

Article

Benthic Diatoms Differentially Regulate Apoptosis, Ferroptosis and Androgenic Gland Signaling Underlying Molecular Pathways of Sex Differentiation in *Hippolyte inermis*

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Abstract

Chemical cues released by benthic primary producers regulate key ecological interactions and life-history traits in marine ecosystems. The caridean shrimp *Hippolyte inermis*, a protandric hermaphrodite inhabiting *Posidonia oceanica* meadows, is a model for chemically mediated sex differentiation. Previous studies showed that the benthic diatom *Cocconeis scutellum* var. *parva* produces a lipophilic metabolite that induces a molecular signature previously associated with androgenic gland degeneration and subsequent feminization in *H. inermis*. Here, we investigated whether this activity is shared by other benthic diatoms. Eleven epiphytic diatom strains isolated from *P. oceanica* leaves were tested on PL5 post-larvae, and the expression of apoptosis, ferroptosis, and insulin-like androgenic gland hormone (IAG)-related genes was analyzed by real-time qPCR. Several strains, particularly *Diploneis* sp., *Cocconeis scutellum* var. *posidoniae*, and *Mastogloia fimbriata*, induced upregulation of programmed cell death genes together with downregulation of IAG-related genes, closely resembling the response elicited by *C. scutellum* var. *parva*. Principal Component Analysis supported these shared molecular responses, whereas other strains activated only early ferroptosis markers. These findings demonstrate that several benthic diatoms induce molecular pathways previously associated with androgenic gland degeneration and feminization in *H. inermis*. Although phenotypic feminization was not assessed in the present study, these results suggest that this molecular regulatory mechanism may be more widespread among benthic diatoms inhabiting *P. oceanica* meadows than previously recognized, highlighting their ecological significance.

Keywords: chemical ecology; epiphytic diatoms; *Posidonia oceanica*; programmed cell death; sex differentiation

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1. Introduction

In marine environments, chemical cues create an intricate network which regulates life. A wide range of organisms release chemical compounds, as secondary metabolites, into seawater to mediate intra- and interspecific communication [1,2]. These substances can be broadly divided into two categories: allochemicals and infochemicals. Allochemicals are often regarded as “chemical weapons” regulating spatial competition and defense, exerting in many cases toxic effects, whereas infochemicals are primarily used for communication, facilitating ecological interactions and habitat structuring [3,4]. In the highly heterogeneous benthic environment, numerous organisms live attached to the sea floor substrate and are therefore largely reliant on chemical mediators for interaction [5,6]. Within this context, diatoms (phylum Bacillariophyta) play a pivotal role. These unicellular microalgae are primary producers characterized by a complex profile of chemicals that work as bioactive compounds, including oxylipins, polyunsaturated aldehydes (PUAs), hydroxyecosapentaenoic acids (HEPEs), volatile organic compounds (VOCs), extracellular polymeric substances (EPS), fatty acid derivatives, and polyphenols [7–11]. Such compounds regulate diatoms’ ecological success, mediating trophic interactions, influencing the reproductive physiology of other organisms, and contributing to the structuring of benthic habitats [12,13]. For instance, oxylipins produced by benthic diatoms such as *Nanofrustulum shiloi* and *Cylindrotheca closterium* are known to induce developmental malformations and apoptosis in the sea urchin *Paracentrotus lividus* [14,15]. Other diatom-derived molecules, including fatty acids, can also affect the physiology and reproduction of marine crustaceans [16].

In recent decades, increasing evidence has highlighted that diatom-derived secondary metabolites, particularly oxylipins and other oxygenated fatty acid derivatives, can profoundly affect the reproductive success and developmental processes of a wide range of marine invertebrates [17]. These compounds, produced through lipoxygenase-mediated pathways, have been shown to interfere with fertilization, embryogenesis, and larval viability in organisms such as copepods, sea urchins, and tunicates, often through the activation of apoptosis and disruption of intracellular signaling pathways [8]. Importantly, similar molecular responses, including the activation of programmed cell death pathways, have been observed across phylogenetically distant taxa, suggesting the existence of conserved cellular targets for diatom-derived infochemicals. While most studies have focused on planktonic systems, recent findings indicate that benthic diatoms associated with seagrass meadows also produce bioactive oxylipins and related metabolites capable of modulating reproductive processes in grazers, thus playing a key role in structuring benthic communities [8,18,19].

Recent studies demonstrated that a specific metabolite produced by the epiphytic diatom *C. scutellum* var. *parva* can regulate the sexual development of the caridean shrimp *H. inermis*, a consecutive protandric hermaphrodite decapod inhabiting seagrass meadows along the Mediterranean sea, the western Europe Atlantic coast and the sea of Marmara [20–23]. Its juvenile diet is based on epiphytic microalgae, and it has been observed that at early developmental stages (immediately after post-larval settlement) the ingestion of *C. scutellum* var. *parva*, particularly during spring, triggers a unique mechanism of direct feminization, whereby individuals skip the male stage of the protandric hermaphroditism and directly differentiate as females [11,21]. The compound responsible for this interaction is a lipophilic 20-carbon metabolite, likely a polyunsaturated fatty acid (PUFA) similar to dihomono- γ -linolenic acid (DGLA; 20:3n-6), which is known to induce programmed cell death (PCD) in *Caenorhabditis elegans* germ cells and in human cancer cells [24–31]. In *H. inermis* post-larvae, this bioactive compound induces ferroptosis and apoptosis in undifferentiated cells of the Androgenic Gland (AG), the endocrine organ responsible for producing the insulin-like androgenic gland hormone (IAG), which regulates the

development and maintenance of male characteristics in decapods [2,22,32–39]. At the molecular level, this process is characterized by the downregulation of IAG pathway and the upregulation of ferroptosis and apoptosis pathways, ultimately leading to AG degeneration and feminization of the shrimp [22,40].

At the ecological scale, the interaction between *H. inermis* and the epiphytic diatoms represents a clear bottom-up regulatory mechanism, in which primary producers influence the life-history traits of consumers. The compound released by *C. scutellum* var. *parva* modulates reproductive timing, sex allocation, and population dynamics in this species [22,41]. Experimental exclusion of these diatoms from the diet of post-larvae has been shown to delay protandrous sex change and reduce reproductive success, highlighting both the specificity and the ecological importance of these chemical cues [16,21,22,27]. This model also provides a compelling natural system for studying endocrine disruption in marine invertebrates. Understanding the mechanisms underlying the action of these bio-active compounds is therefore crucial for interpreting the functioning of benthic ecosystems. Although the role of *C. scutellum* var. *parva* is well documented, it remains unclear whether similar effects are restricted to this species or are shared among other benthic diatoms associated with *P. oceanica* meadows. In this study, ten additional benthic diatom strains from a culture collection were tested on *H. inermis* post-larvae to assess whether other species can induce the same molecular responses previously shown to precede androgenic gland degeneration and feminization. Beyond its ecological relevance, this system provides a unique model to investigate how environmental chemical cues can regulate developmental trajectories and reproductive strategies in marine invertebrates. Understanding such mechanisms may improve our knowledge of population resilience, reproductive dynamics, and the effects of environmental changes on species associated with seagrass ecosystems, while offering a foundation for future investigations into potential applications in marine resource management. Based on previous evidence that *C. scutellum* var. *parva* induces androgenic gland degeneration through activation of apoptosis and ferroptosis pathways, we hypothesized that this molecular response is not restricted to a single diatom species but may represent a more widespread ecological interaction among benthic diatoms associated with *P. oceanica* meadows. Therefore, we tested whether different epiphytic diatom strains induce comparable transcriptional changes in apoptosis-, ferroptosis-, and IAG-related pathways in *H. inermis* post-larvae.

2. Materials and Methods

2.1. Isolation and Selection of Benthic Diatoms

P. oceanica leaves were initially collected from the coastal waters of Lacco Ameno (Ischia, Gulf of Naples, Italy). Samples were collected in spring 2024 from the coastal waters. At the time of collection, seawater temperature was approximately 18 °C and salinity was approximately 38 PSU. These environmental conditions were recorded to provide the temporal and physicochemical context of the epiphytic diatom communities investigated in this study. To extract the associated epiphytic microalgae, the surface of each leaf was gently scraped using a sterile razor blade. The blade was then rinsed into a small crystallizing dish containing filtered seawater to release the detached material. The suspension was examined under an inverted light microscope and target benthic diatom species were carefully isolated using a Leica micromanipulator. This precision tool allowed for the selection and transfer of individual diatoms into wells of a 12-well culture plate, each containing 3 mL of sterile f/2 medium. The culture plates were incubated in a thermostatically controlled chamber at 18 °C and a photoperiod of 12 h with irradiance of 400 $\mu\text{E m}^{-2} \text{s}^{-1}$. These conditions were selected to maintain optimal growth and physiological activity of the isolated benthic diatom strains under controlled laboratory conditions, while remaining within the range of environmental conditions experienced by epiphytic diatoms asso-

ciated with *P. oceanica* meadows in the Mediterranean Sea. After one week, a second round of micromanipulation was performed to re-isolate and ensure the purity of the resulting mother cultures [21,22]. Diatom samples were harvested from the mother culture and fixed using 2.5% glutaraldehyde. Following filtration onto cellulose Millipore filters, the specimens were mounted on aluminum stubs. The samples then underwent a series of three washes and treatment with 1% osmium tetroxide. Subsequently, the material was dehydrated through a graded ethanol series (25%, 50%, 75%, and 100%) and coated with platinum for morphological identification through Scanning Electron Microscopy (SEM, Zeiss EVO MA LS). Selected benthic diatom species were maintained separately and incubated in the previously described conditions. The cultures were grown in Guillard's f/2 medium supplemented with silica (Sigma Aldrich, Milan, Italy). Subculturing was carried out periodically to preserve culture purity and minimize the risk of contamination or stress. The newly isolated strains were maintained alongside previously established cultures (see Table 1), including *N. shiloi* [19], *C. scutellum* var. *parva*, *Diploneis* sp. [14], and *C. neothumensis* var. *marina* [42], which were also identified by 18S rRNA gene.

Table 1. Benthic diatom species identified and cultured in Ischia Marine Centre (Stazione Zoologica Anton Dohrn, Naples).

Species	Code	Taxonomy Author
<i>Cocconeis neothumensis</i> var. <i>marina</i>	CNN	De Stefano, Marino, Mazzella, 2000
<i>Cocconeis neothumensis</i> var. <i>marina</i> (strain isolated from naturally acidified site)	CNA	De Stefano, Marino, Mazzella, 2000
<i>Cocconeis scutellum</i> <i>scutellum</i>	C1	Ehrenberg, 1838
<i>Cocconeis scutellum</i> <i>parva</i>	C2	Cleve, 1895
<i>Cocconeis</i> sp.	C99	-
<i>Cocconeis</i> sp.	C120	Ehrenberg, 1837
<i>Cocconeis scutellum</i> var. <i>posidonie</i>	D	De Stefano, Marino, Mazzella, 2000
<i>Diploneis</i> sp.	CN	Ehrenberg, Cleve, 1894
<i>Mastogloia fimbriata</i> (strain isolated from naturally acidified site)	MFA	Grunow, 1863
<i>Nanofrustulum shiloi</i>	MEL	Round, Hallsteinsen and Paasche, 1999

Some strains included in the culture collection—namely, *Cocconeis neothumensis* var. *marina* and *Mastogloia fimbriata*—were originally isolated from naturally acidified environments. This designation refers exclusively to the environmental conditions of the collection site and not to the laboratory cultivation conditions, as all strains were subsequently maintained under identical culture conditions.

2.2. Intensive Culturing of Benthic Diatoms

After isolation, benthic diatom cultures were transferred, under sterile conditions, from multi-well culture plates to Petri dishes (14 mm in diameter). Once established, diatoms formed a cohesive mat-like layer. Replicate cultures were maintained for 15 days in f/2 Sigma medium at 18 °C within controlled chambers, under a 12:12 h light/dark photoperiod and an irradiance of approximately 140 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 15 days of cultivation, cultures had reached a dense benthic biofilm stage characterized by a cohesive mat-like layer on the bottom of the Petri dishes. Biomass was collected at this standardized stage to ensure comparable physiological conditions among the different diatom strains. At the end of the cultivation period, the culture medium was carefully removed from each dish, and the diatom biomass was preserved by freezing at −20 °C. Subculturing was performed every 15 days, and cultures were examined periodically using inverted light microscopy to ensure purity and monitor growth [43].

2.3. Sampling of *H. inermis*

Ovigerous females of *H. inermis*, identified by the presence of egg masses attached to the pleopods beneath the abdomen, were collected with a 400 µm mesh net (50 cm diameter), dragged by boat through the *P. oceanica* meadow off Lacco Ameno (Ischia Island, Gulf of Naples, Ischia, Italy). Specimens were sorted on board and temporarily held in plastic bags for transport to the laboratory, where females (specimens larger than 15 mm) were transferred to 2 L conical glass flasks, with three individuals per flask. Each flask was filled with 1.8 L of filtered natural seawater and supplemented with small fragments of *P. oceanica* leaves to provide shelter and reduce stress. The flasks were maintained in a thermostatic chamber at 18° C, under a 10:14 h light: dark photoperiod, with a mean irradiance of 250 µmol m⁻² s⁻¹. Larval release was daily monitored [44]. Once an adequate number of larvae were collected, the adult females were carefully returned to their original collection site.

2.4. Culture of *H. inermis* Larvae

Larvae were collected daily from the culture flasks containing ovigerous females. The contents of each flask were gently poured through a 60 µm mesh filter to separate the larvae from the seawater. If present, larvae were retained on the filter and identified under a stereomicroscope. They were then carefully collected using a sterilized Pasteur pipette and transferred into 1 L conical flasks containing 800 mL of filtered seawater (pH 8.2), with each flask accommodating a group of 80 individuals. For the first seven days, larvae were fed a mixture of *Artemia salina* nauplii and *Brachionus plicatilis* at a concentration of 4 individuals per milliliter. From the eighth day onward, *A. salina* nauplii were enriched daily with Algamac (Biomarine, Hawthorne, CA, USA) to enhance their nutritional value. Larval survival was monitored daily in each flask.

2.5. Post-Larval Experimental Cultures

Post-larvae that had completed metamorphosis, approximately 20–30 days after hatching, were randomly pooled and individually transferred into distinct benthic diatom cultures grown in 6-well plates; each well contained a single post-larva and 8 mL of filtered seawater. This setup allowed the post-larvae to feed directly on the benthic diatom substratum grown on the well floor. The negative control group (CBn) was cultured in 14 mm crystalizing dishes and fed daily on 5 mg of a standard diet consisting of equal parts of SHG “Artemia Enriched,” SHG “Microperle,” and SHG “Pure Spirulina” (Super High Group, Ovada, Italy). Three replicates of negative control cultures were set up. The seawater in each dish and 6-well culture plates was replaced daily to maintain optimal rearing conditions. At 5 days after settlement, post-larvae (PL₅) were fixed in RNA later [22].

2.6. RNA Extraction, cDNA Synthesis and Gene Expression by Real Time qPCR

Total RNA was extracted from mature juveniles using the RNeasy Mini Kit (Qiagen, Austin, TX, USA), following the manufacturer’s protocol. Samples were lysed in 350 µL of RLT buffer supplemented with β-mercaptoethanol (10 µL/mL). Homogenization was performed using a TissueLyser (Qiagen) at 20.0 Hz for 3 min with 3 mm sterile aluminum beads, according to the protocol described by Glaviano et al. [40]. The RNA was eluted in 30 µL of RNase-free water and stored at –80 °C. RNA concentration and purity were assessed by measuring the absorbance at 260 nm, and the quality by ratio of absorbance 260/230 nm and 260/280 nm by Nanodrop (ND-1000 UV Vis, NanoDrop Technologies, Wilmington, DE, USA); to check its integrity, 100–200 ng of RNA was loaded on 0.8% agarose gel. Furthermore, for each sample, 600 ng of RNA were reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy) following the manufacturer’s pro-

tolol. For subsequent gene expression analysis across experimental groups, undiluted cDNA (1:1) was employed as template.

The gene expression was followed for the following genes involved in i. ferroptosis (*Gamma glutamylcysteine synthetase*, GSHI; *Glutathione peroxidase 4*, GPX4; *Sterile alpha-motif domain-containing protein 3 metalloredutase*, STEA3; *Spermidine/spermine N1-acetyltransferase*, SAT), ii. apoptosis (*Activating transcription factor*, ATFC; *Cathepsin B*, CATB; *Cytochrome C*, Cyt-c; *Death receptor-associated nemesis-like*, Dronc; *High-temperature requirement A2 serine peptidase*, HTRA2; *Translocator protein*, TSPO) and iii. *Insulin-like secretion* (*Adenylyl cyclase*, AC; *Cholecystokinin A receptor*, CCKAR; *Muscarinic acetylcholine receptor M3*, M3R; *Phospholipase C*, PLC; *Piccolo presynaptic cytomatrix protein*, PCLO; *Synaptosome-associated protein 25*, SNP25; *Vesicle-associated membrane protein 3*, VAMP3) [22,45]. Primers for these genes were tested in Glaviano et al. [40].

The expression level was quantified using Real Time qPCR in QuantStudio™ 5 Real-Time PCR System and 2× Quantitative Master Mix with SYBR Green Low Rox (Genespin Srl, Milan, Italy). The following thermal profile was used, starting with (i) initial denaturation: 95 °C for 20 s; (ii) amplification (40 cycles): 95 °C for 1 s, followed by 60 °C for 20 s; (iii) final elongation: 95 °C for 15 s, followed by 60 °C for 15 s. Fluorescence was measured using QuantStudio™ Design & Analysis Software v1.5.2 (Applied Biosystem, Waltham, Massachusetts, USA). The expression level of each gene was normalized using the Relative Expression Software Tool (REST; REST-MCS©—version 2, Weihsenstephan, Germany), with Actin as a housekeeping gene. Values for the expression levels of the analyzed genes were reported relative to the control. Differences larger than a 2-fold change were considered significant (Tables S1 and S2 for detailed numerical values).

2.7. Statistical Analysis and PCA

To investigate relationships among gene expression profiles in the different experimental groups, a Principal Component Analysis (PCA) was performed. The dataset consisted of Cycle Threshold (CT) values for apoptosis-, ferroptosis-, and IAG-related target genes (Table S3). CT values were organized into a matrix with samples as rows and genes as columns. Before PCA, data were standardized using the StandardScaler function in the scikit-learn library, which transformed each variable to a mean of zero and a standard deviation of one, ensuring that all genes contributed equally to the analysis regardless of their absolute expression levels. PCA was then performed using the scikit-learn library in Python (version 2.14.0) to calculate the principal components. The first two components (PC1 and PC2) explained 58.25% of the total variance and were used for visualization of sample distribution. Samples positioned close to each other exhibit similar gene expression profiles, whereas those located in opposite regions display divergent molecular responses. The PCA represents an exploratory visualization of transcriptional similarities and differences among treatments.

3. Results

3.1. Molecular Responses Induced by Different Benthic Diatom Strains

Gene expression analyses revealed distinct expression patterns among the tested benthic diatom strains with several treatments showing transcriptional profiles consistent with modulation of apoptosis, ferroptosis, and IAG-related pathways in PL₅ post-larvae. Using *C. scutellum* var. *parva* (C2) as a positive control, several strains elicited transcriptional responses consistent with the molecular signature previously associated with androgenic gland degeneration and the onset of feminization. Among the tested strains, *Diploneis* sp. (CN), *C. scutellum* var. *posidoniae* (D) and *M. fimbriata* (MFA) induced the strongest activation of programmed cell death pathways. These strains were characterized by

the upregulation of key apoptotic markers, including *DRONC* and *HTRA2*, together with the modulation of ferroptosis-related genes. In particular, CN and MFA also promoted the expression of *TSPO*, suggesting activation of mitochondrial stress-associated apoptotic mechanisms. A second group of strains, including *C. neothumensis* var. *marina* (CNN), *N. shiloi* (MEL), C99 and C120, elicited a more selective molecular response. These treatments mainly affected ferroptosis-associated genes such as *SAT*, *GPX4*, and *STEAD3*, while inducing only partial activation of apoptotic pathways. Such profiles suggest the initiation of early cellular stress responses without the complete activation of the molecular cascade observed in the positive control.

The IAG-related pathway showed a general tendency toward downregulation in most treatments. *PLC* was suppressed by nearly all strains, whereas genes involved in vesicle trafficking and hormone secretion (*PCLO*, *SNP25* and *VAMP3*) were particularly affected in CNN and D. This pattern is consistent with a progressive impairment of androgenic gland secretory activity resembling that previously reported in C2-fed post-larvae. In contrast, strains such as C1 displayed limited modulation of both programmed cell death and IAG-associated genes, indicating a weaker biological activity compared with the most responsive strains.

The overall transcriptional responses are summarized in Figures 1–3, which highlight distinct molecular signatures among the tested benthic diatoms (see also Tables S1–S3).

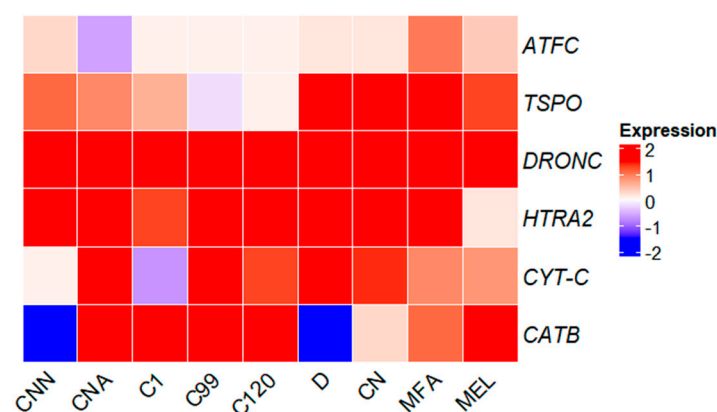


Figure 1. Heatmap showing the gene expression of Apoptosis pathway related to REST analysis using C2 as positive control.

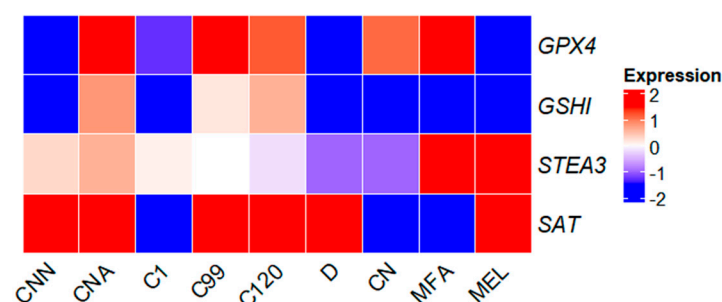


Figure 2. Heatmap showing the gene expression of Ferroptosis pathway related to REST analysis using C2 as positive control.

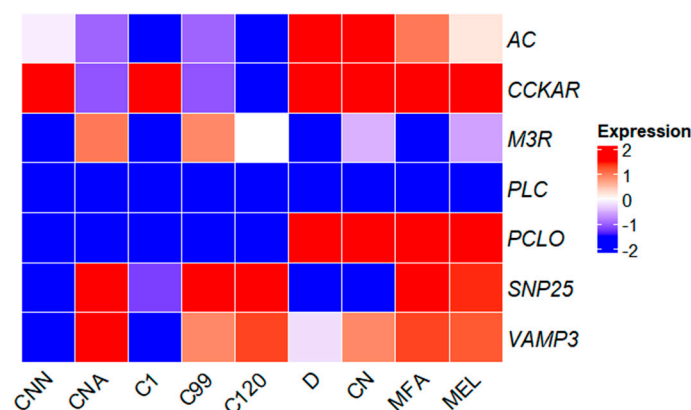


Figure 3. Heatmap showing the gene expression of IAG pathway related to REST analysis using C2 as positive control.

3.2. PCA Analyses

The PCA revealed distinct distribution patterns among the benthic diatom strains according to their gene expression profiles in PL5 post-larvae (Figure 4). These patterns represent similarities and differences in multivariate transcriptional responses and should be interpreted as exploratory relationships rather than statistically defined groups. Strains positioned in the same quadrant as C2, particularly CN and D, displayed gene expression profiles with similarities to those induced by *C. scutellum* var. *parva*, suggesting comparable molecular responses. In contrast, strains located in the opposite quadrants, including CBN, C99, CNA, and C120, exhibited clearly divergent molecular responses. The orientation and magnitude of the gene loading vectors (red arrows) indicated the relative contribution of each gene to sample distribution along the principal components. Samples clustering near C2 were mainly associated with stronger contributions of *ATFC*, *STE3A3*, *SNP25*, *CATB* and *M3R*, and secondly of *TSPO* and *DRONC*, as indicated by the longer loading vectors, whereas *CYT-C*, *HTRA2* and *VAMP3* showed comparatively weaker contributions in this quadrant. Conversely, samples grouped near the control food (CBn) were primarily associated with the expression of *CCKAR* and *PCLO*. A separate group composed of CNN, MFA, and MEL was associated with the expression of *GPX4*, *GSHI* and *SAT*, suggesting the activation of ferroptosis-related molecular responses. In contrast, C1 occupied an intermediate position between the first and second quadrants, opposite to the C2 cluster, indicating a partially distinct and less pronounced regulatory effect.

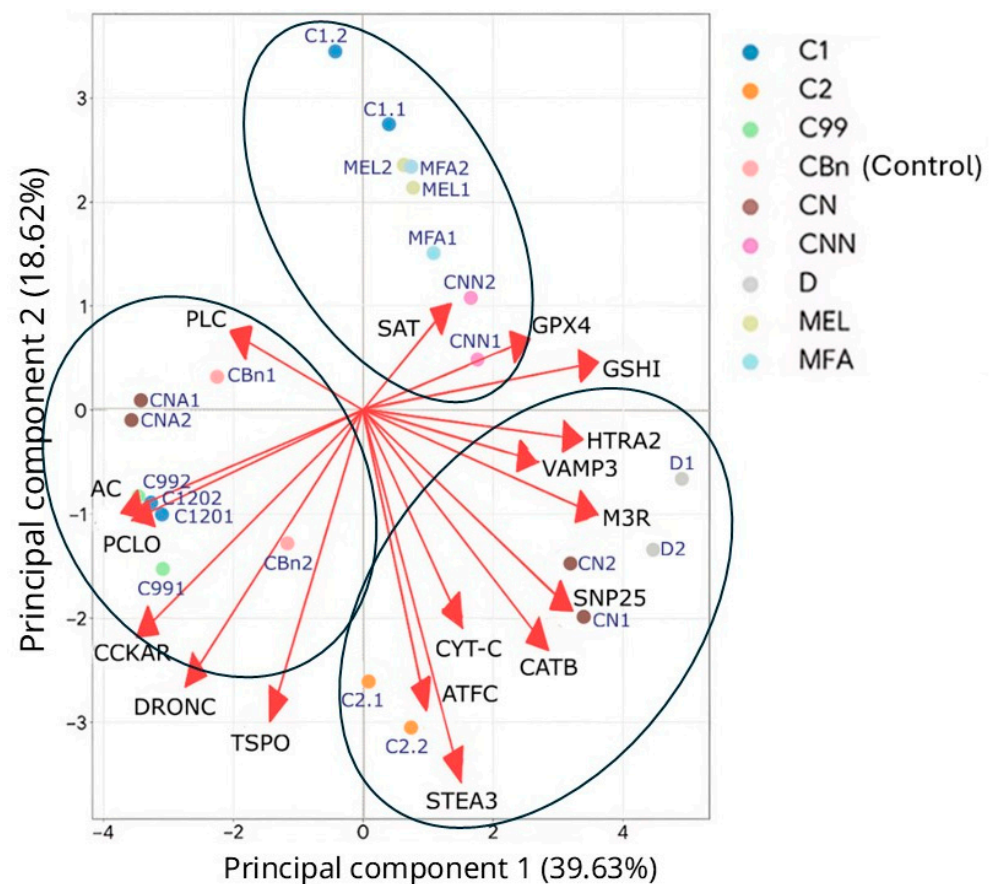


Figure 4. PCA biplot showing gene expression patterns related to apoptosis, ferroptosis, and IAG pathways across all experimental conditions. Samples are distributed according to the first two principal components (PC1 and PC2), which together explain most of the variance in the dataset. Samples positioned close to each other exhibit similar gene expression profiles, whereas those located in opposite quadrants display divergent molecular responses. Red arrows represent gene loading vectors, with arrow length indicating the relative contribution of each gene to the separation of samples along the principal components. Black outlines indicate groups of samples with similar expression profiles.

4. Discussion

The results of this study provide further molecular evidence supporting the role of benthic diatoms as key chemical mediators regulating ecological interactions in seagrass ecosystems. The interaction between the caridean shrimp *H. inermis* and epiphytic benthic diatoms inhabiting *P. oceanica* leaves represents a highly specialized bottom-up regulatory mechanism in which primary producers influence developmental pathways, reproductive dynamics, and ultimately the population structure of their consumers through the release of secondary metabolites [22,27]. While the feminizing activity of *C. scutellum* var. *parva* has already been described, the present study demonstrates that the capacity to induce PCD in AG cells is not restricted to a single species but is instead distributed among several benthic diatoms associated with seagrass meadows. The molecular analyses focused on genes involved in apoptosis, ferroptosis and IAG signaling pathways, which collectively regulate AG degeneration and the consequent suppression of male differentiation in decapod crustaceans. Previous studies demonstrated that the degeneration of the AG in *H. inermis* is associated with the activation of ferroptotic and apoptotic pathways, leading to the loss of IAG production and the subsequent feminization of post-larvae [22,27,31,40,46]. The present study was specifically designed to investigate whether other benthic diatom species elicit the same early molecular responses previously shown to pre-

cede androgenic gland degeneration and phenotypic feminization in *H. inermis*. Accordingly, our conclusions are based on the activation of these molecular pathways at the PL₅ stage and should not be interpreted as direct evidence of complete phenotypic feminization. In agreement with these findings, several of the benthic diatom strains tested here induced transcriptional profiles consistent with the activation of these molecular processes.

Within the apoptotic pathway, *DRONC* emerged as one of the most responsive genes, showing strong upregulation in almost all treatments when compared with the control diet (CBn). Particularly high expression levels were observed in individuals fed with CN, D and MFA strains, indicating an active induction of apoptotic signaling. Similarly, *HTRA2* was markedly upregulated in several strains, especially CN, MFA, C99, and C120 when compared to C2, suggesting mitochondrial involvement in the apoptotic cascade. Conversely, *ATFC* and *CYT-C* exhibited comparatively limited variation, indicating that not all apoptotic mediators respond with the same intensity or timing during AG degeneration. *TSPO* and *CATB* displayed moderate but selective activation, mainly associated with CN and MFA, reinforcing the hypothesis that these strains can induce molecular responses approaching those elicited by C2.

The ferroptotic pathway also showed substantial modulation in response to specific diatom strains. MFA, MEL, CNN, D, and C99 induced expression profiles partially overlapping with those previously described for C2. In particular, MEL showed simultaneous upregulation of *STEAD3* and *SAT*, two genes closely associated with iron metabolism and oxidative stress responses, suggesting activation of early ferroptotic mechanisms. Similarly, D and MEL displayed notable ferroptotic signatures when compared with the control diet, whereas strains such as C1 exhibited pronounced downregulation of ferroptosis-related genes, indicating little or no ability to trigger AG degeneration. These observations support the hypothesis that ferroptosis may represent one of the earliest cellular events initiating the molecular cascade previously associated with sex reversal in *H. inermis*.

The IAG-related pathway further confirmed the differential effects among the tested strains. *PLC* was consistently downregulated in most treatments, suggesting that this gene may represent a sensitive marker of AG functional impairment. Among all strains, CNN induced the strongest downregulation of *PCLO*, *SNP25* and *VAMP3*, closely resembling the molecular pattern observed in C2-fed post-larvae. Since these genes are associated with vesicle trafficking and neuroendocrine secretion, their suppression likely reflects a progressive disruption of AG secretory activity and reduced IAG production. The marked downregulation of *M3R* observed in some treatments further supports the idea that multiple regulatory nodes within the IAG pathway are affected during the sex reversal process. Overall, the molecular analyses indicate that although *C. scutellum* var. *parva* remains the best-characterized inducer of molecular pathways associated with sex reversal in *H. inermis*, other benthic diatoms, particularly CN, D, MFA, and CNN, can activate similar molecular pathways, albeit with lower intensity or partial effects. These findings suggest that the regulation of sex differentiation in natural populations may depend on a consortium of epiphytic diatoms rather than on a single species. Such a scenario is ecologically plausible within *P. oceanica* meadows, where complex and dynamic benthic biofilms expose juvenile shrimps to a diverse mixture of bioactive metabolites.

The PCA further clarified the relationships among the tested strains by revealing different patterns of similarity in molecular responses. The first group of samples, including MEL, MFA, CNN, and partially C1, showed a distribution pattern mainly associated with *GPX4*, *GSH1* and *SAT* expression, together with *DRONC* and *TSPO* expression, suggesting activation of early ferroptotic and apoptotic signaling. These strains may therefore initiate the biochemical cascade leading to AG deterioration without fully completing the downstream apoptotic and endocrine processes required for complete feminization. A second

group of samples, comprising C2 together with CN and D, showed a strong association with genes involved in apoptosis, ferroptosis, and IAG suppression, including *ATFC*, *CYT-C*, *CATB*, *HTRA2*, *SNP25*, *M3R*, *VAMP3* and *STE3*. The proximity of CN and D to C2 in the PCA space strongly suggests that these strains produce metabolites with similar physiological activity, potentially related to the same or structurally analogous lipophilic compounds previously hypothesized to resemble DGLA. The coordinated activation of all three pathways in this cluster is consistent with progression toward AG degeneration and with the molecular signature previously associated with functional sex reversal. In contrast, the third cluster included the control diet and strains characterized by weak or absent activation of PCD-related genes. Their association with genes such as *AC*, *PCLO*, *CCKAR*, indicates that these strains lack the specific chemical signals required to trigger the molecular cascade underlying AG degeneration. This distinction reinforces the specificity of the interaction between *H. inermis* and selected benthic diatoms. The differential responses observed among strains also suggest that the activation of PCD pathways may occur along a temporal and quantitative gradient. Some diatoms appear capable of initiating early ferroptotic responses, whereas only a limited number of strains induce the complete transcriptional profile associated with AG degeneration and sex inversion. This variability highlights the need to identify and chemically characterize the metabolites responsible for these effects. The occurrence of strains inducing only partial molecular responses may reflect differences in the quantity, chemical identity, or bioavailability of the metabolites released by different benthic diatoms. In natural *P. oceanica* meadows, juvenile shrimps are exposed to a complex assemblage of epiphytic microorganisms rather than to a single diatom species, and such variability in chemical signals may contribute to a flexible regulation of developmental responses [47]. Therefore, weaker responses should not necessarily be interpreted as a lack of ecological relevance, but rather as evidence of a heterogeneous chemical landscape in which different diatom species may contribute to varying degrees to the modulation of host physiology. Although the similarity of molecular responses observed among different diatom strains suggests the possible involvement of chemically related metabolites, this hypothesis remains to be experimentally validated. Recent metabolomic approaches applied to *C. scutellum* var. *parva* have identified candidate metabolites potentially associated with androgenic gland degeneration, including a putative lysophosphatidylglycerol (LPG 16:1) and a fatty acid ester; however, the active compound(s) responsible for the biological effect remain to be fully confirmed [48]. Furthermore, the precise chemical identity of the active metabolite(s) remains unresolved, and no comparative chemical analyses have yet been conducted among different benthic diatom species. Future comparative metabolomic and chemical characterization approaches will be required to determine whether other benthic diatoms produce the same compound or structurally related molecules responsible for the observed biological effects.

A broader ecological interpretation of the observed molecular responses emerges when considering the seasonal dynamics of *P. oceanica* meadows and the life cycle of *H. inermis*. Epiphytic benthic diatoms typically reach their maximum abundance during spring, when newly developed seagrass leaves provide extensive surfaces for colonization and environmental conditions favor rapid microalgal growth. Remarkably, this seasonal bloom coincides with the main recruitment period of *H. inermis*, when post-larvae settle within the meadow and begin feeding intensively on epiphytic microalgae. Such temporal synchrony suggests that juvenile shrimps are consistently exposed to diatom-derived metabolites during a critical developmental window, increasing the likelihood that these compounds act as reliable environmental signals rather than accidental stressors. From a demographic perspective, the direct feminization induced by diatom-derived metabolites may confer significant advantages to *H. inermis* populations. As a protandric hermaphro-

dite, this species normally passes through a male phase before becoming female. In the context of a protandric hermaphrodite species, the ability to modulate the timing of sex differentiation could have important consequences for reproductive dynamics. However, the present study does not directly demonstrate that diatom-induced molecular responses result in earlier female differentiation, increased female abundance, or enhanced reproductive output. Long-term experiments following treated individuals through sexual maturation will be required to determine whether the molecular changes observed here translate into measurable demographic effects. Previous studies have suggested that spring cohorts exposed to *Cocconeis*-rich diets show accelerated recruitment of reproductive females, thereby maximizing reproductive output during the most favorable season. In this context, chemically induced sex reversal may represent an adaptive mechanism linking reproductive allocation to habitat quality and food availability [49,50]. These considerations raise the intriguing possibility that the metabolites produced by benthic diatoms should not be viewed exclusively as toxic compounds. Although the activation of apoptosis and ferroptosis pathways clearly involves cellular damage within the androgenic gland, the ecological outcome appears beneficial for the recipient organism at the population level. The compounds may therefore function as environmental cues that provide information about the seasonal state of the seagrass ecosystem. Under this hypothesis, *H. inermis* would have evolved the capacity to exploit diatom-derived metabolites as predictive signals of favorable conditions for female differentiation and reproduction. Similar examples of adaptive responses triggered by naturally occurring chemical cues are known in both marine and terrestrial ecosystems, where organisms use environmental metabolites to regulate developmental trajectories. The widespread occurrence of comparable molecular effects among multiple benthic diatom species observed in the present study further supports the idea that *H. inermis* may respond to a broader chemical landscape rather than to a single species-specific metabolite.

The findings of this study have broader implications for the understanding of chemically mediated interactions in marine ecosystems. By demonstrating that multiple benthic diatom strains associated with *P. oceanica* can induce molecular responses linked to androgenic gland regulation, our results suggest that reproductive development in marine invertebrates may be influenced by complex chemical landscapes generated by benthic primary producers. This perspective is particularly relevant for seagrass ecosystems, where epiphytic microbial communities represent an important component of habitat functioning and trophic interactions. From a conservation perspective, preserving the biodiversity and ecological integrity of *P. oceanica* meadows may be essential for maintaining the chemical interactions that contribute to the regulation of associated invertebrate populations [51–53]. Future investigations integrating metabolomic characterization, long-term developmental experiments, and population-level analyses will be required to determine the ecological consequences and potential broader applications of these chemical-mediated processes.

Finally, it is important to consider that the present study evaluated gene expression at the PL₅ stage, representing an early molecular snapshot of the process. While the observed transcriptional responses strongly suggest activation of sex reversal pathways, confirmation that these molecular responses ultimately result in phenotypic feminization requires long-term biological assays. Future studies should therefore quantify the actual sex ratios induced by the different diatom strains and determine whether early molecular activation consistently translates into stable phenotypic sex reversal in adult shrimps. Such investigations will be essential to fully understand the ecological significance of this interaction and to evaluate its potential applications in sustainable crustacean aquaculture.

5. Conclusions

The present study provides new evidence that the modulation of sexual differentiation in *H. inermis* is driven by a broader assemblage of benthic diatoms than previously recognized. Several epiphytic species associated with *P. oceanica* meadows were able to activate molecular pathways linked to apoptosis, ferroptosis, and suppression of androgenic gland activity, indicating that chemically mediated sex regulation in this shrimp is not restricted to a single diatom species. The combined gene expression and PCA analyses revealed different levels of biological activity among the tested strains, ranging from weak or partial activation of programmed cell death pathways to molecular profiles closely resembling those induced by *C. scutellum* var. *parva*. In particular, strains such as *Diploneis* sp. and *C. scutellum* var. *posidoniae* displayed transcriptional signatures strongly associated with androgenic gland degeneration, suggesting the presence of conserved or functionally similar bioactive metabolites among phylogenetically distinct benthic diatoms. These findings strengthen the view that secondary metabolites produced by benthic microalgae represent ecologically relevant infochemicals capable of shaping reproductive strategies and population dynamics in marine invertebrates. More broadly, the study highlights the importance of chemical interactions in structuring seagrass-associated communities and emphasizes the ecological role of benthic diatoms beyond their contribution as primary producers. From an applied perspective, the identification of naturally occurring microalgal strains capable of influencing sex differentiation opens new perspectives for environmentally sustainable approaches in crustacean aquaculture. The use of diatom-based diets to modulate sex ratios may represent a non-invasive alternative to conventional hormonal or genetic methods, offering innovative opportunities for marine biotechnology while preserving ecological compatibility. Although the molecular responses observed closely resemble those previously shown to precede feminization induced by *C. scutellum* var. *parva*, the present study did not assess the final sexual phenotype. Long-term rearing experiments will therefore be required to confirm whether the tested diatom species ultimately induce phenotypic feminization. Future studies integrating metabolomics, functional assays, and long-term phenotypic analyses will be essential to identify the compounds involved and to evaluate their practical applicability in aquaculture systems.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/jmse14141288/s1>, Table S1: Real-time qPCR average CT values for triplicates of PLs fed on different benthic diatoms; Table S2: Rest values for gene expression of Apoptosis, Ferroptosis and IAG pathways using C2 as negative control; Table S3. Rest values for gene expression of Apoptosis, Ferroptosis and IAG pathways using CB as negative control.

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