biological process category, was then performed.

Acanthaster brevispinus RNA extraction and sequencing

Tube feet were dissected from the *A. brevispinus* specimen, preserved immediately in RNA*later* (Life Technologies) for transportation to the University of the Sunshine Coast and subsequently stored at -80°C. Tube feet were thawed and weighed before homogenisation in Trizol reagent (Invitrogen) and total RNA extracted following manufacturer's instructions. Approximately 1μg total RNA, concentration established by Nanodrop spectrophotometry (Thermo Scientific), was sent to Novogene (Hong Kong) for library preparation and 100 base-pair single-end reads generated using the Illumina HiSeq 2500 platform. Raw sequence data was obtained and deposited into the SRA NCBI GenBank database under PRJNA548418. The *A. brevispinus* transcriptome was *de novo* assembled by Novogene using the Trinity pipeline. CoTS genes were subjected to BLASTp against the *A. brevispinus* tube feet transcriptome to determine presence/absence using an *E*-value threshold of 0 and an identity cut-off of $\geq$ 90%.

Excretory-secretory proteome analysis of CoTS conditioned water

Conditioned water samples (n = 6) were collected from non-reproductive adult CoTS (collected from Cairns in September 2021, refer above) by temporarily ($\sim$ 12 h) holding each isolated individual in a 5 L sterilized glass tank under static conditions (i.e., filtered seawater (FSW), no flow, fully aerated). As handling of CoTS can lead to unintended stress during transfer, an initial FSW exchange of $\sim$ half the tank was done within the first 10 min, and the tank filled with FSW and left static. After $\sim$12 h, 500 mL of conditioned water was collected. FSW controls (i.e., tank without CoTS) were also taken and processed alongside samples. All samples and controls were loaded onto C18 Sep-Pak columns (500 mg bedding) under vacuum (Waters manifold). Loaded columns were washed with 15 mL of aqueous 0.1% trifluoroacetic acid (TFA) then eluted with 10 mL 60% acetonitrile (ACN). Final eluates were lyophilized (Savant) overnight and processed through trypsin digestion, as per manufacturers protocol (Promega Corporation, Madison, USA) in preparation for mass spectroscopic (MS) analysis. Samples were also processed for native MS without trypsin digestion.

Peptides were analysed by uHPLC-MS/MS on an ExionLC liquid chromatography system (AB SCIEX, Concord, Canada) coupled to a qTOF X500R MS (AB SCIEX, Concord, Canada) equipped with an electrospray ion source. Each sample (20 μL) was injected onto a 100 mm × 1.7 μm Aeris PEPTIDE XB-C18 100 uHPLC column (Phenomenex, Sydney, Australia) fitted with a security guard column. Peptides were eluted with the following solvent system: solvent A, 0.1% formic acid (FA) (aq), and solvent B, 100% ACN:0.1% FA, at 400 μL/min flow rate. A linear gradient of 5% to 35% solvent B over 10 min was applied followed by a steeper gradient from 35% to

80% solvent B for 2 min and 80% to 95% solvent B over 1min. Solvent B was held at 95% for 1 min to wash the column then returned to 5% solvent B for equilibration prior to the next sample injection. The ionspray voltage was set to 5500 V, declustering potential 100V, curtain gas flow 30, ion source gas 1 40, ion source gas 2 50 and spray temperature at 450°C. MS data was acquired in the Information Dependant Acquisition mode. Full scan qTOF-MS data was acquired over the mass range 350-1400 *m/z*; product ion MS/MS data was acquired over 50-1800 *m/z*. Ions observed in the qTOF-MS scan exceeding a threshold of 100 cps and a charge state of +2 to +5 were set to trigger the acquisition of product ion. The data was acquired and processed using SCIEX OS software (AB SCIEX, Concord, Canada). Biological triplicates were used for the analysis. The uHPLC qTOF-MS/MS data were imported into the PEAKS studio (Bioinformatics Solutions Inc., Waterloo, ON, Canada, version 7.0) with the assistance of MS Data Converter (Beta 1.3, http://sciex.com/software-downloads-x2110). Peptides were analysed using PEAKS v7.0 (BSI, Canada) against the protein database assembled from the available CoTS transcriptomes and genome data (Hall et al. 2017).

Peptide synthesis

From the spine transcriptomic data, those peptide sequences most highly expressed in both reproductive and non-reproductive stages, as identified from matches to the Okinawa Institute of Science and Technology (OIST) (http://marinegenomics.oist.jp) CoTS genome assembly,[19] and based on their presence in the excretory-secretory proteome, were selected for synthesis (Table 1) and testing. Synthetic peptides (herein referred to as SPs) were prepared to 95% purity by Genscript (New Jersey), and Scrum Inc. (Japan), lyophilized and stored at -20°C until use.

Brine shrimp lethality bioassay

A brine shrimp lethality bioassay was performed according to Meyer et al. (1982),[63] with some modifications. Brine shrimp (*Artemia salina*) eggs were hatched in a hatching container filled with 300 mL of aerated FSW for 48 h at room temperature. At 48 h post-hatch, brine shrimp larvae were transferred via a pipette into a 96-well plate filled with 20 μL/well of aerated FSW (n = 14±3 individuals per well). Brine shrimp larvae were then exposed to a synthetic peptide mixture (SPM) at a final concentration of 100 pM, 10 μM, and 100 μM (5 replicates per dose per extract). For each SPM, the higher concentrations used in the brine shrimp lethality assay were prepared at 1:2 of the original concentration of a given SPM in each replicate. For negative controls, FSW (20 μL) was added into wells. Live brine shrimp larvae were counted at 0, 1, 6, 12, 24, and 48 h post-treatment to calculate percent mortality.

Behaviour flume assay to confirm pheromone bioactivity

CoTS were exposed to SPM in dual two-current flume behavioural assay systems.[64] Dual flumes, run under identical conditions, were used to expedite animal testing. Each flume design comprised identical 6-metre large-scale arenas with honeycomb collimators that create a laminar flow ensuring separation and even distribution of the introduced chemical cue to create two separate flow lanes, a 'cue' and 'non-cue' arm (see Figure 4B). Unlike a traditional Y-maze system,[19] the 'cue arm' maintains a constant concentration of the chemical cue. Parameters from Hall et al., 2017 were modified to suit the flume arena, with the flow rate maintained at 25 L/min, temperature at 23°C, and cue delivered via a peristaltic pump at a 2.2 mL/min flow rate. Naïve non-reproductive adult CoTS (n = 14) were exposed to SPM delivered at 1nM. Controls were exposed to FSW as the delivered cue. Each flume assay was run for 3 h and behaviour was tracked under infrared light using the same system as above. A trial was considered invalid if the animal failed to move at all from the start position over the 3 h and data excluded from further analysis. CoTS movements were recorded and analysed (applying the variables listed above) using EthoVision® XT17 software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Differential gene expression analysis was performed using the CLC Genomics Workbench, which utilises multi-factorial statistics based on a negative binomial Generalized Linear Model. For brine shrimp toxicity assays a two-way ANOVA and subsequent Dunnett's multiple components post-hoc tests were used to establish significance at a confidence threshold of 99% ($P \leq 0.01$). Data is presented in ± standard deviation. For behavioural flume assays, data associated with time spent in cue arm (SPM or FSW) vs non-cue (FSW) and meandering was extracted and analysed using an independent samples t-test. All statistical analyses were conducted in GraphPad Prism 10.2.2 software.