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Tactic-specific antimicrobial activity suggests a parental care function for accessory glands in a marine toadfish

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Males of some species possess extra reproductive organs called accessory glands which are outgrowths of the testes or sperm duct. These organs have a well-established role in reproduction; however, they also appear to have other important functions that are less understood. Here, we investigate the function of the highly complex accessory glands of a marine toadfish, *Porichthys notatus*, a fish with two reproductive male types: large care-providing 'guarder' males and small non-caring 'sneaker' males. While both male types have accessory glands, guarder male accessory glands are much larger relative to their body size. We show that accessory gland fluids strongly inhibit the growth of bacterial genera associated with unhealthy eggs and have no effect on the growth of strains isolated from healthy eggs. This antibacterial effect was particularly pronounced for extracts from guarder males. Furthermore, we demonstrate that both healthy and unhealthy plainfin midshipman eggs have diverse but distinct microbial communities that differ in their composition and abundance. The highly specific inhibitory capacity of accessory gland fluid on bacteria from unhealthy eggs was robust across a wide range of ecologically relevant temperatures and salinities. Collectively, these ecological and molecular observations suggest a care function for the accessory gland mediated by antimicrobial agents.

1. Introduction

Parents often go to great lengths to ensure their offspring survive and thrive: defending their young against predators, sheltering them from harsh environmental conditions and provisioning them with nutrients [1–3]. A less common care strategy involves the production of antimicrobial compounds to protect developing young from infectious diseases. For example, invasive Australian bull ants (*Myrmecia gulosa*) produce formic acid secretions that inhibit fungal growth on their broods [4], and barn swallows (*Hirundo rustica*) transfer bacterial-killing lysozymes to their offspring via egg albumen [5]. Males of two fish species, the redlip blenny (*Ophioblennius atlanticus*) and the peacock blenny (*Salaria pavo*), have large external antimicrobial (lysozyme-like)-producing organs called *accessory glands* that are rubbed on their eggs [6], and removing these glands dramatically decreases egg survival [7].

Accessory glands are outgrowths of the testes or sperm duct and are found in males of many species [8–11]. These morphologically diverse organs have been proposed to contribute to sperm performance, sperm storage and activation, sperm buffering from osmotic and ionic challenges, and production of mating plugs, spermatophores and pheromones [11,12]. Accessory gland fluid can also influence female receptivity and oviposition rates [13–16]. While accessory gland fluid can markedly alter the chemical micro-environment of sperm and

eggs during fertilization, the role of these specialized glands in parental care and antimicrobial protection is still poorly understood. Unattended demersal fish eggs can be quickly decimated by microbial infections [17], and previous work suggests that accessory gland function in fishes may be related to the care of young in these microbe-rich aquatic habitats [6,7,15,18].

The plainfin midshipman fish (*Porichthys notatus*) has both a structurally complex accessory gland (figure 1*a*) and two alternative reproductive tactics [19,20]. Every spring, large 'guarder' males excavate nests underneath rocks and produce a low-frequency hum that attracts females [19]. After laying bright yellow eggs, females desert the rocky nests, leaving the guarder males to protect, aerate and hydrate the developing eggs over a 60-day period—an unusually lengthy and costly care period for fishes [21,22]. During this time, guarder males and eggs experience fluctuations in environmental conditions (i.e. temperature, salinity, oxygen) as the tide height oscillates and the nests are exposed [23]. However, not all male plainfin midshipman fish provide care. Small 'sneaker' males do not excavate nests or court females. Instead, they employ stealth tactics to steal fertilizations from guarder males by either sneaking into the nest or by fanning sperm in from the nest periphery [19]. Following fertilization, sneaker males leave and provide no care for the young.

Our previous work has shown that accessory glands are much larger in guarder males than in sneaker males, and that these glands play a role in sperm performance by increasing sperm velocity [20]; however, other possible functions for these glands have not been investigated. The plainfin midshipman accessory glands have two distinct structures: nodes and lobules (figure 1*a*), with guarder males investing more in the lobule mass than sneakers [20]. Why such differences exist between male types and what function is served by the accessory glands have been long-standing questions [24–26]. Given that guarder male accessory glands increase in size during the caring season while those of sneaker males decrease over this same period, we hypothesized that accessory glands might also have a role in parental care by storing antimicrobial fluids that can be applied to developing eggs [20].

Typically, males guard nests filled with bright yellow or orange translucent eggs (healthy); however, on occasion, the eggs turn white or grey, cloudy and opaque (unhealthy; figure 1*b*). Because bacterial colonization can influence fish egg health [27], we aimed to test whether accessory gland extracts could influence bacterial growth. We collected healthy/clear eggs and unhealthy/opaque eggs (figure 1*b*), cultured a subset of their bacterial communities and assessed whether accessory gland extracts from guarder and sneaker males could affect the growth of these ecologically relevant bacterial species. Furthermore, we investigated how accessory gland extracts affected bacterial growth across environmental conditions and explored the identity of a growth-inhibitory agent associated with guarder male lobule extracts.

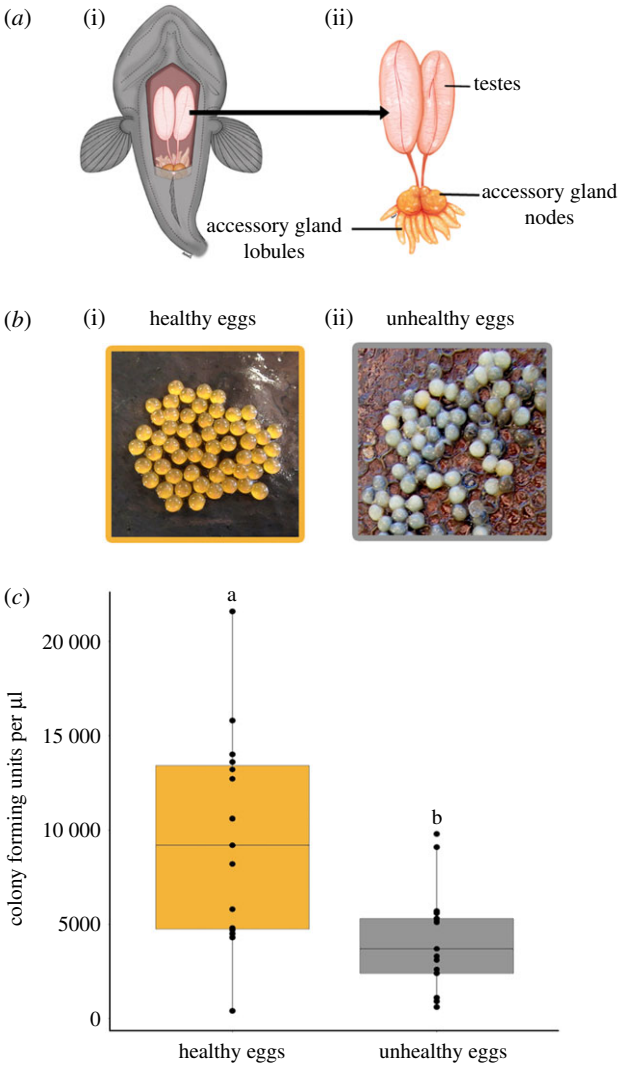

Figure 1. (*a*) (i) Schematic of the ventral surface of a male plainfin midshipman fish dissected to show the testes and accessory glands, and their relative positions in the body cavity. For clarity, other organs are not included. (ii) Close-up view of the testes, accessory gland nodes and accessory gland lobules. Illustration credit: A. Pathak. (*b*) Photographs of healthy (i) and unhealthy (ii) plainfin midshipman eggs. Photograph credit: A.P.H. Bose. (*c*) CFUs associated with healthy eggs (n=18) versus unhealthy eggs (n=19). Mean ± s.e. for healthy eggs: 9560 ± 1453. Mean ± s.e. for unhealthy eggs: 4100 ± 653. Significant differences are denoted by the use of different letters. (Online version in colour.)

2. Methods and results

(a) Healthy and unhealthy midshipman eggs host distinct bacterial communities

To culture bacteria associated with healthy and unhealthy plainfin midshipman eggs (figure 1*b*), eggs were collected from 37 nests (1 egg per nest taken from 18 healthy and 19 unhealthy broods) in the field using sterilized tweezers. The eggs were suspended in glycerol and then stored at −80°C until serial dilutions of the glycerol suspension could be plated on marine agar medium [28]. Diverse microbial communities were observed for all egg suspensions, with colonies of varying size and colour represented. A representative colony of each phenotype (i.e. colonies with distinct colour, size and texture) was profiled using 16S rRNA gene sequencing (see the electronic supplementary material, S1 for details). It should be noted that the sequencing of phenotypically distinct colonies was not done on a per-egg basis, and these isolates do not represent an exhaustive list of bacteria associated with the plainfin midshipman eggs. We cultured 31 distinct isolates, which were identified as bacterial species representing 13 different genera (see the electronic supplementary material S1, table S1). Most of the isolated species were Gram-negative bacteria and many of

the bacterial genera were shared between healthy and unhealthy eggs, including *Aquimarina*, *Cellulophaga*, *Flavobacterium*, *Phaeobacter* and *Psychrobacter*. There were, however, also genera exclusively associated with healthy eggs (e.g. *Algoriphagus*, *Celeribacter*, *Vibrio*), or with unhealthy eggs (e.g. *Ahrensia*, *Leucobacter*, *Maribacter*, *Colwellia*, *Sulfitobacter*).

Total colony-forming units (CFUs) were tallied and compared for each of the healthy and unhealthy eggs examined. We observed a higher abundance of CFUs from the healthy egg samples than from the unhealthy eggs (*t*-test, $t_{30} = 3.57$, est. ± s.e. = 5460 ± 1530, $p = 0.001$; figure 1*c*). Unexpectedly, no fungal colonies were observed, even when the glycerol suspensions were spread on fungal-specific growth media; all but two isolates yielded 16S rRNA products (indicative of bacteria), and the two refractory isolates also failed to yield products when polymerase chain reaction amplifications were conducted with fungal-specific 18S rRNA gene primers (see the electronic supplementary material, S1 for details). Spreading the contents of glycerol-only control vials (made at the same time, using the same procedures and transported together with the vials into which eggs were placed and stored) on marine agar medium did not yield any CFUs, indicating that the bacteria cultured in this study probably originated from the plainfin midshipman nest environment.

(b) Accessory gland extracts inhibit *Leucobacter* growth

Twenty-four guarder and 12 sneaker males were collected, and their accessory glands were removed (see the electronic supplementary material, S2 for details). The accessory glands were divided into nodes and lobules, which were then treated as separate tissues in this study. Fluids extracted and pooled from nine guarder male lobules were tested against all typed (by 16S sequence) bacterial isolates from healthy and unhealthy eggs (electronic supplementary material S1 and table S1) using a disc diffusion bioassay (see the electronic supplementary material, S2 for details). Of the 31 species initially tested, only the growth of *Leucobacter* (an unhealthy egg isolate) was impacted by the guarder lobule extracts, with a clear inhibition zone observed (figure 2*a* inset). These results suggested that the guarder lobule extracts had bactericidal activity with unusual specificity. Lobule extracts from individual sneaker males showed low-level growth inhibition of *Leucobacter*, but this was 3 times less potent than equivalent volumes of lobule extract from individual guarder males (n=6; inhibition zone distances were measured and compared with a *t*-test, $t_4 = 15$, est. ± s.e. = 2.26 ± 0.15, $p < 0.001$; figure 2*a*). Extracts from individual guarder and sneaker male nodes (n=6) were also tested for their ability to inhibit *Leucobacter* growth, but neither had any effect (no inhibition zone was observed; figure 2*a*).

In the wild, plainfin midshipman—and their eggs in the intertidal zone nests—experience wide temperature and salinity fluctuations with the daily incoming and receding tides [23,29]. To explore the antibacterial activity of the guarder lobule extracts under more ecologically relevant conditions, pooled lobule extracts from 12 guarder males were tested for their ability to affect *Leucobacter* growth at temperatures ranging from 4°C to 30°C in 'full salt' (35 ppt) and 'half salt' (17 ppt) media conditions (see the electronic supplementary material, S1 for details). Under all of these conditions, the extracts showed strong growth inhibition (based on the size of the inhibition zone; figure 2*b*).

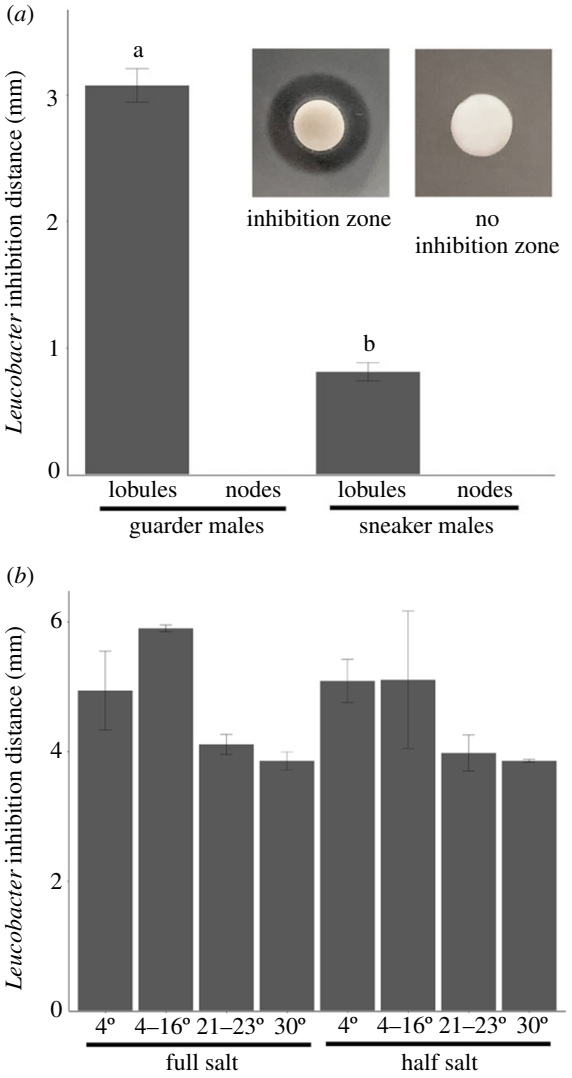

Figure 2. (*a*) *Leucobacter* growth inhibition by the accessory gland lobule versus node extracts, from guarder and sneaker males. n=3 for each male type and accessory gland region (lobule versus node). Significant differences are denoted by the use of different letters and standard error bars are plotted. Inset: photos of the growth inhibition bioassay showing an extract-infused filter disc surrounded by an inhibition zone (dark ring around the filter disc; left) versus a disc with no inhibition zone (right). The inhibition distance (mm) was measured from the edge of the filter disc (see the electronic supplementary material, S2 for details). (*b*) *Leucobacter* growth inhibition by guarder male accessory gland lobule extracts grown across a range of salinities (full salt = 35 ppt, half salt = 17 ppt) and temperatures (°C). Three replicates were performed per environmental condition. Standard error bars are plotted. Extracts from 12 individuals were combined for these experiments (see the electronic supplementary material, S2 for details).

(c) Extracts specifically inhibit the growth of diverse genera from unhealthy eggs

Given the unexpected *Leucobacter*-specificity of the guarder lobule extracts, we wanted to determine whether *Leucobacter* species were commonly associated with plainfin midshipman eggs, and if so, what frequency they were associated with healthy versus unhealthy eggs. We plated glycerol serial dilutions from the same 37 eggs as in our initial screen. From these plates, we cultured 10 additional isolates (five from unhealthy eggs and five from healthy eggs) with phenotypic similarity to *Leucobacter* (small, circular, pale yellow colonies) and sequenced their 16S rRNA gene fragments.

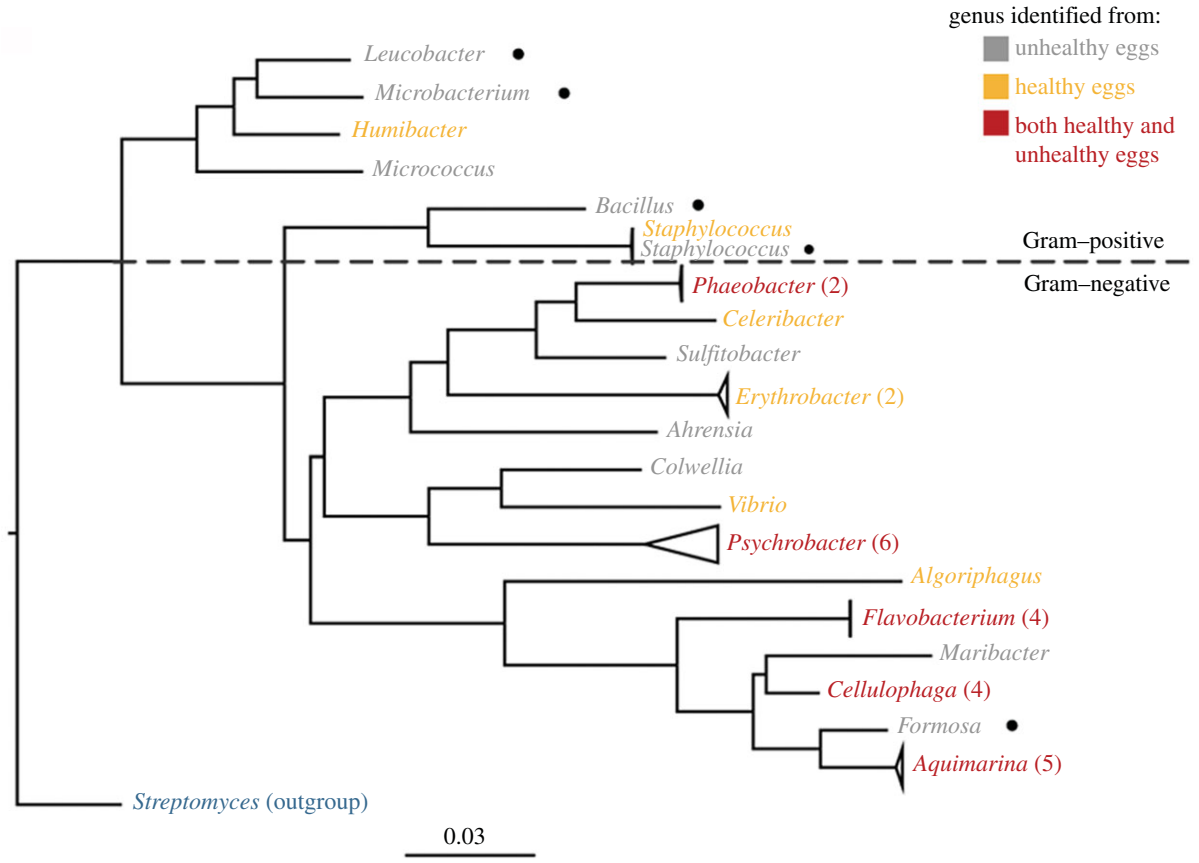

Figure 3. Phylogenetic tree of microbial isolates from healthy and unhealthy egg samples, based on partial 16S rRNA gene sequences. Species within the same genus are grouped (number of sequences per genus are shown in brackets), with the variances indicated by the size of triangles. Fluid extracted from the guarder male accessory gland lobules had antibacterial activity against a number of isolates from unhealthy eggs (indicated with black circles). Bootstrap values of each branch: greater than or equal to 50% (based on 1000 resampled trials). The scale bar (labelled 0.03) represents substitutions per nucleotide position.

We found that none of these isolates were *Leucobacter*, but instead were phylogenetically diverse bacterial species from the *Bacillus*, *Erythrobacter*, *Formosa*, *Humibacter*, *Microbacterium*, *Micrococcus*, *Psychrobacter* and *Staphylococcus* genera (electronic supplementary material S1, table S2). Each of these isolates was also tested for their sensitivity to the guarder male lobule extracts (using pooled samples from 12 males). Intriguingly, none of the genera isolated from healthy eggs were affected by the lobule extracts, while the growth of all but one of the genera (*Micrococcus*) isolated from unhealthy eggs were inhibited by the lobule extracts. Most of the susceptible unhealthy egg isolates were Gram-positive bacteria, with the exception of the Gram-negative *Formosa* isolate belonging to the Flavobacterium family. This was unexpected, considering all other species from the Flavobacterium family isolated in this study (*Cellulophaga*, *Maribacter*, *Aquimarina*) were resistant to the guarder lobule extracts (figure 3). Hence, guarder lobule extracts inhibit the growth of specific bacterial genera within diverse families and phyla and have no discernible effect on culturable bacteria associated with healthy eggs (figure 3).

(d) Inhibitory activity of lobule extracts is mediated by non-proteinaceous molecule(s)

To further test the antimicrobial specificity of the guarder lobule extracts (pooled from 12 males), we assessed whether those extracts could impact the growth of *Micrococcus luteus*: a close relative of *Leucobacter* (same actinobacterial phylum). *Micrococcus luteus* is generally susceptible to many antibiotics [30] and is known to be exquisitely sensitive to the effects of lysozyme [31]. We found *M. luteus* growth was unaffected by the guarder lobule extract, which suggested that the active molecule in the guarder lobule extracts was not a lysozyme.

To test whether the growth-inhibitory compound(s) was a lysozyme-like protein, or some other protein, the same pooled lobule extracts from 12 guarder males were treated with proteinase K (a protease with flexible cleavage capabilities). We first confirmed that proteinase K alone did not affect *Leucobacter* growth using the growth inhibition bioassay, before testing whether proteinase K treatment of guarder lobule extracts led to a loss of antimicrobial activity. We found the protease-treated extracts retained their *Leucobacter* growth-inhibitory activity (figure 4*a*), suggesting that the inhibitory molecule was not a protein. To ensure that proteinase K could cleave proteins in the guarder lobule extracts, we separated proteinase K-treated and -untreated lobule extracts using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and observed effective degradation of the proteins in the lobule extract (figure 4*b*). While we cannot exclude the possibility that the inhibitory activity was owing to a low abundance protease-resistant protein that was undetectable on our gels, our results suggest that the antimicrobial properties associated with lobule extracts are unlikely to be lysozyme-like or protein-mediated.

We further assessed the composition of the lobule extracts using liquid chromatography mass spectrometry (LC/MS) to compare the metabolic profiles of lobule extracts from sneakers (pooled from four males) and guarders (pooled from 12 males). The metabolic profiles of these extracts were consistently distinct: some peaks differed in abundance but

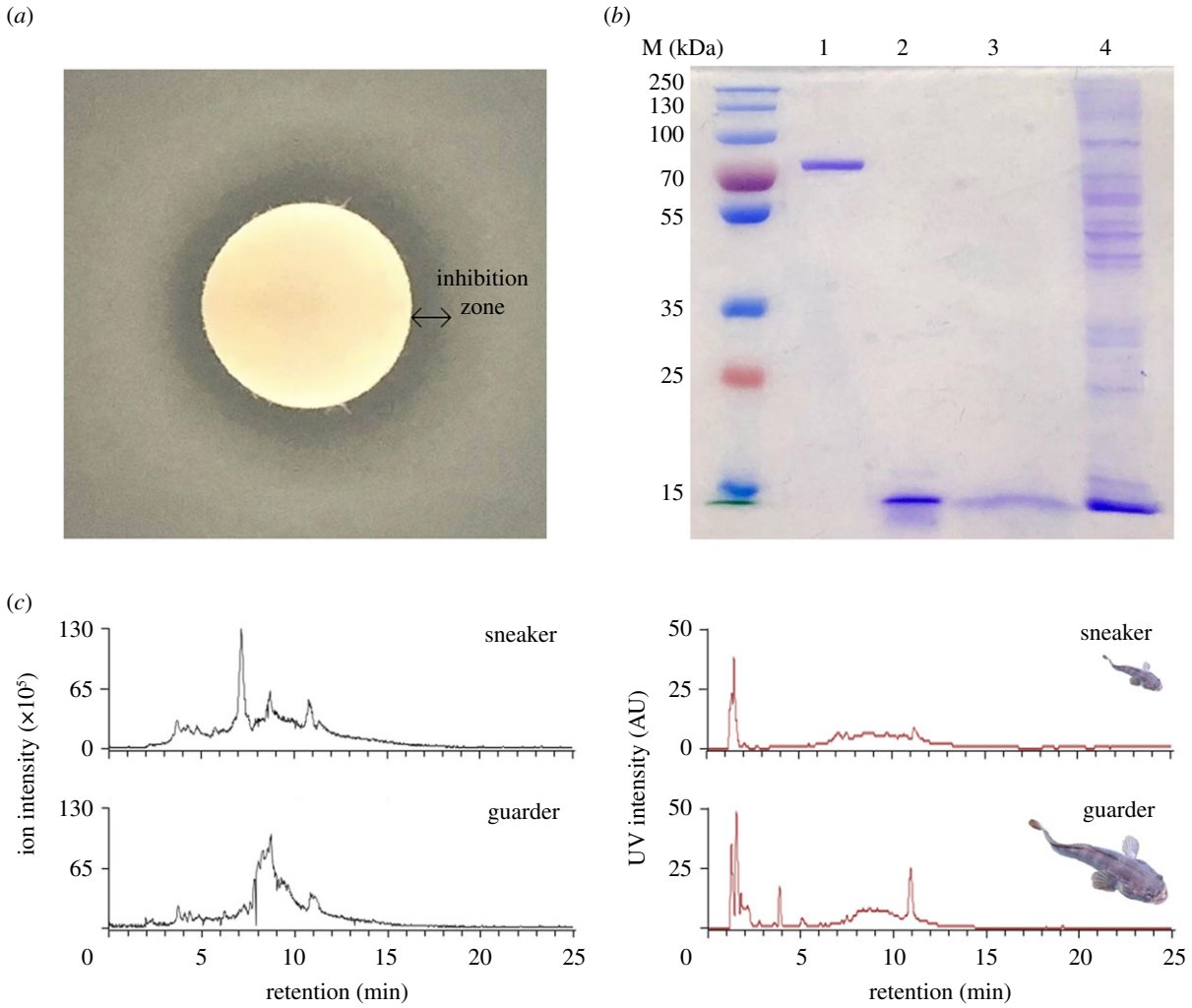

Figure 4. (*a*) Growth inhibition bioassay using proteinase K-treated guarder male lobule extracts. The inhibition zone around the filter disc is indicated. (*b*) Ten per cent denaturing SDS–PAGE showing the effects of proteinase K on guarder male lobule extracts and control proteins. Lane 1: bovine serum albumin (BSA) alone (negative control). Lane 2: BSA treated with proteinase K (positive control). Lane 3: BSA and lobule extract treated with proteinase K. Lane 4: 1 : 50 dilution of undigested lobule extract (negative control). (*c*) Chromatograms of guarder male and sneaker male accessory gland lobule extracts based on total ionization (black lines) and UV spectra (red lines). Sneaker lobule extracts were pooled from four individuals and guarder lobule extracts were pooled from 12 individuals in these experiments (see the electronic supplementary material, S2 for details). (Online version in colour.)

seemed to be shared between samples, while others appeared unique to each male type (figure 4*c*). Comparing the mass spectra of compounds within the lobule fluids did not reveal any match with known antimicrobials. Collectively, these results suggest that the compound(s) capable of inhibiting the growth of unhealthy egg-associated microbes may be a unique small molecule(s).

3. Discussion

Here, we report on an effective but understudied form of parental care: producing antimicrobial compounds and presumably secreting them onto developing eggs [2,3]. We show that fluids contained within the plainfin midshipman accessory glands inhibited the growth of bacteria isolated from unhealthy eggs but did not affect the growth of bacteria isolated from healthy eggs. Importantly, the antimicrobial activity was confined to the lobules and this activity was significantly more pronounced—and more potent—for caring guarder males compared with non-caring sneaker males. The guarder male accessory gland lobules increase in size throughout the caring period [20], which, when considering the antimicrobial activity associated with these lobules, is consistent with the idea that guarder males invest in their accessory glands to protect their developing young. Furthermore, given that guarder male lobule and node extracts were previously found to have similar impacts on sperm performance (i.e. both lobule and node extracts increased guarder male sperm velocity), this functional difference between the lobules and nodes in the context of parental care is notable. Further characterizing these fluids to understand the components responsible for each function is a priority for future investigations.

The growth of five phylogenetically disparate genera from unhealthy eggs was inhibited by the extracts from guarder male lobules. These susceptible bacteria represented three phyla: the Gram-positive Actinobacteria (*Leucobacter* and *Microbacterium*) and Firmicutes (*Bacillus* and *Staphylococcus*), and the Gram-negative Bacteroidetes (*Formosa*). Importantly, growth inhibition was not observed for all members of these phyla, with *M. luteus*, a common 'indicator' (antibiotic-susceptible) bacterium and close relative of *Leucobacter* and *Microbacterium*, exhibiting resistance to the inhibitory agent.

An obvious question is whether the unhealthy egg-associated bacteria are the cause or the consequence of egg death/

disease. These bacteria may directly infect plainfin midshipman eggs, and this action could provide strong selective pressure for the production of an antimicrobial agent that inhibits their growth. Among the genera susceptible to the guarder extracts, only *Staphylococcus* and *Microbacterium* are known to cause disease in fishes [32,33], while *Leucobacter*, *Formosa* and *Bacillus*, to the best of our knowledge, have not been investigated in relation to fish disease. Indeed, *Bacillus* spp. have been more commonly studied for their probiotic/protective potential in aquatic environments than for their pathogenic properties [34–36]. While *Leucobacter* is not a known fish pathogen, it is a pathogen of the nematode *Caenorhabditis elegans*, owing in part to its ability to produce robust biofilms in the *C. elegans* uterus [37]. Whether *Leucobacter* forms deleterious biofilms on plainfin midshipman eggs remains an important question to address in the future. There are many other ways that bacterial colonization could adversely affect developing fish embryos, including penetrating the eggshell and causing disease; releasing toxins and enzymes that are detrimental to embryonic development; and generating a damaging hypoxic environment for the developing embryo [27]. Whether, and how, any of these extract-inhibited genera impact egg development remains to be determined.

Alternatively, some bacterial species associated with plainfin midshipman eggs may be beneficial to egg development. These beneficial isolates may have originated from parent reproductive microbiomes in the male accessory glands or from the female reproductive system [38]. A growing body of literature is suggesting that reproductive microbiomes can have positive—and negative—effects on reproductive success and offspring survival [38]. The antimicrobial properties of the lobule extracts may function to maintain beneficial bacteria on the plainfin midshipman eggs by specifically inhibiting the growth of other (possibly environmental in origin) bacteria. This possibility may be supported by our observations that more culturable bacteria were associated with healthy eggs (figure 1*c*), although it should be noted that our data do not take into account any unculturable bacteria that may be associated with healthy and unhealthy eggs.

To address this question, our future work will involve more broadly defining the microbiome of healthy and unhealthy plainfin midshipman eggs using metagenomic shotgun sequencing. This could allow us to identify both culturable and unculturable species and facilitate quantitative analyses of microbial diversity and abundance. We are also interested in addressing why egg death (and presumed bacterial infection) is commonly observed in wild nests under the care of guarder males, given the observed antimicrobial properties of their accessory glands. Do these fluids need continual application? Could fluid depleted males lose their broods because the efficacy of their gland fluid diminishes? Future investigations will allow us to address these questions and shed additional light on accessory gland function and an apparently unique mode of parental care and microbial community modulation.

The specificity and apparent non-proteinaceous nature of the lobule-associated antimicrobial activity is particularly intriguing, as all previously characterized antimicrobial secretions from caring fish parents have been lysozyme-like compounds [6,7,15]. LC/MS analyses revealed distinct metabolic profiles of the fluids extracted from accessory gland lobules of guarder and sneaker males. Some peaks were present in both male types but were more abundant in guarder male lobule extracts (e.g. peak at 11 min in the UV spectra; figure 4*c*); these are of interest, as they are correlated with the difference in antimicrobial potency between the two male types (figure 2*a*). Notably, none of the associated mass spectra matched those of known antimicrobial compounds. Further isolation and characterization of the inhibitory molecule(s) will be necessary to determine its identity and the mechanism underlying the specificity of its growth inhibition.

In summary, by combining ecological marine fieldwork with microbiological laboratory studies, we have uncovered an ecologically relevant, potentially novel antibacterial agent employed by a marine toadfish from the intertidal zone of the North American coast. Extracts from the guarder male accessory gland lobules inhibited the growth of bacteria associated with unhealthy eggs with remarkable specificity, having no effect on the growth of bacteria associated with healthy eggs. Our findings suggest that these reproductive glands function in paternal care, as only the caring males invest heavily in the antimicrobial-containing lobule structures. A better understanding of the function of accessory glands broadly across taxa may aid in our understanding of the evolution of these extra reproductive glands, and in the case of our particular study, may reveal a source of new antimicrobial agents.

Ethics. The plainfin midshipman fish is a common intertidal species and its populations are not endangered or threatened [39]. All fish were collected in accordance with permits issued by Fisheries and Oceans Canada (XR-58-2017 and XR-48-2018). All research procedures were approved by the McMaster University Animal Research Ethics Board (AUP's nos 13-12-52 and 18-01-02) and the University of Victoria Animal Care Committee (AUP Juanes-20).

Data accessibility. The datasets and codes for statistical analysis supporting this article are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.kkwh70s3m [40].

Authors' contributions. M.A.P. carried out microbiological laboratory work, participated in data analysis, helped design the study and co-wrote the first draft of the manuscript; H. carried out microbiological laboratory work, conducted the phylogenetic analysis, participated in designing the study and critically revised the manuscript; J.S.M. carried out the statistical analyses and made all figures, collected field data, curated the data, helped conceive the study design and co-wrote the first draft of the manuscript; M.A.E. supervised all microbiological laboratory work, conceived of the study design and critically revised the manuscript; S.B. conceived of the study design, collected and curated field data, and co-wrote the first draft of the manuscript. All authors edited and revised the manuscript and gave final approval for publication.

Competing interests. We declare we have no competing interests.

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