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Proteomic changes associated with predator-induced morphological defences in oysters

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Abstract

Inducible prey defences occur when organisms undergo plastic changes in phenotype to reduce predation risk. When predation pressure varies persistently over space or time, such as when predator and prey co-occur over only part of their biogeographic ranges, prey populations can become locally adapted in their inducible defences. In California estuaries, native Olympia oyster (*Ostrea lurida*) populations have evolved disparate phenotypic responses to an invasive predator, the Atlantic oyster drill (*Urosalpinx cinerea*). In this study, oysters from an estuary with drills, and oysters from an estuary without drills, were reared for two generations in a laboratory common garden, and subsequently exposed to cues from Atlantic drills. Comparative proteomics was then used to investigate molecular mechanisms underlying conserved and divergent aspects of their inducible defences. Both populations developed smaller, thicker, and harder shells after drill exposure, and these changes in shell phenotype were associated with upregulation of calcium transport proteins that could influence biomineralization. Inducible defences evolve in part because defended phenotypes incur fitness costs when predation risk is low. Immune proteins were downregulated by both oyster populations after exposure to drills, implying a trade-off between biomineralization and immune function. Following drill exposure, oysters from the population that co-occurs with drills grew smaller shells than oysters inhabiting the estuary not yet invaded by the predator. Variation in the response to drills between populations was associated with isoform-specific protein expression. This trend suggests that a stronger inducible defence response evolved in oysters that co-occur with drills through modification of an existing mechanism.

KEYWORDS
inducible defence, local adaptation, oyster, phenotypic plasticity, predation, proteomics

1 | INTRODUCTION

Predation risk is an environmental factor that can vary considerably across space and time, and consequently, prey populations can experience differing strengths of selection on predator defence mechanisms. Constitutive (or canalized) defences are favoured when predation pressure is high and constant, causing undefended phenotypes to suffer severe fitness declines (Clark & Harvell, 1992; Edgell et al., 2009; Moran, 1992). However, defences that are expressed only when predators are present become advantageous when predation pressure is variable, and the costs associated with producing and maintaining defensive traits exceeds their benefit during periods of low predation risk (Jarrett, 2018; Mitchell et al., 2017; Nunes et al., 2014). Under these conditions, inducible

prey defences can evolve in which organisms exhibit plastic changes in phenotype in response to the presence of a predator or a predator attack (Harvell, 1990).

Anthropogenic activities are driving species introductions that result in contact between organisms that do not share a common evolutionary history, including new interactions between predators and prey (Capinha et al., 2015; Cox, 2004; Doherty et al., 2016; Freeman & Byers, 2006; Strauss et al., 2006). In the absence of historical exposure, invasive predators can cause declines or even extinctions among native prey populations because these species may not have evolved appropriate defence systems (Cox & Lima, 2006; Paolucci et al., 2013; Salo et al., 2007; Sih et al., 2010). However, introduced predators also impose strong selection that can cause native prey to evolve new defences that reduce predation risk (Freeman & Byers, 2006; Mooney & Cleland, 2001; Nunes et al., 2014). Furthermore, if there is persistent spatial or temporal variation in predation pressure across the biogeographic range of prey, there may be adaptive divergence in the defensive responses of prey populations (Large & Smee, 2013; Reger et al., 2018). For example, populations of marine mussels (*Mytilus edulis*) that vary in historical exposure to an invasive crab (*Hemigraspus sanguineus*) also differ in the strength of inducible defences against this predator. Mussel populations co-occurring with the invasive crab rapidly evolved an inducible defence response, growing thicker shells when exposed to cues from the predator. In contrast, mussel populations inhabiting regions not yet invaded by the crab did not mount an inducible defence when exposed to the same predator cues (Freeman & Byers, 2006).

The Atlantic oyster drill (*Urosalpinx cinerea*) is a predatory snail native to estuaries on the East coast of North America. In the late nineteenth century, the drill was introduced to the Pacific coast of North America (Carlton, 1992), where it encountered new prey species, including the only native oyster found on this coastline, the Olympia oyster (*Ostrea lurida*) (Polson & Zacherl, 2009). Distribution of the invasive drill is presently uneven across the *O. lurida* range, resulting in oyster populations that experience varying levels of predation risk that might lead to differences in the strength of selection for defences against this predator. The presence of drills in the Tomales Bay estuary of Northern California has decreased Olympia oyster abundance in this location (Kimbro et al., 2009). In contrast, drills are absent from the Elkhorn Slough estuary in Central California (Figure 1; Wasson et al., 2001, 2014). Consequently, oyster populations within Tomales Bay probably experience greater selection pressure to evolve defences that reduce drill predation compared with oyster populations inhabiting Elkhorn Slough.

Inducible defences of Olympia oysters originating from Tomales Bay and Elkhorn Slough differ in a pattern that is consistent with local adaptation to predation pressure from Atlantic drills. Oysters from either Tomales Bay or Elkhorn Slough grow smaller, thicker, and harder shells when exposed to drills consuming conspecifics (Bible et al., 2017). These data indicate that predator diet cues or prey alarm cues released during drill predation trigger an existing inducible defence system in Olympia oysters, one that probably evolved to protect oysters from native predators (such as the drilling whelk *Acanthinucella spirata*). However, the magnitude of the inducible defence response is not uniform between these two oyster populations. Oysters from Tomales Bay grow significantly smaller (but not thicker or harder) shells and tend to be preyed upon less frequently compared with oysters living in drill-free Elkhorn Slough. Since these experiments were performed using second generation, common garden raised oysters having never been exposed to predators, variation in the response between these populations probably reflects natural selection modifying existing inducible defence mechanisms in association with an increased risk of drill predation in Tomales Bay (Bible et al., 2017).

Evolution of distinct inducible defences among Olympia oyster populations provides an opportunity to identify molecular mechanisms involved in plastic responses to predators. Inducible prey defences are widespread among taxa and ecosystems (Kishida et al., 2009; Tollrian & Harvell, 1999), yet few investigations have

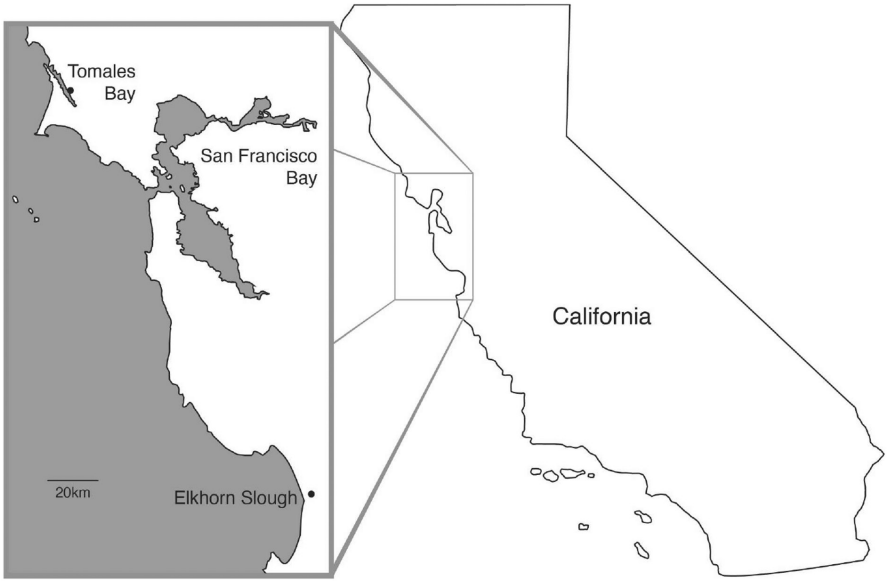

FIGURE 1 Map of oyster populations sampled in California, USA

sought to identify the pathways and processes that contribute to phenotypic change in response to predators (Mitchell et al., 2017; Weiss, 2019). Molecular and evolutionary underpinnings of predator-induced changes in shell phenotype are also unknown, despite this being a common means of reducing predation among marine and freshwater molluscs (Auld & Relyea, 2011; Bourdeau et al., 2013; Leonard et al., 1999; Trussell & Smith, 2000). Past efforts to resolve molecular bases of inducible prey defences have focused on water fleas of the genus *Daphnia*, a classic model for studying plastic responses to predators (e.g., Hales et al., 2017; Orsini et al., 2018; Otte et al., 2015; Tams et al., 2020). Mechanisms having evolved in *Daphnia* are unlikely to be similar in prey that encounter predators using different prey capture methods (e.g., gape-limited predators of *Daphnia* vs. crush or boring predators of bivalves) or that respond with markedly different types of morphological defence (e.g., neck teeth in *Daphnia* vs. shell thickening in bivalves).

In this study, the proteomic response to invasive Atlantic drills was compared between Olympia oysters from Tomales Bay and Elkhorn Slough. Quantifying abundance across the proteome represents an unbiased screen to identify proteins that contribute to conserved and divergent features of inducible defences in Olympia oyster populations. Proteins with three expression patterns were of interest. Proteins upregulated by both populations following exposure to drills consuming oysters are most likely to facilitate plastic changes in shell phenotype and highlight aspects of inducible defences that are conserved in Olympia oysters. Proteins downregulated by both oyster populations in the presence of drills point toward costs associated with inducible defences. A tenet of inducible prey defences is that defended phenotypes incur trade-offs that prevent their canalization (Harvell, 1990). These trade-offs can drive non-consumptive effects on prey populations (Peacor et al., 2013), but such consequences of inducible defences are unknown for Olympia oysters (Bible et al., 2017). Lastly, proteins exhibiting opposing directions of change between Tomales Bay and Elkhorn Slough oysters are most likely to have been acted on by varying natural selection associated with local differences in predation risk.

2 | MATERIALS AND METHODS

2.1 | Oyster husbandry and predator exposure

Oysters were collected from the wild, spawned, and raised through two generations in a laboratory common garden as described previously (Bible et al., 2017). Broodstock (F_0) were collected in September 2011 from two Olympia oyster populations that differ in historical exposure to *Urosalpinx*: Marshall in Tomales Bay, where oysters co-occur with invasive drills; and Elkhorn Slough, an estuary that has not yet been invaded by the predator (Figure 1). Broodstock from each location were transported to Bodega Marine Laboratory (Bodega Bay, CA, USA), maintained in separate 19 L tanks at room temperature (18–24°C) with daily water changes, and fed microalgae culture (*Isochrysis* at 400,000–900,000 cells/ml) or Shellfish Diet (300,000 cells/ml; Reed Mariculture, Inc.). Broodstock were held in these conditions for 16 days before spawning, after which F_1 larvae were filtered (37 μm nylon mesh) and transferred to 100 L culturing cones. F_1 oysters from each population were grown for 16 months under common garden conditions, after which these oysters were spawned to produce second-generation (F_2) oysters. Second-generation, lab-reared oysters were allowed to settle onto 10 × 10 cm polyvinyl chloride (PVC) tiles, and cultured under common garden conditions for 3 months before being used in experiments.

The predator exposure experiment distributed F_2 oysters from each population (Tomales Bay and Elkhorn Slough) between two treatment conditions (control and exposed to predator cues) with six replicates per population-by-treatment combination (Figure S1). F_2 oysters from each population were first distributed into separate 4 L mesocosms containing between 2–4 PVC tiles with either 12 or 13 oysters attached per tile. Two of these mesocosms (one per population) were then connected to a single upstream 7 L container that delivered seawater with either cues from 10 *Urosalpinx* consuming Olympia oysters (i.e., predator cues) or seawater without animals (i.e., control). This system was replicated six times and oysters were maintained in each treatment for 6 weeks before being sampled.

A pilot study indicated that exposure to this density of *Urosalpinx* was sufficient to reduce oyster growth within 6 weeks and published results confirm changes in oyster growth, shell thickness, and shell hardness from this experimental design (Bible et al., 2017). Oysters within each mesocosm were kept at ambient temperature (mean temperature ± SD = 12.6 ± 1.1°C) and were fed shellfish diet at approximately 450,000 cells/ml. *Urosalpinx* in upstream containers originated from Tomales Bay, were fed Olympia oysters for 1 week after being brought into the laboratory, and then starved for 1 week prior to the beginning of the experiment. Drills were fed a mixture of laboratory-reared oysters from multiple sites in San Francisco Bay and Tomales Bay ad libitum during the six-week experiment.

A portion of oysters from this experiment were assessed for growth, hardness, and shell-to-tissue weight ratios as reported elsewhere (Bible et al., 2017). Here, a subset of 12 oysters were sampled from each population-by-treatment combination and used for proteomics: Tomales Bay oysters exposed to drills (n = 12), Elkhorn Slough oysters exposed to drills (n = 12), Tomales Bay oysters exposed to control seawater (n = 12), and Elkhorn Slough oysters exposed to control seawater (n = 12). Each set of 12 oysters was collected from between 7–9 different tiles representing either five or six of the replicate exposure systems. During sampling, whole oysters were removed from PVC settlement tiles, wrapped in aluminium foil, flash frozen in liquid nitrogen, and then stored at −80°C.

2.2 | Proteomics

Protein extraction and in-solution digestion with bead-immobilized trypsin (Princeton Separations EN-251) were performed as described previously (Kültz et al., 2013). Whole oysters were dissected from shells and refrozen in liquid nitrogen, then crushed into a fine

powder and mixed with an ice-cold solution of 10% trichloroacetic acid, 90% acetone, and 0.2% dithiothreitol (DTT). Samples were incubated in this solution for 1 h before being centrifuged at 18,000 *g* for 5 min at 4°C. Precipitated proteins were washed twice in acetone containing 0.2% DTT and the protein pellet dissolved in buffer containing 8 M urea and 0.2% DTT. After centrifugation at 18,000 *g* for 5 min, the supernatant was removed and stored at −80°C. Protein concentration was determined using a 660 nm protein assay compatible with the dissolution buffer (Thermo-Pierce 22660). Protein extracts were reduced and alkylated for 30 min each in 10 triethanolamine buffer (pH 8.0, final concentration 100 mM) using 16 mM DTT and 16 mM iodoacetamide, respectively. Immobilized trypsin was added to each sample at a 1:25 ratio relative to total protein and incubated for 16 h at 35°C.

Next, 2 µl of tryptic peptides (200 ng) from each sample were injected into a nanoAcquity sample manager (Waters Corp.), desalted for 1 min using a trap column (15 µl/min flow rate; Symmetry, Waters Corp. 186003514), and separated by reversed phase chromatography using a 1.7 µm particle size BEH C18 column (250 mm × 75 µm, Waters Corp. 186003545). A nanoAcquity binary solvent manager (Waters Corp.) was used to generate a 2 h linear gradient of 3%–35% acetonitrile with 0.1% formic acid in the aqueous phase. Online nano-electrospray ionization (nanoESI) was achieved by zero-dead-volume connection of a pico-emitter tip (New Objective FS360–20-10-D-20) to the end of the column, which was connected to the nanoESI source of the mass spectrometer.

Tandem mass spectrometry (LCMS2) was performed with an ImpactHD UHR-QTOF mass spectrometer (Bruker Daltonics). ESI-L low concentration tuning mix (Agilent G1969–85000) was used for mass calibration. Batch processing of samples was controlled with HYSTAR 4.0 software (Bruker Daltonics). All peaklists were generated with DATAANALYSIS 4.2 (Bruker Daltonics) and proteins identified using PEAKS 8.5 (Zhang et al., 2012a). The following parameters were used: enzyme specificity trypsin, missed cleavages permitted = 2, fixed modification Cys carbamidomethylation, variable modifications Met oxidation, Pro hydroxylation, N-terminal acetylation, precursor ion mass tolerance = 10 ppm, and fragment ion mass tolerance = 0.02 Da. A threshold score of 5% probability that a protein identification is incorrect was used for accepting individual MS/MS spectra. A database containing 46,755 protein sequences comprising the entire Pacific oyster (*Magallana gigas*; formerly *Crassostrea gigas*; Salvi & Mariottini, 2021) reference proteome was downloaded from NCBI RefSeq on 30 September 2017. An expanded version of this database was generated with PEAKS 8.5 that included randomly scrambled decoy sequences for each protein entry and more than 200 potential contaminant proteins such as human keratins and porcine trypsin. This expanded decoy database was used for all protein identification searches to allow consistent assessment of protein identification false discovery rate. Redundancy in assigning peptides to protein identifications and ambiguity in protein identifications were eliminated by PEAKS 8.5.

Relative protein abundances were determined using the quantitative module of PEAKS 8.5 and the MS1 peak area for the top three peptides per protein. The internal PEAKS algorithm, which takes into account isotopic distribution of masses, was used for protein quantitation to generate relative abundance differences between experimental conditions for each protein (Zhang, Xin, et al., 2012). A mass error threshold of 30 ppm and a retention time threshold of 3 min was enforced for matching MS1 isotope peaks in all quantitative analyses over their entire retention time range. Samples were normalized to their respective total ion chromatograms to account for small differences in total sample peptide concentrations.

2.3 | Statistical analyses of protein abundance

Proteins identified in at least eight of the 12 samples within each treatment-by-population combination were used in statistics (Kültz et al., 2016). Protein abundance data were analysed using singular value decomposition (SVD; Alter et al., 2000). SVD is a data-reduction technique in which the complete dataset is simplified into a series of "eigenvectors," with each eigenvector corresponding to a particular protein expression pattern. The first eigenvector represents the expression pattern accounting for the largest proportion of total variation in the data set, the second eigenvector describes the next largest proportion, and so on. This statistical approach has proven effective in resolving meaningful patterns of variation within proteomic data sets (e.g., Vohradsky et al., 2007). SVD was performed using the National Institute on Aging's Array Analysis tool (Sharov et al., 2005), which identifies eigenvectors within the protein expression matrix and then uses Pearson's correlation coefficients to find proteins strongly correlated with each eigenvector. Only proteins strongly correlated with the first three eigenvectors (Pearson's correlation coefficients >.8; Evans et al., 2017) and with mean expression changes >1.5-fold were analysed. Hierarchical clustering (i.e., heatmaps) was used to illustrate changes in protein abundance between oyster populations and experimental treatments. Clustering was based on the Spearman correlation dissimilarity matrix and the Ward agglomerative linkage method using $\log_2$ of protein abundance. Heatmaps were created using the GPLOTS package in R (Warnes et al., 2009). Amino acid sequence alignment of differentially expressed proteins was performed using CLUSTAL OMEGA (Sievers et al., 2011) accessed through the Universal Protein Resource (UniPRot) database (The UniProt Consortium, 2021).

2.4 | Over-representation analysis and protein functional annotation

Over-representation analysis was used to determine if proteins with particular functions were more numerous among proteins that changed abundance in oysters exposed to drills. Over-representation analysis uses functional information to identify categories of proteins (called ontologies; The Gene Ontology Consortium, 2019) found in a greater-than-expected proportion within a user-defined list. Significance is determined by the probability that the number of proteins from a given

ontology in the user-defined list is greater compared with the number of proteins from this same ontology in a larger background list. Over-representation analysis was performed with the R package TopGO using the "classic" algorithm (Alexa et al., 2006). The background list was formed by proteins that could be identified in at least eight of the 12 samples within each treatment-by-population combination. Ontologies with unadjusted Fisher's exact test *p*-values <.05 and containing at least two differentially expressed proteins were considered significantly over-represented (correction for multiple testing is not recommended in TopGO; Alexa & Rahnenfuhrer, 2019).

Subsets of oyster proteins were also functionally evaluated using the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa & Goto, 2000). Amino acid sequences from select proteins were uploaded to the KEGG GhostKoala tool, which searches the KEGG database for orthologous sequences, and when a match is found, returns a K-number identifier that is linked with functional annotations (Kanehisa et al., 2016). Resulting K-numbers where then uploaded to the KEGG Mapper Tool to retrieve functional annotations for each protein of interest (Kanehisa & Sato, 2020).

Functional annotations in KEGG and TopGo are derived primarily from studies performed in vertebrate model organisms (Gaudet & Dessimoz, 2017; Haynes et al., 2018). Consequently, proteins involved in mollusc processes that lack vertebrate orthologues, such as proteins involved in shell formation and immune responses, could be overlooked by these annotations. Statistically significant over-representation in TopGo can result from as few as two proteins annotated with the same ontology and most proteins annotate to multiple ontologies within KEGG and gene ontology databases. Given these caveats, KEGG annotations and TopGo over-representation were supplemented with protein functional information derived from the scientific literature. This approach provided a more comprehensive understanding of the functions of oyster proteins.

2.5 | Protein network analysis

Network analysis was used to identify potential interactions among differentially expressed proteins. Protein–protein interaction networks were developed using STRING, a database of known and predicted protein–protein interactions (Szklarczyk et al., 2021). Names of differentially expressed proteins were entered into the STRING search function and the resulting gene network generated using a minimum interaction score of 0.6, which is more stringent than the default setting of 0.4 and greater than that used in other proteomic studies (Levitan & Kültz, 2021). K-means clustering (number of clusters = 3) within STRING was used to identify subnetworks of interacting proteins.

2.6 | Prediction of 14-3-3 binding sites

14-3-3 Pred was used to identify putative 14-3-3 binding sites within proteins differentially expressed in oysters. 14-3-3 proteins are signalling molecules that bind to phosphorylated consensus sequences on client proteins in order to regulate their activity (Pennington et al., 2018). 14-3-3 Pred software is based on an analysis of known 14-3-3 binding site consensus sequences contained in the 14-3-3 interactome (ANIA) database (Tinti et al., 2014). The software uses a combination of artificial neural networks, position-specific scoring matrix, and support vector machines to identify binding site sequences among the proteins of a user-defined list. Following 14-3-3 Pred recommendations, only binding sites with consensus scores >0.5 were considered here (Madeira et al., 2015).

3 | RESULTS

3.1 | General patterns of proteomic change

Quantitative proteomics identified 1189 oyster proteins across all samples and treatments. After removal of duplicate proteins, 609 unique proteins remained. Among these 609 proteins, 330 were present in at least eight of the 12 samples within each treatment-by-population combination and were used in statistical analyses (Table S1). Singular value decomposition revealed three expression patterns accounting for the vast majority of variation in protein abundance among oyster populations and treatments. Eigenvector 1 explained 42% of the variation in protein expression and consisted of proteins that decreased in abundance in both Tomales Bay and Elkhorn Slough oysters after exposure to *Urosalpinx* (Table S2). Eigenvector 2 accounted for 32% of the variation in protein expression and was formed by proteins that increased in abundance in both populations after drill exposure (Table S3). Proteins upregulated in Tomales Bay oysters, but downregulated in Elkhorn Slough oysters after predator exposure comprised eigenvector 3 (Table S4). Eigenvector 3 accounted for 26% of the total variation in protein abundance. Proteins that were upregulated in Elkhorn Slough and downregulated in Tomales Bay oysters represented a small fraction of the total variation in the data set (<1%), and no proteins with this expression pattern met criteria for being differentially expressed (i.e., Pearson's correlation coefficient >.8; mean fold-change >1.5).

3.2 | Eigenvector 1: Proteins downregulated by both populations

Twenty-nine proteins were strongly correlated with eigenvector 1 and downregulated in both oyster populations following exposure to drills (Figure 2). Over-representation analysis demonstrated that functions relating to cell signalling (e.g., signal transduction) and protein degradation (e.g., peptidase activity) were significantly over-represented among these 29 proteins (Table 1; Table S5). Cross-referencing proteins annotated with cell signalling and proteolysis ontologies with functional descriptions in the scientific literature suggests that these proteins are involved in oyster immune responses. Over-representation of protein degradation functions were caused

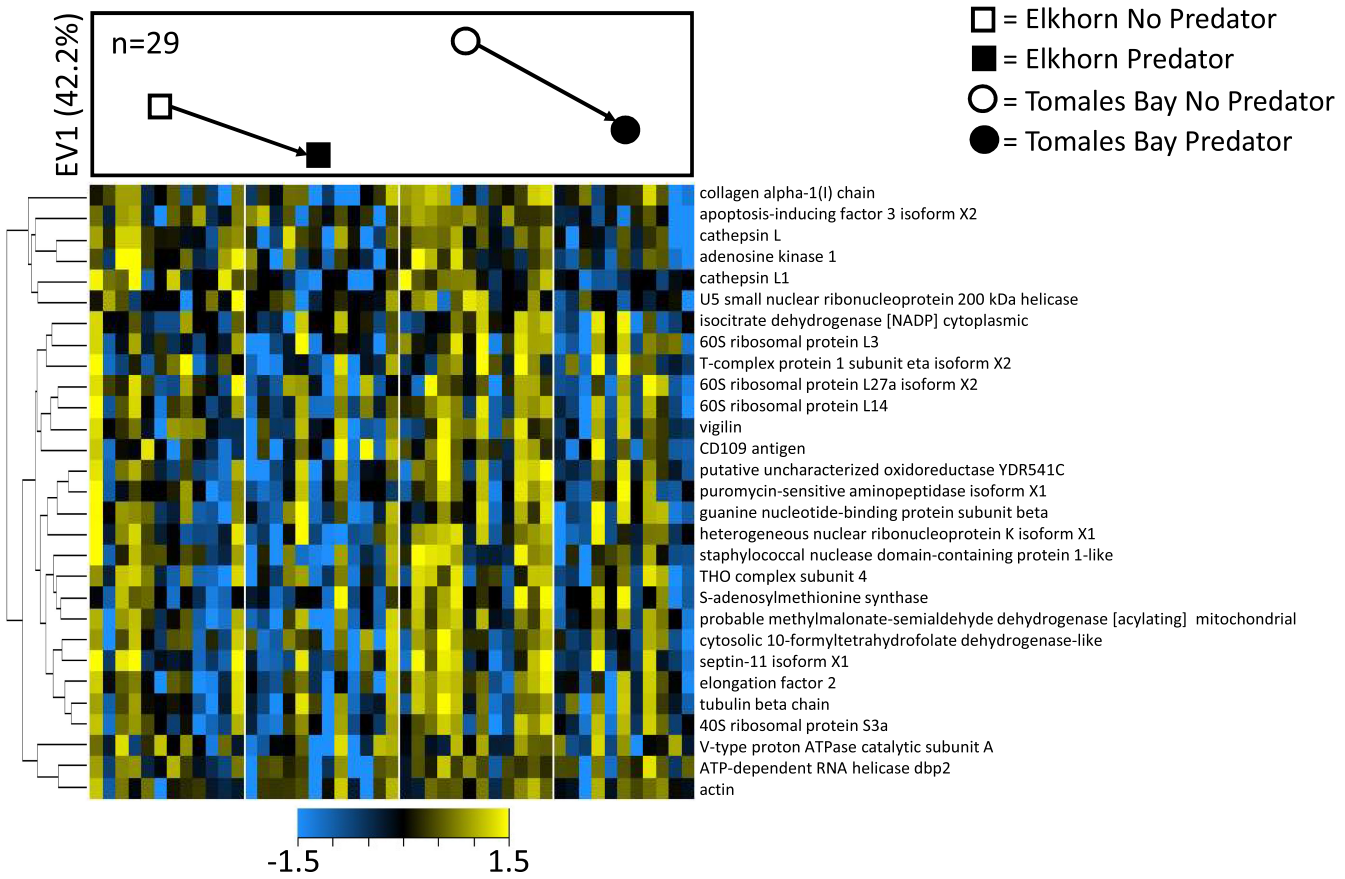

FIGURE 2 Changes in abundance among proteins downregulated by both oyster populations following exposure to drills ($n = 29$). Upper graph displays relative variation in average protein abundance (y-axis = eigenvector 1 [EV1]; 42.2% of variation; arbitrary units) among each population-by-treatment combination (x-axis). Square symbols denote average protein abundance in oysters originating from Elkhorn Slough. Circles denote average protein abundance in oysters originating from Tomales Bay. Filled symbols denote average protein abundance in oysters exposed to predator cues. Empty symbols denote average protein abundance in oysters exposed to control seawater. Lower hierarchical clustered heatmap displays variance scaled $\log_2$ transformed protein abundance. Each row illustrates the expression profile of a single protein and each column individuals from a given population-by-treatment combination. Yellow coloration is indicative of comparatively higher expression and blue of lower expression

Over-represented ontology	Database	Number of proteins	*p*-value
Signal transduction (GO:0007165)	Biological Process	2	.018
Signalling (GO:0023052)	Biological Process	2	.018
Cell communication (GO:0007154)	Biological Process	2	.018
Peptidase activity, acting on L-amino acids (GO:0070011)	Molecular Function	3	.045

TABLE 1 Representative significantly over-represented ontologies among proteins down-regulated by both oyster populations after drill exposure. Gene ontology identifiers shown in parenthesis. Number of proteins column refers to number of proteins strongly correlated with eigenvector 1 that annotate to that ontology

by downregulation of two cathepsin proteases, and cathepsins have well-established roles in oyster immune systems (Jiang et al., 2018; Ma et al., 2010). Cathepsin L mRNA increases in the pearl oyster *Pinctada fucata* following pathogen infection (Ma et al., 2010) and transcripts are also elevated in oyster phagocytes that engulf and destroy microbial pathogens (Jiang et al., 2018). Reduced expression of staphylococcal nuclease domain-containing protein 1 and THO complex subunit 4 caused over-representation of cell signalling ontologies. These two proteins are also linked with bivalve immune functions. Staphylococcal nuclease domain-containing protein 1 mRNA increases in Manila clam (*Venerupis philippinarum*) hemocytes following *Vibrio* infection (Moreira et al., 2014) and THO complex protein is induced by Pacific oysters after exposure to *Staphylococcus aureus* or *Vibrio splendidus* pathogens (Wang et al., 2018a).

Searches of the scientific literature confirm that other proteins correlated with eigenvector 1 also function within oyster immunity.

CD109 antigen is part of the oyster complement system, which contributes to innate immune responses and mediates processes such as phagocytosis and cell lysis (Wang et al., 2017; Zhang et al., 2015). Apoptosis-inducing factor 3 isoform X2 stimulates cell death upon contact with pathogens or parasites and is an important aspect of oyster immune responses (Gervais et al., 2018; Hughes et al., 2010; Sokolova, 2009). Septin 11 isoform X1 was also strongly correlated with eigenvector 1. Septins have proapoptotic functions (Zhou et al., 2018), and in oysters, septins change abundance following pathogen exposure (Modak & Gomez-Chiarri, 2020).

Proteins strongly correlated with eigenvector 1 were also repeatedly referenced in studies investigating the composition of oyster mucus, a substance with important functions in pathogen recognition and defence (Allam & Pales Espinosa, 2016; Fernández-Boo et al., 2020; Pales Espinosa et al., 2016). Twenty-five of the 29 proteins comprising eigenvector 1 (86%) were included among 1514 proteins identified within mucosal secretions of the Eastern oyster, *Crassostrea virginica* (Pales Espinosa et al., 2016). In contrast, of the 330 Olympia oyster proteins analysed for differential expression here, only 133 appeared on the list of 1514 *C. virginica* oyster mucus proteins (40%). A Fisher's exact test confirmed that proteins strongly correlated with eigenvector 1 contained a significantly higher than expected proportion of mucus proteins ($p < .0001$). This result further emphasizes that a disproportionate number of immune response proteins were downregulated by Olympia oyster populations after exposure to *Urosalpinx*.

3.3 | Eigenvector 2: Proteins upregulated by both populations

Nine proteins were strongly correlated with eigenvector 2, which consisted of proteins upregulated by both oyster populations following exposure to drills (Figure 3). Two of these proteins, sarcoplasmic calcium-binding protein isoform X1 and troponin C isoform X2, bind calcium, which caused calcium-binding functions to be significantly over-represented within this protein set (Table 2; Table S6). Olympia oyster shells are constructed by combining calcium ions with carbonate minerals from seawater. Given that oysters alter shell morphology in the presence of drills, a plausible explanation for elevated abundance of calcium-binding proteins is to modify biomineralization (Sillanpää et al., 2016, 2018). KEGG mapping supported this hypothesis, showing that four of the nine proteins strongly correlated with eigenvector 2 were annotated with the "exosome" function (Table S7). Exosomes are membrane-bound vesicles that deliver carbonate minerals and shell matrix proteins to sites of biomineralization in oysters (Song et al., 2019; Wang et al., 2013).

Searches of the scientific literature confirm that other proteins strongly correlated with eigenvector 2 also function within shell biomineralization pathways. Olympia oysters increased expression of mammalian ependymin-related protein 1 after exposure to drills. Originally identified in mammals (Apostolopoulos et al., 2001), this calcium-binding protein is hypothesized to play a direct role in the organization of the shell matrix in molluscs (Ganss & Hoffmann, 2009; Marie et al., 2010; Miyamoto et al., 2013). Lamin Dm0 was also upregulated by both oyster populations and expression of this protein is associated with variation in deposition of calcium carbonate in the silver lip oyster (*Pinctada maxima*; McDougall et al., 2021).

3.4 | Eigenvector 3: Proteins upregulated in Tomales Bay oysters and downregulated in Elkhorn Slough oysters

Eigenvector 3 was formed by proteins that increased in abundance in oysters from Tomales Bay, which co-occur with drills and grew the smallest shells in the presence of these predators, but decreased in abundance in oysters from Elkhorn Slough, where drills are absent and oyster shells grew comparatively larger after drill exposure (Figure 4). Nine proteins were strongly correlated with

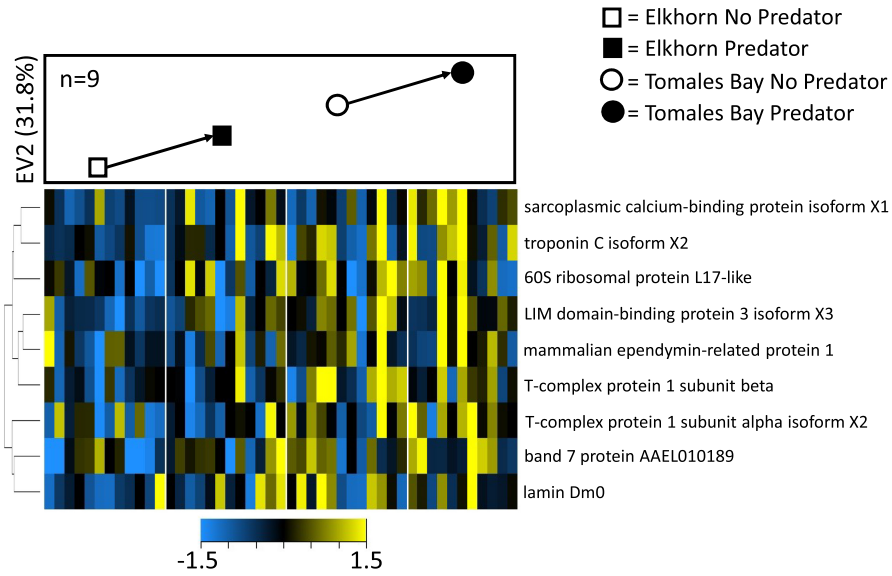

FIGURE 3 Changes in abundance among proteins upregulated by both oyster populations following exposure to drills ($n = 9$). Upper graph displays relative variation in average protein abundance (y-axis = eigenvector 2 [EV2]; 31.8% of variation; arbitrary units) among each population-by-treatment combination (x-axis). Lower hierarchical clustered heatmap displays variance scaled $\log_2$ transformed protein abundance. Rows, columns, colours, and symbols are as described in Figure 2

Over-represented ontology	Database	Number of proteins	*p*-value
Calcium ion binding (GO:0005509)	Molecular Function	2	.002
Cation binding (GO:0043169)	Molecular Function	2	.013
Metal ion binding (GO: 0046872)	Molecular Function	2	.013

TABLE 2 Representative significantly over-represented ontologies among proteins upregulated by both oyster populations after drill exposure. Gene ontology identifiers shown in parenthesis. Number of proteins column refers to number of proteins strongly correlated with eigenvector 2 that annotate to that ontology

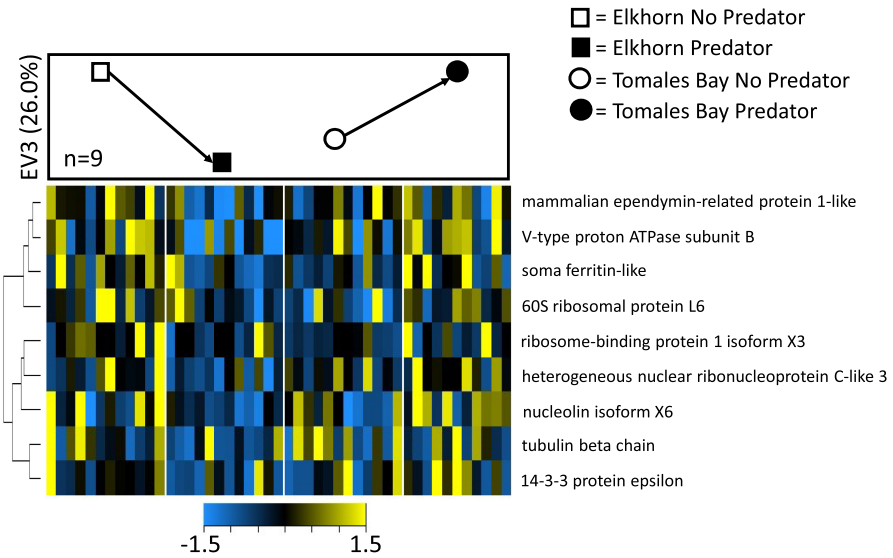

FIGURE 4 Changes in abundance among proteins upregulated by oysters from Tomales Bay and downregulated in oysters from Elkhorn Slough ($n = 9$). Upper graph displays relative variation in average protein abundance (y-axis = eigenvector 3 [EV3]; 26.0% of variation; arbitrary units) among each population-by-treatment combination (x-axis). Lower hierarchical clustered heatmap displays variance scaled $\log_2$ transformed protein abundance. Rows, columns, colours, and symbols are as described in Figure 2

this expression pattern. No ontologies were significantly over-represented in this protein set (Table S8). Several of the nine proteins strongly correlated with eigenvector 3 were different isoforms or subunits of proteins correlated with eigenvectors 1 or 2. Among the 330 proteins analysed here, two were annotated as mammalian ependymin-related protein 1. One of these ependymin isoforms was strongly correlated with eigenvector 2 (upregulated by both populations), while the other isoform was strongly correlated with eigenvector 3 (upregulated in Tomales Bay and downregulated in Elkhorn Slough oysters). Protein sequence alignments demonstrate significant deviation in the amino acid composition of these ependymin isoforms despite identical annotations: the proteins share only 25% sequence identity (data not shown). Oyster populations also exhibited opposing expression patterns for isoforms of beta tubulin. Two proteins analysed here were annotated as tubulin beta chain, one of which was correlated with eigenvector 1, while the other was correlated with eigenvector 3. Sequence alignment shows that these two tubulin isoforms have amino acid sequences that are 96% similar (Table S9).

3.5 | Protein interaction networks and 14-3-3 signalling

14-3-3 protein epsilon was among the nine proteins expressed at higher levels in Tomales Bay oysters compared with oysters from Elkhorn Slough after predator exposure (eigenvector 3). 14-3-3 proteins regulate a wide range of cellular processes by binding to client proteins and modifying their activity (Pennington et al., 2018). Thousands of 14-3-3 binding partners have been identified to date (Madeira et al., 2015). Differences in 14-3-3 activity may be important to the evolution of divergent inducible defences among Olympia oysters given the ability of this protein to modulate large numbers of downstream molecules. In support of this hypothesis, 14-3-3 epsilon was part of the largest subnetwork of interactions among proteins that changed abundance in the presence of drills, including connections with four ribosomal proteins that regulate protein synthesis (Table S10). 14-3-3 binding sites were predicted to occur in 24 of the 29 proteins downregulated by both populations after exposure to drills (eigenvector 1), nine of nine proteins that increased abundance in both populations after exposure to drills (eigenvector 2), and eight of nine proteins upregulated in Tomales Bay oysters, but downregulated in Elkhorn Slough oysters having been exposed to drills (eigenvector 3) (Table S11).

Adaptive changes to protein amino acid sequences that result in gain or loss of 14-3-3 binding sites could contribute to phenotypic differences in the response to drills between oyster populations. This hypothesis was investigated by searching for differences in the number of 14-3-3 binding sites among proteins that exhibited isoform-specific expression between oyster populations after drill exposure (i.e., beta tubulin and mammalian ependymin-related protein 1). The beta tubulin isoform correlated with eigenvector 3,

and only upregulated in Tomales Bay oysters, contained an additional 14-3-3 binding site compared with the beta tubulin isoform correlated with eigenvector 1 that was downregulated by both populations. This additional binding site was the result of a single amino acid substitution: methionine to isoleucine at position 316 (Figure 5). Ependymin isoforms also differed in the number of 14-3-3 binding sites. Two 14-3-3 binding sites were identified in the ependymin isoform correlated with eigenvector 2, whereas no binding sites were predicted in the ependymin isoform correlated with eigenvector 3 (Table S11).

4 | DISCUSSION

Proteins represent the functional machinery inside cells, and changes in protein abundance form the basis of adaptive phenotypic change (Tomanek, 2014). Comparative proteomics indicates that differences in protein abundance contribute to plastic changes in shell phenotype that occur following exposure of Olympia oysters to predatory Atlantic drills. The minimum drill exposure time needed for oysters to develop smaller, thicker, or harder shells, is unknown. A previous study has demonstrated that 6 weeks exposure of oysters to drill cues is sufficient to cause significant changes in these aspects of shell phenotype (Bible et al., 2017). Being that oysters were only sampled at the end of this 6 week period for experiments here, variation in protein abundance among treatments and populations could be involved in producing phenotypic change or in maintaining phenotypic differences that developed earlier in the predator exposure period.

4.1 | Conserved mechanisms of inducible prey defences in Olympia oysters

Although Olympia oysters from Tomales Bay and Elkhorn Slough have evolved adaptive differences in the strength of their inducible defence response to drill predation, oysters from both populations grow smaller, thicker, and harder shells when exposed to *Urosalpinx* compared with unexposed oysters (Bible et al., 2017). Proteins that exhibit parallel increases in both populations after exposure to drills most likely contribute to these conserved changes in shell phenotype. Functional analysis of upregulated proteins suggests that cues released during drill predation stimulate calcium ion transport and secretion of skeletal matrix proteins so that oysters can modify rates of biomineralization and adjust the size and physical properties of their shells.

Uptake and transport of calcium is necessary for the formation of calcium carbonate ($CaCO_3$) crystals used in oyster shell growth, maintenance, and repair (Marin et al., 2012). Ionic calcium (Ca^{+2}) used in biomineralization is absorbed from seawater by cells in the gills and in the mantle, a tissue layer that encloses the internal organs and whose outermost edge, the outer mantle epithelium, interfaces with the shell forming area (Sillanpää et al., 2016, 2018). Movement of calcium from the gills and mantle toward the outer mantle epithelium is facilitated by proteins upregulated in Olympia oysters exposed to drills. Both intracellular and extracellular (within the haemolymph) transport of calcium toward the biomineralization zone is enabled by calcium-binding proteins (Nair & Robinson, 1998; Richards et al., 2018; Sillanpää et al., 2018; Xue et al., 2012). Olympia oysters with smaller, thicker, and harder shells increased expression of several calcium-binding proteins, such as sarcoplasmic calcium-binding protein isoform X1. In the pearl oyster (*Pinctada fucata*), this protein is hypothesized to help concentrate calcium ions at biomineralization sites (Zhu et al., 2021). Troponin C, another calcium-binding protein upregulated in Olympia oysters exposed to drills, may also facilitate biomineralization by regulating calcium transport. Proteins involved in the production of nacre, the calcium carbonate innermost layer of oyster shells (Song et al., 2019), possess domains with homology to troponin C and are activated by calcium (Chang et al., 2016; Jain et al., 2017). Olympia oysters also increased expression of ependymin, a protein that is expressed in the mantle tissues of several mollusc species (Li et al., 2017; Mann et al., 2012; Yarra et al., 2016), contains a calcium-binding domain, and is believed to participate in biomineralization through a calcium dependent mechanism (Jackson et al., 2006; McDougall et al., 2018).

Calcium carbonate crystals and skeletal matrix proteins within oyster shells are secreted by cells of the outer mantle epithelium and by haemocytes using vesicles called exosomes (Kalluri & LeBleu, 2020; Song et al., 2019; Wang et al., 2013). Four of the nine proteins that increased in abundance after exposure of Olympia oysters to drills were annotated with the "exosome" ontology, implying that these proteins assist with delivery of matrix proteins and calcium carbonate needed to modify shell phenotype. Consistent with this supposition, 61 of 259 proteins identified from shells of the Pacific oyster matched proteins in the exosome database (Zhang et al., 2012b). Exosome-like vesicles containing calcite crystals have been observed at regions of biomineralization in Eastern oysters (Johnstone et al., 2015; Mount et al., 2004), suggesting that exosomes influence biomineralization through secretion of both calcium carbonate and matrix proteins.

4.2 | Nonconsumptive effects of Olympia oyster inducible defences

Prey defences that are inducible, rather than constitutively expressed, evolve in part because there are costs, or nonconsumptive effects, associated with permanently expressing defended phenotypes (Creel & Christianson, 2008; Sheriff et al., 2020). For example, Eastern oysters exposed to a predatory snail increase shell thickness, but grow more slowly compared with unexposed oysters (Gosnell et al., 2017). Nonconsumptive effects most frequently associated with inducible defences include changes in developmental rate, growth rate, fecundity, or timing of life-history events (Kishida et al., 2010). However, constraining analyses to these organism-level metrics obscures changes occurring at the molecular or cellular level

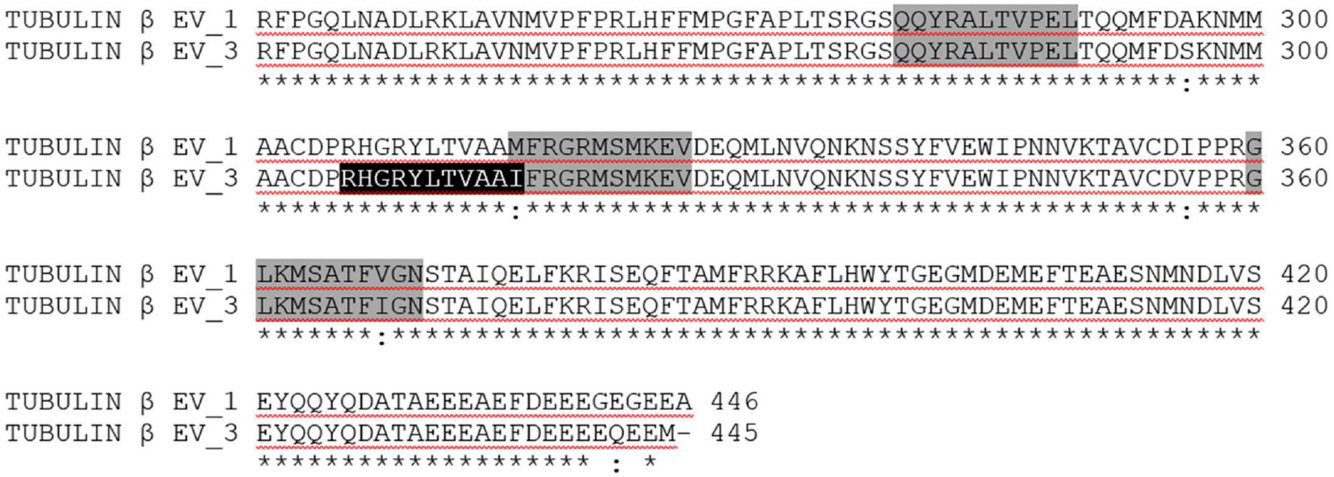

FIGURE 5 Partial amino acid sequence alignment for the beta tubulin isoform downregulated in both oyster populations (TUBULIN β EV_1) and the beta tubulin isoform upregulated in oysters from Tomales Bay, but downregulated in oysters from Elkhorn Slough (TUBULIN β EV_3). Grey areas denote predicted 14-3-3 binding sites conserved between populations. Black area shows a methionine to isoleucine substitution at position 316 resulting in an additional 14-3-3 binding site in the beta tubulin isoform upregulated by oysters from Tomales Bay, but downregulated in oysters from Elkhorn Slough

and prevents discovery of novel trade-offs resulting from phenotypic plasticity.

Changes in protein abundance in oysters exposed to drills imply a trade-off between biomineralization, needed to modify shell phenotype, and the immune system, used to protect against pathogens and disease (Ivanina et al., 2018). A larger than expected number of immune defence proteins were downregulated by both oyster populations after exposure to drills (eigenvector 1), suggesting that immune pathways were repressed at a time when biomineralization was stimulated (eigenvector 2). This potential trade-off can be explained by predator cues causing a shift in oyster haemocyte abundance, such that haemocytes specializing in biomineralization (and expressing biomineralization-related proteins) increase in number, while haemocytes involved in immune responses (and expressing immunity-related proteins) decrease in number. Such shifts in haemocyte abundance may represent a form of resource reallocation that is needed to bring about phenotypic change and that often accompanies inducible defence responses (Clark & Harvell, 1992). The total number of haemocytes, as well as proportions of haemocyte subtypes, can shift in response to environmental factors, suggesting that haemocyte abundance is a plastic trait in oysters (Lambert et al., 2007; Oliver & Fisher, 1995).

Bivalve haemocytes have been traditionally divided into two groups, hyalinocytes and granulocytes; however, populations of cells within these categories exhibit morphological diversity and functional specialization (Huang et al., 2018; Ivanina et al., 2017, 2018; Lau et al., 2017). In oysters, certain granulocyte subtypes participate in biomineralization, while other subtypes contribute to immune responses (Huang et al., 2018; Ivanina et al., 2017). Granulocytes play an important role in oyster shell formation by delivering minerals to the site of calcification (Clark, 2020; Huang et al., 2018; Ivanina et al., 2017; Ivanina et al., 2018; Mount et al., 2004; Song et al., 2019). Subpopulations of granulocytes increase in number during oyster shell rebuilding (Mount et al., 2004), transport and secrete calcium carbonate (Li et al., 2016), and express proteins and mRNAs indicative of specialization in biomineralization, including genes involved in calcium transport (Huang et al., 2018). Other granulocytes have immune specializations, including recognition of foreign bodies, destruction of pathogens through phagocytosis, and production of antimicrobial compounds (Wang et al., 2018b). These immune granulocytes express a greater abundance of mRNAs and proteins involved in immune responses (Ivanina et al., 2017), including cathepsins, septins, and apoptosis-inducing factors (Huang et al., 2018), that were downregulated in Olympia oysters after exposure to drills. Immune granulocytes are also abundant within the mucus covering the mantle and gills (Lau et al., 2017), and Olympia oysters reduced abundance of a disproportionate number of mucosal proteins when drills were present.

Pacific oysters (*M. gigas*) and Eastern oysters (*C. viginica*) exhibit differences in shell properties and disease resistance that support a trade-off arising from dual functions of haemocytes in biomineralization and immunity (Ivanina et al., 2018). Shells of Eastern oysters are mechanically superior to those of Pacific oysters, exhibiting greater microhardness, stiffness, and maximum load (Ivanina et al., 2018). This disparity in shell strength is associated with differences in haemocyte function between the two species: haemocytes from Eastern oysters contain a higher concentration of calcium and express higher levels of biomineralization genes compared with haemocytes from Pacific oysters (Ivanina et al., 2018). While Eastern oysters may possess a greater capacity for biomineralization than Pacific oysters, this species suffers from a comparatively weaker immune system, a trait that can also be linked with hemocyte specialization (Guo et al., 2015; Ivanina et al., 2018). Eastern oysters commonly experience mass mortalities due to parasitic, bacterial, or viral infections; however, mortality in Pacific oysters is less often the result of epizootic diseases, despite serving as a host to a variety of

potential pathogens (Barbosa Solomieu et al., 2015; Chu et al., 1996; Elston, 1993; Ford, 1996; Ivanina et al., 2018). Circulating haemocytes in Pacific oysters express higher levels of immune defence transcripts and are more capable of parasite destruction than haemocytes from Eastern oysters (Foster et al., 2011; Goedken et al., 2005; Hughes et al., 2010; Ivanina et al., 2018; Sunila & LaBanca, 2003).

4.3 | Mechanisms of divergent inducible defences between Olympia oyster populations

Proteins exhibiting opposing directions of change between oyster populations exposed to drills provide clues as to how natural selection has modified inducible defences in response to local predation risk. Several of the proteins expressed at comparatively higher levels in Tomales Bay oysters (eigenvector 3) were different isoforms of proteins modified by both populations after exposure to drills (eigenvector 1 or 2). These data suggest that the more robust defence response of Tomales Bay oysters arose from evolutionary modification of an existing inducible defence mechanism present in both populations. Populations of mussels (*Mytilus edulis*) having evolved divergent responses to an invasive predator are also hypothesized to have done so through adaptive modification of an ancestral response to a longstanding predator. Mussels from both the northern and southern regions of the New England coast of the United States develop thicker shells in response to cues from the green crab (*Carcinus maenas*), which has occupied these regions for more than two centuries. However, only mussels from southern New England thicken shells in response to a more recent crab invader, the Asian shore crab (*Hemigraspus sanguineus*), which was introduced to southern New England in the 1980s but had not yet co-occurred with mussels inhabiting regions further north. Rapid evolution of an inducible defence to the new predator was hypothesized to involve adaptive changes to cue specificity or thresholds within the existing predator recognition mechanism (Freeman & Byers, 2006).

Increased expression of an additional isoform of mammalian ependymin-related protein 1 may act to couple sensory systems responsible for predator detection with changes in biomineralization that cause oysters from Tomales Bay to grow smaller shells compared with oysters from Elkhorn Slough. Both oyster populations upregulated mammalian ependymin-related protein 1 following exposure to drills (eigenvector 2); however, Tomales Bay oysters increased abundance of an additional ependymin isoform that did not change abundance in oysters from Elkhorn Slough (eigenvector 3). In fish, ependymins are neurotrophic factors that assist in forming new neural pathways during processes such as memory and learning (Adams et al., 2003; McDougall et al., 2018). Isoform-specific expression of ependymin in Olympia oysters from Tomales Bay may indicate evolution of a new sensory pathway needed to detect and respond to a novel predator. In aquatic organisms, ependymin isoforms change abundance in the presence of predators, corroborating a role for these proteins in predator defence systems (Sneddon et al., 2011). Rainbow trout induce ependymin during periods of increased predation risk and upregulation of this protein is correlated with a subsequent reduction in boldness behaviours (Thomson et al., 2012). Crown-of-thorns starfish release multiple ependymin isoforms into the surrounding seawater as part of an alarm response to a predator, implying that these proteins are involved in conspecific communication during predation events (Hall et al., 2017). Much like the isoforms differentially expressed between Olympia oyster populations, ependymin proteins vary markedly in sequence and expression pattern within other taxa (Hall et al., 2017). Evolutionary radiation of ependymin proteins has been observed in multiple animal lineages, including molluscs (Hall et al., 2017; McDougall et al., 2018; Suárez-Castillo & García-Arrarás, 2007), and rapid evolution of ependymin isoforms is expected to have created species-specific repertoires of signalling molecules that regulate predator defences (Hall et al., 2017). Evidence that ependymins are also involved in oyster biomineralization implies that these proteins could connect sensory systems involved in predator detection to changes in shell morphology that reduce predation risk. Ependymins interact with components of the extracellular matrix in a calcium dependent manner and are expressed in the mantle tissue of abalone *Haliotis asinina* (Jackson et al., 2006; Marie et al., 2010; McDougall et al., 2018).

Oysters from Tomales Bay and Elkhorn Slough also varied in their expression of beta tubulin isoforms following exposure to predators, providing additional evidence that natural selection is modifying existing inducible defence pathways to match responses with local predation risk. Two tubulin isoforms were among the 330 proteins analysed here, one isoform was downregulated by both populations following exposure to drills (eigenvector 1), while the other was upregulated only in oysters from Tomales Bay that mount a more robust defence response compared with that of oysters from Elkhorn Slough (eigenvector 3). Expression of an additional tubulin isoform in Tomales Bay oysters could contribute to development of even smaller shells in the presence of drills. Pearl oysters that vary in growth rate and in the mechanical properties of their shells express differing levels of tubulin in their mantle tissues, suggesting that tubulin can contribute to changes in shell structure (Xu et al., 2019; Yang et al., 2018). Some insight into the role of the cytoskeleton in shell growth comes from studies of single celled foraminifera that, like oysters, produce highly structured calcium carbonate shells. In these organisms, formation and dissociation of microtubules is required for construction of individual chambers within the shell, with tubulin-based microtubules supporting cellular extensions upon which carbonate crystals are deposited (Tyszka, 2006; Tyszka et al., 2005, 2019). Tubulin, microtubules, and/or the cytoskeleton network could alter the physical properties of oyster shells by influencing stability of the organic extracellular matrix upon which calcium carbonate crystals are deposited.

Following exposure to drills, Tomales Bay oysters express higher levels of 14-3-3 protein epsilon than do oysters from Elkhorn Slough. An ability to post-translationally regulate a range of essential processes, including cell division, transcription, apoptosis, protein trafficking, and protein degradation (Gardino et al., 2006; Madeira et al., 2015; Pennington et al., 2018; Sluchanko, 2018;

Yaffe, 2002), suggests that differences in 14-3-3 abundance could play a major role in generating phenotypic change. In support of this hypothesis, 14-3-3 was part of the largest network module among proteins that changed in abundance in Olympia oysters exposed to drills, and 14-3-3 protein binding sites were identified in 41 of 47 proteins differentially expressed in this study. Comparison of 14-3-3 binding sites among proteins exhibiting opposing directions of change between Tomales Bay and Elkhorn Slough oysters illustrates evolutionary mechanisms by which inducible defences could diverge between these populations. The beta tubulin isoform expressed at higher levels only in Tomales Bay oysters is 96% similar to the beta tubulin isoform upregulated by both populations after exposure to drills. However, one of the 11 amino acid substitutions that distinguishes this isoform results in the formation of an additional 14-3-3 binding site. Regulatory change that arises from small changes in amino acid sequence may be particularly important for rapid evolution of inducible defences. A similar framework can be applied to ependymin isoforms that differed in abundance between populations. Two 14-3-3 binding sites are present in the ependymin isoform up-regulated by both populations after exposure to drills; however, in the isoform that increased in abundance only in Tomales Bay oysters that mount a more robust defence response, no 14-3-3 binding sites were identified.

4.4 | Implications of inducible defences in wild oysters

Olympia oysters are an estuarine foundation species that provides habitat for a community of other species and valuable ecosystem services such as water filtration and nutrient cycling (Kimbro & Grosholz, 2006; Newell, 2004). There is a growing recognition that nonconsumptive effects of prey defence responses can have important population-, community-, and ecosystem-level consequences (Preisser et al., 2005; Sih et al., 2010). Concomitant downregulation of immune proteins and upregulation of biomineralization proteins as part the inducible defences of Olympia oysters adds to evidence for a trade-off between immune functions and shell construction (Ivanina et al., 2018). Olympia oyster populations inhabiting the North American west coast are exposed to many potentially pathogenic bacteria, viruses, and protists (Pritchard et al., 2015). Most recent analyses suggest these disease-causing agents are not a major factor influencing the abundance of Olympia oysters (Wasson et al., 2014). However, Olympia oyster populations co-occurring with drills may become more susceptible to pathogens as energy and resources are diverted away from the immune system and toward predator defence. Disease outbreaks have caused mass mortality in other oyster species (Pernet et al., 2014; Petton et al., 2021).

Olympia oysters exposed to drills upregulate biomineralization-related proteins, build thicker and harder shells, and tend to have higher shell-to-tissue weight ratios compared with oysters not exposed to the predator. These data indicate increased investment in biomineralization is required to reduce predation risk (Bible et al., 2017). Ocean acidification caused by increasing human emissions of carbon dioxide may make such changes in shell phenotype more difficult (Sanford et al., 2014). Synthesis of calcium carbonate needed for biomineralization produces hydrogen ions ($Ca^{+2} + HCO_3^- = CaCO_3 + H^+$). The so-called "proton flux hypothesis" posits that shell construction is impaired among organisms living in more acidic seawater because the rate at which H^+ can be removed from regions of biomineralization is constrained (Jokiel, 2011; Tresguerres, 2016). In a variety of marine calcifiers, V-type H^+-ATPases pump protons away from regions of biomineralization in order to elevate local pH and ensure saturation of carbonate for use in shell construction (Ivanina et al., 2017, 2018; Li et al., 2016; Ramesh et al., 2019, 2020). Modulation of V-type H^+-ATPase A and B subunits by Olympia oysters after exposure to drills emphasizes that additional proton efflux is needed to modify shell size and strength for predator defence (Figures 2 and 4). As ocean acidification progressively worsens, Olympia oysters may be challenged to construct shells that are more resistant to drill predation because of the inability to regulate pH at sites of biomineralization (Sanford et al., 2014). Even if the required biomineralization rates can be sustained in future oceans, energetic costs of Olympia oyster inducible defences may increase as more protons must be transported using the ATP dependent V-type H^+-ATPase.

AUTHOR CONTRIBUTIONS

Experiments were conceived and designed by Tyler G. Evans, Jillian M. Bible, Kaylee R. Griffith, Eric Sanford, and Dietmar Kültz. Experimental protocols were performed by Tyler G. Evans, Jillian M. Bible, Ashley Maynard, Kaylee R. Griffith, and Dietmar Kültz. Tyler G. Evans analysed the data. Tyler G. Evans, Jillian M. Bible, Eric Sanford, and Dietmar Kültz wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as previously described.

DATA AVAILABILITY STATEMENT

Proteomic raw data and metadata are publicly accessible in MaSSIVE (MSV000088386) and ProteomeXchange (PXD029757) repositories.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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