Cassiosomes are stinging-cell structures in the mucus of the upside-down jellyfish Cassiopea xamachana

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Snorkelers in mangrove forest waters inhabited by the upside-down jellyfish Cassiopea xamachana report discomfort due to a sensation known as stinging water, the cause of which is unknown. Using a combination of histology, microscopy, microfluidics, videography, molecular biology, and mass spectrometry-based proteomics, we describe Cassiopea xamachana stinging-cell structures that we term cassiosomes. These structures are released within Cassiopea xamachana mucus and are capable of killing prey. Cassiosomes consist of an outer epithelial layer mainly composed of nematocytes surrounding a core filled by endosymbiotic dinoflagellates hosted within amoebocytes and presumptive mesoglea. Furthermore, we report cassiosome structures in four additional jellyfish species in the same taxonomic group as Cassiopea xamachana (Class Scyphozoa; Order Rhizostomeae), categorized as either motile (ciliated) or nonmotile types. This inaugural study provides a qualitative assessment of the stinging contents of Cassiopea xamachana mucus and implicates mucus containing cassiosomes and free intact nematocytes as the cause of stinging water.
Jellyfish, along with corals, anemones, hydroids, and myxozoa, belong to the phylum Cnidaria, the earliest diverging venomous animal lineage\textsuperscript{1,2}. These diploblastic animals have two so-called epithelial layers, outer ectoderm and inner endoderm, separated by a gelatinous extracellular matrix called mesoglea\textsuperscript{3,4}. Despite their seemingly simple morphology, cnidarians have adapted globally to most saltwater habitats and some freshwater environments\textsuperscript{1,2,5}. As such, cnidarians have evolved a remarkable envenomation mechanism that involves the deployment of subcellular stinging capsules called nematocysts from cnidian-specific cells called nematocytes, which vary in size, morphology, and bioactive contents\textsuperscript{6-9}. Sea anemones possess unique nematocyte-rich structures (e.g., acrorhagi, acontia)\textsuperscript{9,10} and employ strategies such as tentacle and column contraction and expansion to enhance nematocyte deployment for prey capture and protection, while in medusae (i.e., jellyfish) the first line of defense is their extendable nematocyte-laden tentacles that envenomate prey and predators they encounter in the water column\textsuperscript{5}, as well as humans participating in marine recreation. In addition to direct stings caused by jellyfish, indirect stings have also been reported. Some possible explanations for indirect jellyfish stings are contact with tentacle fragments in the water (e.g., jellyfish stings in offshore fishers\textsuperscript{11}), envenomation by juvenile venomous jellyfish (e.g., Irukandji-like syndrome in United States Military combat divers\textsuperscript{12}) or Sea Bathers Eruption caused by microscopic jellyfish life forms (e.g., Linuche unguiculata\textsuperscript{13}).

Another indirect stinging mechanism is through mucus, such as in medusae of the upside-down mangrove jellyfish Cassiopea xamachana Bigelow 1892 (Class Scyphozoa; Order Rhizostomeae), an emerging cnidian model for its relevance to the study of co-evolution as well as symbiosis-driven development (reviewed in ref. \textsuperscript{14}). Additionally, the ubiquity of Cassiopea medusae in healthy mangroves has earned the upside-down jellyfish status as a potential bioindicator species for coastal management and conservation efforts\textsuperscript{15,16}. Cassiopea is known to release large amounts of mucus into the water column\textsuperscript{17-24}, which has been referred to as toxic mucus due to reports of nematocysts found freely suspended in the viscous substance\textsuperscript{17,20,25}. For instance, Cassiopea mucus is known to kill certain species of fish on contact\textsuperscript{24}. Cassiopea is an exception to the iconic image of a jellyfish in that it lacks marginal tentacles and, instead of swimming in the water column, lies apex-down on the substrate in mangrove forests, seagrass beds or other coastal waters with its relatively short oral arms facing upward (reviewed in ref. \textsuperscript{26}). Despite this benthic lifestyle, warnings have been published alerting sea bathers of the stinging water or toxic mucus phenomena blamed on unidentified potent little grenades in the water column surrounding Cassiopea medusae\textsuperscript{26,21}. In general, Cassiopea stings are categorized as mild to moderate in humans, but crude venom extracted from the nematocysts displays hemolytic, cardiotoxic and demeronocropic properties\textsuperscript{27-30}, suggesting that excessive exposure may be detrimental for humans.

During the course of this study, a review of the old literature on Cassiopea revealed a probable explanation for the grenades reported in stinging water. First, Perkins\textsuperscript{37} discovered in the mucus of Cassiopea undeployed nematocysts and ciliated innumerable minute spherical bodies, the latter of which were dismissed as non-coelenterate (i.e., non-cnidarian) in nature. Next, a brief description was published by Smith\textsuperscript{40} of peculiar structures found in the oral vesicles (i.e., vesicular appendages) of the oral arms of C. xamachana and conspecific C. frondosa medusae that were ‘shot’ at prey, which he dubbed small bags of mesogela and nematocysts and suggested might play a role in predation. Finally, Larson\textsuperscript{43} reported polygonal-shaped bodies on the flattened sides of the oral vesicles (i.e., vesicular appendages) of the oral arms of C. xamachana and C. frondosa corresponding to nematocyst clusters that released upon contact, to which he attributed a role in defense. The sum of these reports suggests that an investigation of the contents of Cassiopea mucus is needed to test the hypothesis that undeployed nematocysts and/or another nematocyst-bearing structure(s) present within the mucus of the upside-down jellyfish together are responsible for the phenomenon of stinging water experienced by humans in the vicinity of Cassiopea medusae.

In this study, we used a combination of microscopy, microfluidic devices, molecular biology techniques, mass spectrometry-based proteomics, and other experimental assays to provide the first detailed description, to our knowledge, of the contents of the mucus liberated from lab-reared Cassiopea xamachana medusae. Released within the mucus, we discovered three types of undeployed nematocysts, as well as microscopic, motile, cellular masses composed of nematocytes that we formally call cassiomes. While cassiosomes bear some resemblance to another cnidian structure originating in mesenteries of the starlet sea anemone Nematostella called nematosomes\textsuperscript{31}, the unique traits of cassiosomes in C. xamachana include their release into the water column within mucus, the ability to trap and kill prey as mobile grenades outside of the medusa, their organization as an outer epithelial layer surrounding a mostly empty core (rather than a solid ball of cells), and the presence of centrally-located endosymbiotic Symbiodinium dinoflagellates. We document the presence of cassiosomes in five species spanning four families of the order Rhizostomeae, while also confirming their absence in the moon jellyfish Aurelia (Semaeeostomeae), a representative of the sister lineage, and discuss the possibility of a single evolutionary event behind this envenomation strategy which, to our knowledge, is unique. Despite the growing body of work on C. xamachana from an organismal biology perspective\textsuperscript{14,26}, this study is the first to directly investigate stinging properties of the mucus of this jellyfish and the potential ecological and evolutionary relevance.

**Results**

**Study overview.** Observations were made on lab-reared Cassiopea xamachana (see the “Methods” section) and on medusae in their natural habitat in waters of Florida Keys mangrove forests (Fig. 1a, b). In both cases, medusae were observed releasing copious amounts of mucus into the water when surrounding water was disturbed (by jellyfish aquarists and/or snorkelers), or when prey items were provided (e.g., Artemia nauplii in aquarium-reared medusae). Stinging water phenomenon was experienced by the authors while handling lab-reared and/or wild C. xamachana and other rhizostome jellyfish examined in this study (species list provided below).

**Cassiopea xamachana overview.** Life cycle and endosymbiosis C. xamachana medusae start out like most scyphozoan jellyfish, as an asexual microscopic polyp that metamorphosizes into a sexually reproducing medusa via a process known as strobilation\textsuperscript{19}. However, they differ from most jellyfish in that they host endosymbiotic dinoflagellates (also called zooxanthellae)\textsuperscript{26,32}. Colonization of polyps by algal endosymbionts is the most common type of intracellular mutualism among cnidarians of the class Anthozoa (e.g., corals and anemones), and although it is less common in jellyfish species\textsuperscript{33}, endosymbiosis triggers the start of C. xamachana polyp strobilation\textsuperscript{18,19}. During the sessile life stage, these polyps engulf dinoflagellates (unialgal algae called Symbiodinium) via the manubrium (feeding tube), which are then phagocytosed by endodermal cells\textsuperscript{18,33}. Bound by a membrane complex that combines host and infecting cell membranes (called a symbiosome\textsuperscript{35}), Symbiodinium spp. migrate to the polyp mesoglea and remain there housed in endodermal cells,
transformed into amoebocytes. Shortly after infection with endosymbionts, *C. xamachana* polyps undergo strobilation, and the apical portion metamorphoses into an ephyra (juvenile medusa) which then develops into a sexually mature male or female medusa, with multiple color variants based on endosymbionts (Figs. 1a, b, 2a).

Symbiodinium-generated photosynthates support the jellyfish host metabolism, growth, reproduction and survival. This promotes conservation and recycling of essential nutrients, given their strategic presence amidst downwelling light, which is of unrivaled ecological importance for coral reefs and *Cassiopea* populations alike.

**Medusa feeding.** Feeding studies on *Cassiopea* medusae show that prey capture occurs as a result of perpetual medusa pulsation that carries the prey into the subumbrellar space and then onto the oral arms where they are held by nematocyst-rich digitate fringed lips and vesicular appendages (i.e., small oral vesicles) (Fig. 1a–f), eventually being reduced to fragments. Finally, food particles are then forced into the oral ostia of secondary mouths, and ingested via ciliary action. *Cassiopea* are opportunistic predators, feeding on a broad range of prey items (e.g., crustaceans, nematodes, eggs) in the field (Larson), while in the lab polyps and medusae are fed *Artemia salina* (1–3 days old, lab reared).

**Cassiosomes overview.** Cassiosomes morphology: Numerous, motile cellular structures, which we call cassiosomes, were observed suspended within mucus released by *C. xamachana* medusae (3.0–8.8 cm umbrella diameter) (Fig. 2a–f) in response to feeding or mild disruption with short bursts of seawater from a pipette. Herein, we describe cassiosomes in *C. xamachana* as microscopic (100–550 μm in diameter), irregularly-shaped cellular masses whose peripheral cell layer is primarily composed of nematocytes and other irregular ectodermal cells that surrounds a space containing amoebocytes—some hosting *Symbiodinium* and others lacking them—among presumptive mesoglea (Fig. 2f).

Cassiosome motility: When multiple *C. xamachana* medusae were placed together and agitated by directing water at their oral arms using a glass pipette, they consistently released cassiosome-laden mucus within 5–10 min (Fig. 2a–c) for periods lasting several hours. When collected mucus was transferred to a small glass dish, cassiosomes moved around within the mucus for about 15 min and then descended to the bottom of the dish, leaving the neutrally buoyant mucus. This permitted efficient isolation of numerous cassiosomes (Fig. 2b–f), which remained in constant motion by rotating and displacing in various directions along the bottom, but never elevating from the bottom of the dish (Supplementary Movie 1). Isolated cassiosomes remained motile for up to 10 days, gradually losing their corrugated appearance...
after 5 or 6 days and shrinking in size to a smooth spherical shape until movement ceased and cassiosomes disintegrated. Additionally, custom-designed microfluidic devices with channels equal to or slightly bigger than the cassiosomes were used to observe cassiosomes of *C. xamachana* to gain a better understanding of their motility, and three-dimensional irregular, popcorn-shaped structure. These microscopic observations revealed motile cilia extending from the periphery that propel cassiosomes.

**Cassiosomes kill prey**: We conducted assays to determine if cassiosomes were capable of trapping *Artemia* nauplii provided as food in aquarium-reared medusae since so-called non-penetrant *O-isorhiza* nematocysts are the only type found in cassiosomes of *C. xamachana*. For this purpose, microfluidic chambers provided an arena in which to document immobilization and rapid trapping of *Artemia* nauplii (Supplementary Movie 2), cassiosomes that encountered the underside of the nauplii carapace immediately immobilized and killed the prey items (Fig. 3a–c). In cases where *Artemia* nauplii came into contact only briefly with cassiosomes, prey were able to escape rapid immobilization and death (Supplementary Movie 5). Furthermore, during discharge assays when either FASW (Filtered Artificial Seawater) or mucus containing no cassiosomes, following manual removal, was added (see the "Methods" section), *Artemia* were not affected and continued to swim around in the dish (Supplementary Movies 4 and 3).

**Cassiosome ultrastructure**: In order to better understand their organization, live cassiosomes were isolated from *C. xamachana* mucus. After fixing, dehydrated specimens were examined using scanning electron microscopy (SEM) (Fig. 4a–f). The cassiosome perimeter was found to be lined with nematocyst capsules and
numerous long, spiny tubules (Fig. 4a–f) extruded from abundant O-isorhiza nematocysts in the periphery following spontaneous deployment (likely during the dehydration process (see the "Methods" section)). More abundant on the surface, however, were much thinner filaments corresponding to abundant cilia connected to ectodermal cells (Fig. 4a–c). Close observation of several cassetomes via SEM revealed along the outer layer emptied out regions appearing as collapsed cell membrane remnants of deployed nematocysts (Fig. 4a–f), underneath which could be seen an amorphous thick central extracellular matrix-like substance (Fig. 4e, f). Confocal microscopy on fixed cassetomes with labeled nuclei, nematocysts and cilia (see the “Methods” section) (Fig. 5a–d) corroborated these findings of an organized cell mass. Cassiosomes are composed of a peripheral layer of nematocytes bearing O-isorhiza nematocysts (Fig. 5a) patterned with presumptive ectoderm cells that lack nematocysts, from which numerous cilia protrude (Fig. 5a–d). This outer layer surrounds centralized clusters of Symbiodinium endosymbionts (i.e., hosted by amoebocytes) within an apparently acellular region (Fig. 4e, f).

**Cnidome of C. xamachana.** The term cnidome refers to the dynamic repertoire of nematocyst types in a cnidarian species.\(^5,8\) The cnidome, a species-specific trait, often changes throughout the life cycle of the jellyfish as it undergoes metamorphosis from a sessile polyp, to strobila, and then to juvenile and sexually mature medusa. Given reports implicating nematocysts, or tiny little granules, within Cassiopea mucus as the cause of stinging water, we sought to characterize the cnidome of this species at several life stages, and within the mucus and cassiosomes (isolated from mucus) (Fig. 6a–d). Measurements of undischarged nematocysts of each type in the corresponding subsample revealed that O-isorhizas nematocysts are absent in polyps, but appear in medusae from the onset of ephyrae development during strobilation (Fig. 6; Supplementary Table 1). We also observed penetrant nematocytes, birhopaloids and euryteles, which cannot be distinguished in the undeployed state (intact) within C. xamachana tissue using light microscopy. Therefore in this study, these two nematocyst types were analyzed together as rhopaloids (Fig. 6a–d) (as per ref. \(^36\) in C. andromeda); hence, rhopaloids account for a larger proportion of the cnidome in the medusa and mucus than distinct isorhiza types. An assessment of the inventory of nematocysts freely suspended within the mucus yielded a similar nematocyst profile to that of the medusa (Fig. 6a), albeit with a proportionately higher number of rhopaloids, which are implicated in envenomation.\(^28,29\) Conversely, isolated cassiosomes of C. xamachana contain exclusively O-isorhiza nematocysts which are a ubiquitous type in jellyfish tentacles, functioning in prey capture and predation.

**C. xamachana toxin proteins in cassiosomes.** Over a century ago, Perkins\(^17\) documented that disturbed C. xamachana medusae produced mucus containing ciliated structures as innumerable minute spherical bodies containing unicellular zooxanthellae within the interior, which he considered to be parasitic larvae, and claimed it was “impossible to regard [these structures] as of coelenterate [Cnidaria] affinities”. These details suggest that Perkins observed what we have herein identified as cassiosomes, but mistook them for entirely unique, non-cnidarian organisms. In order to test this theory, and properly classify cassiosomes as belonging to C. xamachana, rather than being unknown organism, we used real-time PCR (qPCR) assays to target species-specific cnidarian toxins, employing three custom-designed

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**Fig. 3 Cassiosomes are capable of killing brine shrimp.** a Dead 2-day old Artemia nauplii (orange arrow) with cassiosome (green arrow) attached to carapace, imaged within a microfluidic chamber. b Cassiosomes (green arrow) lodged into two different 1-day old Artemia nauplii (orange arrow). c 1-day old Artemia nauplii (orange arrow), immobilized following cassiosome (green arrow) attachment to the carapace with visibly discharged nematocysts (fuchsia arrows). Scale bars = 200 μm.
primer pairs (Supplementary Fig. 1e–g) that we designed from the publicly available *C. xamachana* genome (see Data Availability section).

We targeted a cnidarian-restricted CrTX/CaTX family toxin gene in DNA extracted separately from *C. xamachana* medusa tissue and isolated cassiosomes, and also from tissue of the jellyfish *Aurelia sp.* (Class Scyphozoa) and a more divergent jellyfish species *Alatina alata* (Class Cubozoa) for comparison. (Fig. 4 Cassiosome organization revealed via SEM. a An individual cassiosome poised in a 100-μm mesh opening, revealing the irregular ‘popcorn’ shape of the cassiosome bearing numerous cilia (pink arrows) protruding from the peripheral layer. White rectangles correspond to magnified region shown in (b) and (c). b, c Close up of the cassiosome reveals cilia (pink arrows), and thicker tubules (white arrows) of discharged O-isorhiza nematocyst capsules (blue arrows) in the periphery which is lined with collapsed nematocytes (cyan arrows). Spiny nematocyst tubules (white arrows) are outnumbered by the abundant cilia (thinner filaments) (pink arrows). d Depressions fringed by deflated cell membranes outlining nematocytes (cyan arrows) in *C. xamachana* medusa tissue and isolated cassiosomes, and also from tissue of the jellyfish *Aurelia sp.* (Class Scyphozoa) and a more divergent jellyfish species *Alatina alata* (Class Cubozoa) for comparison. Amplification of the qPCR gene target was observed for both *C. xamachana* tissue and cassiosome samples using primers for the CrTX/CaTX gene, herein named CassTX-C (see Data Availability section below). Conversely, failure to amplify the target in non-*Cassiopea* medusozoans used in this study (despite reports of CrTX/CaTX genes documented in both *Aurelia sp* and *A. alata*14,39) validates the specificity of our primers to a *C. xamachana*-derived gene target, indicating that cassiosomes originate in *Cassiopea* medusae.

To validate the potential for envenomation by cassiosomes, rather than solely by suspended intact nematocysts in the mucus released by medusae, we used LC-MS/MS analyses to confirm the presence of the same CrTX/CaTX toxin proteins in two *C. xamachana* sample types: cassiosomes isolated from mucus released from ~20 medusae over a 7-h period, and several vesicular appendages containing cassiosome nests, dissected from multiple medusae (e.g., Fig. 1c–f). A shotgun proteomic analysis identified three isoforms of the target toxin family encoded in the *C. xamachana* genome14, which we call CassTX-A, CassTX-B and CassTX-C (Fig. 7). Each toxin protein was identified with
multiple unique peptides and ≥17.0% protein coverage (Fig. 7; Supplementary Fig. 3; Supplementary Table 2), with the exception of CassTX-C, which in the cassiosomes was not assigned with sufficient confidence (based on Mascot Score). Peptides identified and aligned to the three Cassiopea toxin homologs are shown in Supplementary Fig. 3, along with the protein gel (Supplementary Fig. 4).

Cassiosome provenance and development. A visual inspection of C. xamachana oral arms during mucus release revealed that cassiosomes occur as warty clusters within a shallow pocket on the vesicular appendages (Fig. 8a–g) (Supplementary Fig. 2c, d) which are formed of ectoderm, endoderm and mesoglea; the cavity of these appendages communicates with the canals of the oral arms. Vesicular appendages are capable of independent movement, and during feeding of lab-reared C. xamachana medusae, when Artemia nauplii approach the oral arms, the vesicular appendage bends to cover the shrimp, thereby trapping the prey item; this trapping method was also reported in the conspecific C. frondosa (see ref. 32). Clusters of cassiosomes (i.e., 30–100 individuals) line the surface of the numerous, variably sized vesicular appendages present in C. xamachana (Figs. 1, 8). Bigelow (1900) called these appendages nettle batteries, referring...
to their functional role in subduing prey, and possibly also in defense.

Images of semithin sections of five separate vesicular appendages revealed that cassiosomes develop within a depression externally on one side of a vesicular appendage, but occasionally on both sides (Figs. 8, 9). During development, cassiosomes originate proximally as protrusions of the epithelium (ectoderm) of the vesicular appendage, and then spread out distally as they develop, incorporating presumptive amoebocytes (endoderm cells that have migrated into the mesoglea), some of which host Symbiodinium (Figs. 8, 9). Early developing cassiosome protrusions are connected peripherally to the pocket surface of the vesicular appendage by their shared ectoderm epithelial layer, whereas fully developed cassiosomes awaiting deployment are only loosely attached to the pocket and neighboring cassiosomes (Figs. 8, 9). This development process results in irregular popcorn-shaped cassiosomes, as shown in the 3-D reconstruction of their organization within the vesicular appendages, based on semithin images (Fig. 8).

The peripheral layer (nematocytes and ectoderm) surrounds a central space containing clusters of amoebocytes often hosting Symbiodinium, randomly interspersed among clear empty patches that exhibit substantially different refractive index properties (as seen in DIC) (Figs. 5, 8, 9) reminiscent of the small bags of mesoglea and nematocytes witnessed being released in conspecific C. frondosa by Smith (see ref. 40). These findings corroborate those of our SEM and confocal analyses, and suggest the central region of cassiosomes is amorphous, containing only

Fig. 6 Nematocyte type proportion (cnidome) varies within different life stages and structures of C. xamachana. a Figure displaying life cycle stages of C. xamachana (observed in this study) and associated cassiosome-laden mucus release by the medusae. Pie charts indicate proportion of nematocyte types for polyps (n = 3 distinct polyps), strobila/ephyrae (n = 3 distinct ephyrae), medusa (n = 3 distinct medusae), mucus (from n = 4 distinct medusae), and cassiosomes (from n = 4 distinct medusae), based on measurements of multiples of each nematocyst type per life stage (see details in Supplementary Table 1). b, c Different nematocyst types isolated from C. xamachana medusae oral-arm filaments corresponding to colors in pie charts in (a): a-isorhiza intact (light blue arrow), O-isorhiza intact (green arrows) and deployed (dashed green arrow), and rhopaloid intact (lavender arrow) and deployed (dashed lavender arrow); and Symbiodinium (brown arrows). d Mucus contents of C. xamachana containing a triplet of rhopaloid nematocysts (lavender arrows) intact within nematocytes, and Symbiodinium (brown arrow) disassociated from jellyfish tissue but still within amoebocytes (pink arrows). Scale bar: b, d = 10 μm; c = 20 μm.
some loose cells—likely amoebocytes—many of which host *Symbiodinium* (Figs. 8, 9).

**Cassiosomes in other rhizostome jellyfish species.** Jellyfish of the taxonomic order Rhizostomeae, including *Cassiopea xamachana*, all lack marginal tentacles, possessing instead oral arms covered with minute vesicular appendages. Although the main focus of this study is to provide a detailed description of cassiosomes in *C. xamachana*, in an effort to ascertain if cassiosome production is a possible apomorphy of the rhizostome jellyfish clade, we examined the mucus of additional rhizostome jellyfish taxa and documented cassiosomes in a total of four rhizostome jellyfish lineages (five different species) (Fig. 10). Cassiosomes from all six species are classified into two main types: motile, bearing cilia that propel them in the water column and non-motile, bearing no apparent motile cilia. Mucus was directly examined (using light microscopy) from three additional rhizostomes, *Mastigias papua*, *Phyllorhiza punctata*, *Catostylus mosaicus* (Fig. 10d–f, g–i), and a single Semaestomeae (sister group) *Aurelia* sp. (Fig. 10p), all reared at the National Aquarium (Baltimore, USA). Additionally, we obtained a video from the author of a citizen scientist blog post showing abundant motile particles reportedly released by another rhizostome *Netrostoma setouchianum*, collected in Japan (Fig. 10j). Although we were unable to directly examine these cellular masses from *N. setouchianum* (see ref. 41), their motility, the irregular shape they possess (Fig. 10k, l) when released from the oral arms, and the eventual loss of bumpiness and disappearance after several days matches the general description of cassiosomes we first discovered in *C. xamachana*. Cassiosomes of *M. papua* and *P. punctata* medusae are highly motile, and share the same fundamental structure, albeit exhibiting slight variations with respect to nematocyst types present within the peripheral nematocyte layer of each type (Fig. 10d–f, g–i). Superficially, *N. setouchianum* cassiosomes (Fig. 10k, l) appear to match the morphology of the two aforementioned species, however, as we were not able to examine them directly using microscopy (solely via video), the presence of associated dinoflagellates could not be confirmed. Conversely, cassiosomes in *Catostylus mosaicus* (Fig. 10n, o) exhibit several differences in that neither motility nor centralize *Symbiodinium* was observed but, rather, unidentified microalgae are distributed homogenously throughout the cell mass. No cassiosomes were found in the mucus of the semaeostome *Aurelia* sp. which lacks both vesicular appendages and endosymbiotic algae (Fig. 10p).

**Discussion**

Jellyfish are remarkable aquatic animals that diverged over 600 mya and have in spite of, or possibly because of, their diploblastic nature, evolved a remarkable envenomation system in the

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*Fig. 7 LC-MS/MS identification of jellyfish toxin proteins in cassiosomes. a* Alignment of peptides (black boxes) identified in two sample types (C = cassiosomes; VA = vesicular appendages) by shotgun proteomics to the full-length toxin homologs (CassTX-A, -B and -C; black bars) of the cnidarian-restricted CrTX/CaTX toxin family identified in *C. xamachana*. Each protein was identified with a confidence score > 0.05 and with at least two unique peptides in each sample, except for CassTX-C in the cassiosome sample. *b–e* Representative mass and annotated tandem mass spectra of CassTX-A tryptic peptides: (*b*) and (*c*) peptide sequence VAPIPESGLEEGK; (*d*) and (*e*) peptide sequence TEIELSTDLLGDVK.
form of stinging cells—nematocytes—for prey capture and defense. In this work, we reported the findings of an extensive investigation into the provenance, development and ultrastructure of cassiosomes, a newly described cnidarian stinging-cell structure. Based on these findings, we hypothesize that cassiosomes evolved within a single lineage of jellyfish, Rhizostomeae, to further weaponize the jellyfish by sequestering nematocytes (and other cells) into grenade-like structures that are freely released into the water within

Fig. 8 Characterization of the ultrastructure of the vesicular appendages during cassiosome production and development in Cassiopea xamachana. Line drawing of vesicular appendage demonstrates how early developing cassiosome protrusions (pro) are connected peripherally to the pocket surface of the vesicular appendage by their shared epithelium (epi vap), whereas fully developed cassiosomes (cass) awaiting deployment are only loosely attached to the pocket and neighboring cassiosomes. a–e Semithin sections (~1 μm) of resin-embedded vesicular appendages corresponding to arrows labeled a–e in the line drawing of the vesicular appendages (va) extending from the oral arms (arm) of the medusae. Clusters of cassiosomes (pink arrows) developing from protrusions (pro) in the epithelium of the concave vesicular appendage pocket (epi vap) give rise to the cassiosome peripheral layer comprising nematocytes bearing O-isorhiza nematocysts (dark spheres stained with 1% toluidine blue) interspersed with other ectodermal cells. Clusters of amoebocytes hosting Symbiodinium (green arrows) move into the cassiosome core at protrusions points. Cassiosome core containing presumptive mesoglea indicated by difference in diffractive index with DIC. f Partial 3-D reconstruction showing protrusions developing from epithelium of the vesicular appendage pocket (epi vap) into popcorn-shaped cassiosomes. Reconstruction based on sections from a different vesicular appendage than seen above but corresponds to the region between sections a–d, revealing the empty core (core) of cassiosomes (cass) (3-D image orientation is vertical with respect to cross sections in the line drawing). arm = medusa oral arm, cass = cassiosomes(s), core = presumptive mesoglea; pro = protrusion(s); va = vesicular appendage(s), epi vap = epithelial layer of the vesicular appendage pocket. Scale bar = 250 μm.
exuded mucus. Our findings strongly implicate cassiosomes as a major contributor to the stinging water phenomenon reported by sea bathers and aquarists when interacting with rhizostome jellyfish species.

In this inaugural study on cassiosomes, we used extensive microscopy techniques, video-documentation and microfluidics to describe these cnidarian innovations first in C. xamachana, and then in taxa belonging to four additional Rhizostomeae jellyfish families. Our preliminary findings suggest there are motile and non-motile types of cassiosomes among the rhizostomes we examined in this study, and that some host endosymbiotic algae *Symbiodinium* while at least one bears microalgae instead. However, the fundamental trait distinguishing cassiosomes from nematosomes, analogous cell masses deriving from the mesenteries of the sea anemone *Nematostella* [31], is that the structure of cassiosomes is organized into a distinct outer epithelial layer surrounding a central, mostly empty, core. Further studies are needed to elucidate the role of these photosynthetic endosymbionts in cassiosomes. The complete absence of cassiosomes in the mucus released by the semeastome *Aurelia* sp. supports our theory that cassiosomes are a rhizostome evolutionary novelty. However, a comparative examination of the mucus contents

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**Fig. 9** Characterization of the ultrastructure of mature cassiosomes in Cassiopea xamachana. **a** Line drawing, and **b, c** thin sections of fully developed popcorn-shaped cassiosomes from semithin sections (~1 µm) of resin-embedded vesicular appendage (see Fig. 8). Cassiosome peripheral layer comprising nematocytes (cyan arrows) bearing O-isorhiza nematocysts (as peripheral dark spheres stained blue with Richardson’s stain in **b** and **c**) interspersed with patches of oddly shaped ectoderm cells (red arrows), and motile cilia (pink arrows); blue-stained nuclei (yellow arrows) visible below the base of large nematocysts capsule in nematocytes, and also in non-nematocyte ectoderm cells. Cassiosome core containing presumptive mesoglea (gray central region in **a**), gray arrow in **b** and **c**), speckled with amoebocytes (purple arrows)—hosting *Symbiodinium* (green arrows) or empty. Rigid stereocilia/cnidocil complex (orange arrows) visible as a point at the nematocyst apical portion, and deployed tubules (black arrows in **a**) on surface present as long, thick spiny threads. epi vap = epithelial layer of the vesicular appendage pocket. Scale bars = 50 µm.
across all rhizostome lineages, including the eight nominal *Cassiopea* species\(^{26,42}\) is needed to test this hypothesis.

Furthermore, we identified the provenance of cassiosome production and release from oral arm vesicular appendages, corroborating earlier works suggesting these vesicles (as oral vesicles\(^{17,40,43}\)) function in defense and predation. These previous works noted similar structures in the oral vesicles of *Cassiopea* species, dubbed either gray bodies, bags of nematocysts and mucous cells, minute spherical bodies, or grenades, that shot when contacted. Although those reports fell short of providing an adequate description, we are confident that the structures mentioned therein correspond to what we described herein as cassiosomes. The mechanism of cassiosome deployment may vary across different rhizostome taxa, or even between conspecifics, as according to Smith\(^{40}\) when prey was provided to *Cassiopea frondosa*, an aperture opened at the tip of the vesicular appendages releasing gray bodies (putative cassiosomes) (Supplementary Fig. 2a, b). Conversely, in this study on *C. xamachana*, upon disturbance, cassiosomes spontaneously detached from the surface of vesicular appendage pockets, which lack a terminal aperture (Supplementary Fig. 2c, d).

All jellyfish have envenomation capabilities due to bioactive proteins comprising the venom cocktail of the cnidome (i.e., repertoire of nematocysts types). The cnidarian-specific pore-forming CrTX/CaTX toxin family is one of the most potent toxin groups, and represents the main proteinaceous component of the venom of cubozoans (box jellyfish), a clade that includes species whose sting results in a deadly cardiovascular condition\(^{37}\). In this study, the presence of *C. xamachana*-specific CrTX/CaTX toxin family homologs was confirmed in cassiosomes at the DNA and protein level (i.e., CassTX), validating their expression in cassiosomes and their contribution to the stinging water phenomenon.

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**Fig. 10 Cassiosomes observed in jellyfish species of the order Rhizostomeae.** Cladogram of species examined in this study from two orders Rhizostomeae: a–c *Cassiopea xamachana*, d–f *Mastigias papua*, g–i *Phyllorhiza punctata*, j–l *Nestostoma setouchianum*, and m–o *Catostylus mosaicus*, and p *Semaeostomeae Aurelia sp.*, and their respective cassiosome structures, when present. Abbreviations: iso = isorhiza nematocysts; rhp = rhopaloid nematocysts; * = could not confirm type of nematocysts. Blue symbols: star = motile via ciliary movement; hexagon = non-motile; circle = endosymbiotic dinoflagellates within cassiosomes confirmed; oval = microalgae on the surface of cassiosomes confirmed; asterix = could not confirm presence or absence of algal symbionts; X = no cassiosomes witnessed within the mucus. Scale bar: Scale bar: a = 1.5 cm; d, g, j, m, p = 2.5 cm; b, e, h, k, n, o = 300 \(\mu\)m; c, i, l = 200 \(\mu\)m; f, o = 100 \(\mu\)m.
Although *C. xamachana* in Florida waters is considered a mild stinger, reports exist of painful human envenomation resulting in diminished in size and gradually lost the irregular popcorn structure (Fig. 2b).

**Methods**

*C. xamachana* live animal culture. *C. xamachana* polyps were obtained from medusae (Supplementary Fig. 1a) cultured in National Aquarium (Baltimore, USA) and maintained in the Aquaroom wet culture room, Department of Invertebrate Zoology, National Museum of Natural History (NMNH), Smithsonian Institution (Washington D.C.). Polyps were kept in petri dishes until undergoing strobilation. Metamorphosed medusae were kept either in hanging baskets within a 55-gallon (Washington D.C.). Polyps were kept in petri dishes until undergoing strobilation.

**Isolation and preparation of cassiosomes.** *C. xamachana* medusae were placed into 150-ml glass dishes containing 45 ml of FASW (Filtered Artificial Seawater). Transferring medusae from tank to dishes caused them to release mucus within 5 min. Medusae were further agitated (for 5–10 min) by gently pipetting water onto their oral arms using a Pasteur pipette until cassiosomes were identified within the mucus as small white flecks under a dissecting microscope (Fig. 2b). Translucent mucus was collected using a transfer pipette (Supplementary Fig. 1b) and placed in a small dish of FASW for observation (Fig. 2d), or into a 1.5 ml low-binding microcentrifuge tube for molecular analysis. In addition to containing abundant cassiosomes and nematocytes, particles found suspended within the mucus included microscopic artifacts originating from other marine life inhabiting the tank (e.g., sponge spicules, hair algae, diatoms, *Artemia* and other zooplankton), and occasionally the microscopic muscular jellyfish *Cassiopeia coleoptrata*. Mucus and isolated cassiosomes (which sink to the bottom of the dish after 15 min.) were subsequently transferred into new dishes, microfluidic devices (Supplementary Fig. 1d), or microcentrifuge tubes. Their constant motility (spinning, linear displacement, reversal, and turning) persisted for about 10 days when kept in natural light, but diminished in size and gradually lost the irregular popcorn structure (Fig. 2b–f) after 3–5 days, eventually becoming spherical by days 8–10.

**Live medusa observations.** Live medusa observations were conducted on a Nikon Stereoscope; images were captured using a Nikon D7000 Camera and strobes as needed, and analyzed in SharpShooter3 software. Occasionally, for photo-documentation purposes, medusae were temporarily immobilized using 10% MgCl₂, but the reagent had no effect on the motility of isolated cassiosomes. Here we acknowledge that all images appearing in this work were captured using Adobe Illustrator software.

**Cnidome preparation.** *C. xamachana* squash preps made from excised oral arm tissue in medusae, bell margin tissue in ephyrae, and tentacles in polys, cassiosomes and mucus (Fig. 6) were examined with a Nikon Eclipse e80i compound microscope and imaged with a Nikon D7000 Camera using SharpShooter3 software. Nematocyte identification and measurements (as per 12) were conducted using Fiji version of ImageJ software (Version 2.0.0-rc-681-2SF; https://imagej.net). Mean, range and standard deviations were calculated using the R base package (https://www.R-project.org/) (Supplementary Table 1).

**Discharge assays.** To observe the effects of cassiosomes on potential prey items in relatively confined spaces, discharge assays were conducted within a microfluidic device into which *Artemia nauplii* (1–2 days old) were introduced along with cassiosomes either isolated from or still within the mucus in FASW. This 6-chambered microfluidic device, with chambers 7-mm wide × 400-μm tall × 3-mm long, modified from a version originally designed as a behavioral microarena for imaging *Hydra*, was used to immobilize large cassiosomes (400-μm diameter) or to slow down the movement of smaller cassiosomes (<400 μm) to conduct observations over periods of up to two days. Furthermore, *Artemia* nauplii (1–3 days old) were introduced into petri dishes (100-ml) containing either isolated cassiosomes, mucus lacking cassiosomes or a FASW control. Video-documentation was performed using Nikon ShmooScope with a Nikon DXM1200F camera connected to a SharpShooter3 software or at the Imaging Lab at NMNH, Smithsonian Institution (Washington D.C.) using an Olympus BX63 upright microscope, and viewed with cellSens software. All movies were trimmed using iMovie and are available as Supplementary Movies.

**Scanning electron microscopy.** Cassiosomes were imaged at the Imaging Lab at the NMNH, Smithsonian Institution (Washington D.C.), with an Apo FESEM (Field Scanning Electron Microscope) at 5.00 KV. Thousands of isolated cassiosomes were concentrated into 1.5 ml Eppendorf tubes and fixed in freshly mixed 2.5% glutaraldehyde in FASW (33 ppt.). The following day, cassiosomes were transferred to plastic BEEM® capsules (13 mm diameter) with the bottom 1/3 cut off and replaced with a modified bottom with a 100-μm mesh filter. Samples were washed with 1x PBS (3x, 10 min), concentrated in 1x PBS (3x, 10 min), and brought through an ethanol dehydration series, 25% (5 min), 50% (5 min), 75% (5 min), 95% (60 min), 100 (3 x 15 min). BEEM® capsules containing dehydrated cassiosomes were covered with modifed lids with a 100 μm mesh filter and subjected to critical point drying. Each mesh filter containing dehydrated cassiosomes was mounted onto an SEM stub (13 mm) using an adhesive carbon strip and sputtered coated with gold-palladium.

**Confocal microscopy.** Imaging of live and fixed cassiosomes was conducted at the Naval Research Laboratory (Washington D.C.), on a Nikon A1R Confocal (Nikon Instruments) microscope using both DIC and confocal laser scanning. Highly motile cassiosomes were immobilized on MatTek glass bottom dishes coated with either Poly-L-Lysine (Sigma Aldrich) or Cell Tak adhesive (Corning), and on live tissue, NucBlue-Hoescht 33342 (1,100) (ThermoFisher) was used to stain nuclei. Images were collected with ×60 objective (oil).

Fixed material was prepared by fixing cassiosomes in 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 h at 4 °C. Fixative was removed from the cassiosomes with three washes in PBS with 1% Triton X-4 for up to 48 h before staining. Cassiosomes were stored up to four days before processing. To identify tubulin, actin and nuclei in the tissue, fixed material was stained for confocal analysis using the following protocol. Tissue was blocked in 5% goat serum (Jackson ImmunolResearch, 005–000–001) for 1 h, and rinsed three times with 10-min washes in PBS with 1% Triton X-4. Tissue was incubated in 1:400 acetylated alpha Tubulin Antibody (6–11B-1) Alexa Fluor *546 for 90 min in PBS with 1.5% goat serum. After removing, tissues were stained with ActinGreenTM ReadyProbesTM Reagent, 2 drops/ml for 15 min, and then with NucBlueTM Fixed Cell ReadyProbesTM, 2 drops/ml for 30 min. Tissues were then washed five times with PBS before slides of PBS and 0.1% Triton-20, and then mounted with an 80% glycerol in PBS on glass slides for imaging. Imaging was performed with laser lines at 405, 488, 561, and 640 nm, and collected with a Plan Apo 100x objective. Each image was measured via channel series to minimize fluorescence overlap between channels. All images were analyzed with NIS-Elements AR imaging software (Nikon Co. Ltd).

**Whole mount semithin sections for histology.** Five vesicular appendages (~1.5 mm wide) full of cassiosomes (n = 30–100 individuals) were dissected from medusae (~5 cm umbrella diameter). Tissue was fixed overnight in 2.5% glutaraldehyde in a 0.1 M phosphate buffer with 9% sucrose at pH 7.4 at 4 °C. After three washes with the same buffer, the samples were fixed at room temperature for 2.5 h in 1% OsO₄ in the phosphate buffer at 4 °C. Samples were dehydrated through a series of ethanol and infiltrated with Spurrier’s resin. After curing, the resin block was cut to 995 nm thick sections using the procedure described by 18 to create ribbons. These ribbons were mounted on slides and stained with 1% toluidine blue or Richardson’s stain (as per ref. 19). Slides were analyzed using the application AMIRA® for 3D reconstructions (as per ref. 18).

**Real-time PCR (qPCR) analysis.** Genomic DNA was extracted (Qagen DNAeasy Blood and Tissue kit) from *C. xamachana* cassiosomes preserved in ethanol and homogenized using a handheld biorvortexer with stirring rods for microcentrifuge tubes (RPI International Corp., USA), and from medusa tissue of *C. xamachana* (target), *Asurita sp.* and *Alatina alata* (two off-target taxa). All DNA extracts were quantified using a Qubit 2.0 fluorometer with the dsDNA HS Assay Kit. Species-specific primers were designed using PrimerQuest online software (https://www.idtdna.com/PrimerQuest/) to amplify the cnidarian-restricted toxin gene family (i.e. CaTX/GTX) (primer sequences available in Supplementary Fig. 1e).
LC-MS/MS analysis. Isolated cassiosomes and vesicular appendages in FASW (33 ppt) were pelleted (4000 rpm for 10 min) and resuspended in 20 μL NuPAGE LDS sample buffer (Thermo Fisher Scientific, Carlsbad, CA) and 10 mM dithiothreitol (DTT). Exclusively LCMS grade reagents were used. After sonication with a handheld biovortex (30 s, maximum bursts for 3 min) to break down the nematocysts capsule minicollagen double wall, and then in a sonication bath (10 min) to remove bubbles, samples were heated to 70 °C for 10 min and separated on a 10% NuPAGE Bis-Tris mini gel for 35 min at constant voltage (200 V). Proteins were stained with BioSafe Coomassie (Bio-Rad, Hercules, CA) and de-stained overnight with water. Ten bands were excised from the gel according to molecular weight (gel image available as Supplementary Fig. 4). Proteins in all bands were reduced and alkylated, and then digested in gel with sequencing grade trypsin (Promega, Madison, WI). Peptides were extracted from gel by two treatments with a handheld biovortex (30 s, maximum bursts for 3 min) to break down 100% acetonitrile. All extraction fractions were concentrated by speed-vac, and then final wash with 100% acetone. All extraction fractions were concentrated by speed-vac, and then resuspended in 20 μL of 0.1% formic acid. One μL of each sample was injected into an LC-MS/MS system (U3000 LC coupled to Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific, Waltham, MA)) for proteomic analysis. Resulting spectra were extracted, merged, and searched by Mascot (Matrix Science Inc, London, UK) against a database containing common standards and contaminants, i.e., trypsin, keratin, etc. (190 protein sequences). A searchable database was also created in-house of amino acid sequences corresponding to predicted open reading frames (ORFs), created using TransDecoder® (v.5.5.0), for the three cnidarian toxin proteins (CassTX-A, CassTX-B and CassTX-C) (Supplementary Fig. 3) expressed in the C. xamachana transcriptome (see Data Availability section).

Statistics and reproducibility. The mean length and width, and range, of multiple of each nematocyst type comprising the cnidome of Cassiopea xamachana, were determined in triplicate for three separate medusae (or other species for comparison). Additionally, thousands of cassiosomes were extracted from the mucus of at least 20 individual medusae over the course of this project, and imaged using the various microscopy methods described herein. Harvested mucus and/or isolated cassiosomes from multiple medusae (more than five biological replicates) were combined for LC-MS/MS proteomics, and tissue subsamples were taken from three different medusae for genetic molecular analysis (PCR and qPCR). Five separate vesicular appendages were analyzed with histological methods. Detailed explanations provided within the Methods section are sufficient to ensure reproducibility of the experiments conducted herein.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The authors declare that all relevant data supporting the findings of this study are available within the manuscript and its supplementary materials. Additional data are available from the corresponding authors upon request. The following datasets are publicly available:

| Datasets                        | Accession Numbers                                                                 |
|--------------------------------|-----------------------------------------------------------------------------------|
| Cassiopea xamachana transcriptome | PMCID: PMC5932825 http://rnylanl.whitney.ufl.edu/downloads/Cnidaria_transcriptomes/ |
| Cassiopea xamachana genome      | NCBI Accession: OLM000000000.1                                                     |

Additional information on data availability is provided in the Data Availability section. Complete metadata is also available from the corresponding authors upon request.

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Statistics and reproducibility. The mean length and width, and range, of multiple of each nematocyst type comprising the cnidome of Cassiopea xamachana, were determined in triplicate for three separate medusae (or other species for comparison). Additionally, thousands of cassiosomes were extracted from the mucus of at least 20 individual medusae over the course of this project, and imaged using the various microscopy methods described herein. Harvested mucus and/or isolated cassiosomes from multiple medusae (more than five biological replicates) were combined for LC-MS/MS proteomics, and tissue subsamples were taken from three different medusae for genetic molecular analysis (PCR and qPCR). Five separate vesicular appendages were analyzed with histological methods. Detailed explanations provided within the Methods section are sufficient to ensure reproducibility of the experiments conducted herein.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The authors declare that all relevant data supporting the findings of this study are available within the manuscript and its supplementary materials. Additional data are available from the corresponding authors upon request. The following datasets are publicly available:

| Datasets                        | Accession Numbers                                                                 |
|--------------------------------|-----------------------------------------------------------------------------------|
| Cassiopea xamachana transcriptome | PMCID: PMC5932825 http://rnylanl.whitney.ufl.edu/downloads/Cnidaria_transcriptomes/ |
| Cassiopea xamachana genome      | NCBI Accession: OLM000000000.1                                                     |

Additional information on data availability is provided in the Data Availability section. Complete metadata is also available from the corresponding authors upon request.
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Author contributions

K.M., M.K. and C.L.A. made initial observations on cassiosomes, conceived of the experiments and drafted the initial manuscript. Experimental design for this final study, including epifluorescence microscopy, qPCR, data collection and analysis were conducted by A.M.L.K., K.M. and C.L.A.; samples were prepared for LC-MS/MS by J.S. and C.L.A. with substantial instruction by D.L.; J.S. conducted LC-MS/MS analysis with supervision by D.L.; microfluidic devices were designed and constructed by K.B., with substantial instruction by J.R., who also provided training on their use for A.M.L.K., K.M. and C.L.A.; SEM preparation and imaging was carried out by A.R. and C.L.A.; fixation, histology, and imaging of semithins and 3-D reconstructions were done by A.R.; illustrations of vesicular appendages and cassiosomes were conducted by N.B. and C. xamachana life stages by A.M.L.K.; confocal microscopy was performed by L.D.F., M.M. and C.L.A.; J.D.J. provided lab-reared Cassiopea polyps, which were reared to medusa stage by K.M., M.K., A.M.L.K., C.L.A. and A.G.C.; J.D.J. assisted with mussel extraction from additional rhizostome species provided by J.D.J.; A.M.L.K. and C.L.A. drafted the manuscript and produced figures and movies; A.G.C., P.C. and G.J.V. supervised the project from start to finish. All authors revised and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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