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Communication

Atypical Membrane-Anchored Cytokine MIF in a Marine Dinoflagellate

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Abstract: Macrophage Migration Inhibitory Factors (MIF) are pivotal cytokines/chemokines for vertebrate immune systems. MIFs are typically soluble single-domain proteins that are conserved across plant, fungal, protist, and metazoan kingdoms but their functions have not been determined in most phylogenetic groups. Here we describe an atypical multidomain MIF protein. The marine dinoflagellate *Lingulodinium polyedra* produces a transmembrane protein with an extra-cytoplasmic MIF domain, which localizes to cell wall-associated membranes and vesicular bodies. This protein is also present in the membranes of extracellular vesicles accumulating at the secretory pores of the cells. Upon exposure to biotic stress, *L. polyedra* exhibits reduced expression of the MIF gene and reduced abundance of the surface-associated protein. These findings indicate that the transmembrane MIF may contribute to intercellular communication and/or interactions between free-living organisms in multispecies planktonic communities and raise the question of possible analogies in MIF functions between cells of metazoan organisms and protist communities.

Keywords: MIF; *Lingulodinium polyedra*; transmembrane protein; dinoflagellate; stress response; secretion

1. Introduction

Macrophage Migration Inhibitory Factors (MIF) are pivotal cytokines/chemokines for vertebrate immune systems. They are involved in numerous infectious diseases and pathological disorders such as septic shock, cancer, rheumatic and cardiovascular diseases [1–4]. These multifunctional proteins control major cell functions such as migration, proliferation and p53-mediated apoptosis [1,5,6]. MIFs are conserved across plant, fungal, protist, and metazoan kingdoms [7] but their functions have not been determined in most phylogenetic groups. The MIF proteins that have been described to date are small, conserved single-domain proteins (approx. 12.5 kDa). In a recent survey across 800 species of plants, protists, fungi and metazoan, we reported the existence of typical MIF proteins in all kingdoms and phylogenetic groups [7]. Interestingly, we also identified few atypical MIF proteins,

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including a putative membrane-anchored MIF protein from the Genbank TSA database (Genbank accession number JO713726.1) in the dinoflagellate *Lingulodinium polyedra* but their existence remained uncertain as they may result from assembly or automatic annotation errors [7]. The possible occurrence of a transmembrane cytokine in a free living unicellular eukaryote would be unexpected as MIFs are typically soluble proteins that are stored in vesicles of immune and non-immune cells, and massively secreted upon challenge [3,8–11]. In addition, previous studies on MIFs from unicellular species have been restricted to parasitic protists [12] such as *Plasmodium* species [13], *Leishmania*, [14], or *Entamoeba histolitica* [15]. MIFs from these species are secreted into the blood of the vertebrate host and participate in immune evasion and pathogenesis [12,16]. The aim of this study was therefore to investigate the existence of a transmembrane MIF protein in the dinoflagellate *L. polyedra*, and to start exploring its potential function.

3. Results and discussion

3.1. *Lingulodinium polyedra expresses a transmembrane MIF protein.*

The cDNA of *L. polyedra* MIF (LpMIF) was re-sequenced and validated (Genbank accession number MN911288), thereby confirming the existence and expression of this transcript. The open reading frame encodes a 246 amino-acid protein (Figure 1a) with a predicted molecular mass of 25,07 kDa and a theoretical pI of 8.88. A MIF domain corresponding to the Interpro domain IPR001398 and PFAM domain PF01187 is predicted in the C-terminal part of the protein (positions 135 to 245) with e-values of 5.3^{e-33} and 3.2^{e-27}, respectively. This domain shows 60% amino-acid similarity with the human MIF protein (Genbank accession number NP_002406.1) and harbours amino acids that have been shown to determine specific functions or activities in human MIF. Importantly, the MIF tautomerase active sites, encompassing residues Pro-2, Lys-33, Tyr-37, Ile-65 and Tyr-96, (symbol ■ in Figure 1a) are conserved, suggesting potential tautomerase activity of the LpMIF protein. Similarly, LpMIF harbours the CXXC motif (Cys-57 and Cys-60) (o symbol in Figure 1a), previously shown to determine the human MIF oxidoreductase (TPOR; thiol-protein oxidoreductase) activity [17]. This site is associated with the regulation of cellular redox homeostasis. In humans, the biological functions of these two enzymatic activities remain largely unknown, in particular because their endogenous substrates have not been identified yet [3]. However, there is increasing evidence for their roles in the pro-inflammatory effects of MIF [18]. Mutations in or inhibition of the enzymatic active sites lead to the loss of major MIF biological functions [18], possibly due to conformational changes in the catalytic domains [19]. Conservation of these active sites in LpMIF indicates that the protein could have typical MIF biological activities.

In addition to the MIF domain, transmembrane (TM) helix and general hydrophobicity predictions support the existence of three TM helices in the N-terminal part of the protein (Figure 1b). Although the exact positions of the transmembrane regions are determined slightly differently (Figure 1b), all prediction tools agree on the calculated topology, which predicts a non-cytoplasmic localization for the MIF domain (Figure 1c). As a first approach to ascertain the transmembrane nature of LpMIF, we prepared the membranes of *L. polyedra* cells and observed a major protein band (Figure 1d). The presence of an LpMIF peptide in this fraction was confirmed by mass spectrometry

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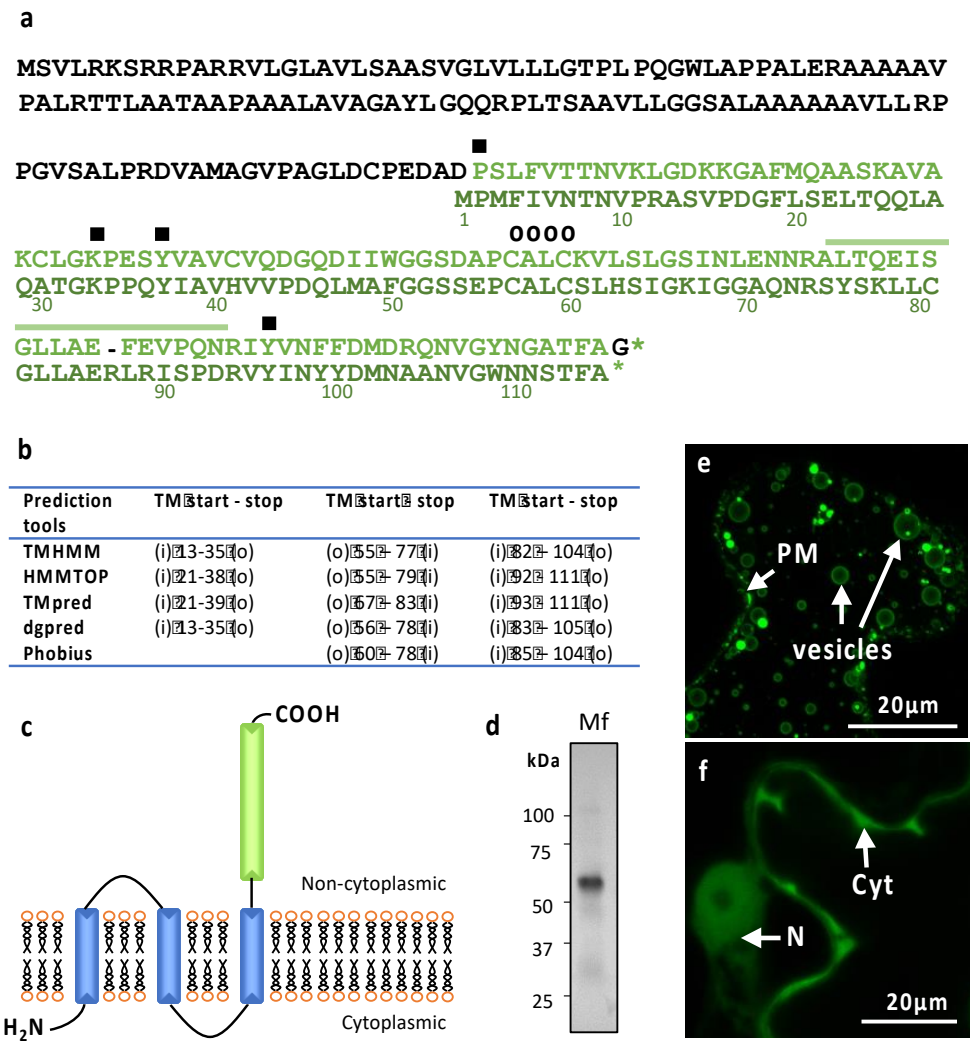

Figure 1:*Lingulodinium polyedra* expresses a transmembrane MIF. (a) Alignment of the *L. polyedra* MIF (LpMIF) sequence with human MIF (dark green). Amino acid numbers of the human MIF are given above the sequence. Hyphens (-) indicate a gap in the respective sequence. Amino acids shown to determine the tautomerase activity (■) and oxidoreductase activity (CXXC motif; **O**) in human MIF are shown by symbols above the sequence. The peptide identified by mass spectrometry is indicated by a line above the LpMIF sequence. (**b**) Location (LpMIF amino-acid number) and orientation of the transmembrane helices predicted by 5 prediction tools, where « i » refers to inside (cytoplasmic) and « o » to « outside » (non-cytoplasmic). (**c**) Schematic representation of the predicted LpMIF topology. The transmembrane domains are represented in blue and the MIF domain in green. The membrane is represented as a lipidic bilayer. (**d**) Western blot analysis of the membrane fraction (Mf) of *L. polyedra* proteins. The membrane extract (10 µg of total proteins) reveals a major protein with an apparent molecular mass of about 60 kDa. The presence of LpMIF in the most prominent band of the membrane fraction was confirmed by mass spectrometry (see 1a). (**e, f**) Subcellular localization of LpMIF fused to GFP in the N-terminal region, with (e) or without (f) the transmembrane domains in *Nicotiana benthamiana*. Leaves were inoculated with *A. tumefaciens* harboring the LpMIF-GFP constructs, 2 days prior to observations. Complete LpMIF proteins localize to the membranes of vesicles and the plasma membrane (PM) (e), while LpMIF without the transmembrane domains (in f) shows a nucleo-cytoplasmic expression in *N. benthamiana* cells, which is typical for small, soluble proteins. (N) nuclei, (Cyt) cytoplasm.

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(Figure 1a). The apparent molecular mass for LpMIF was revealed to be approximately 60 kDa in the immunoblots, which might correspond to a dimer (expected at 50 kDa) as estimated molecular masses of proteins are not accurate under non-denaturing conditions. Typical MIF proteins can co-exist in several oligomeric forms (mono-, di-, or trimers) although MIF trimers represent the most prominent and stable form [10,20,21]. Future structural studies should reveal whether homodimers are the predominant form of the transmembrane LpMIF. Further evidence for the membrane association of LpMIF was provided by *Agrobacterium tumefaciens*-mediated transient expression assays to produce LpMIF in cells of *Nicotiana bentamiana* leaves. The use of this heterologous plant expression system confirmed that LpMIF has a non-cytoplasmic localization and accumulates in the membranes of vesicles and in the plasma membrane of the plant cells (Figure 1e). In contrast, LpMIF without the transmembrane domains shows a nucleo-cytoplasmic expression in *N. benthamiana* cells, which is typical for small, soluble proteins (Figure 1f).

3.2. *LpMIF is present in the membranes of vesicular bodies, cell wall and extracellular vesicles*

The subcellular localization of LpMIF in *L. polyedra* cells was investigated by transmission electron microscopy (TEM) with immunogold labelling. LpMIF-bound gold particles were observed in the membrane of vesicular bodies (Figure 2a), further supporting the membrane localization of this atypical MIF protein. Extensive observation of *L. polyedra* cells did not reveal the presence of labelled LpMIF in any particular organelle (Figure 2a and S2b) other than the vesicular bodies. The highest concentrations of LpMIF-bound gold particles were observed in the cell wall or theca (Figure 2b), particularly in the vicinity of the apical pore (Figure S2a). The cell wall of thecate dinoflagellates (or theca) consists of multiple membrane layers strengthened by polysaccharides [22]. This membranous composition therefore supports the presence of membrane-anchored proteins such as LpMIF. Confocal laser-scanning microscopy (CLSM) confirmed the presence of LpMIF in the theca, and further showed specific accumulation at the apical pore and at secondary thecal pores (Figure 2c, d; compare with scanning electron micrographs in Figure 2e, f). Dinoflagellates are extensively studied for their toxicity because of their roles in marine red tides and shellfish poisoning [23,24], yet their ultrastructure and physiology remain poorly understood. However, dinoflagellate pores, and apical pores in particular, are known to be involved in mucilage excretion on the cell surface [25]. In addition, thecal pores extrude fibrous and mucous trichocysts (cf Fig. S1b) over the entire cell surface, which contribute to dynamic streamlining and defence against grazing [26].

To determine if LpMIF is secreted, we repeated the immunofluorescence labelling using milder washing conditions (identical procedure but without Triton). Confocal analyses subsequently showed MIF-labelled extracellular vesicles at the surface of *L. polyedra* cells (Figure 2g) with particular accumulations in mucus outside the apical pore (Figure 2h).

MIF proteins of metazoa are soluble and present in the cytoplasm of numerous cell types, stored in vesicular bodies and released in extracellular fluids (blood, haemolymph) in response to various stimuli such as bacterial lipopolysaccharides (LPS), tumour necrosis factor or hypoxia [8,11]. Despite the decades of functional studies on human MIF, the exact mechanism of MIF secretion remains unclear. Human MIF, which does not localize to the endoplasmic reticulum or Golgi apparatus, is secreted through unconventional secretory pathways [11]. Some studies showed that MIF secretion was sensitive to inhibitors of ATP-binding cassette (ABC) transporters, while others reported the

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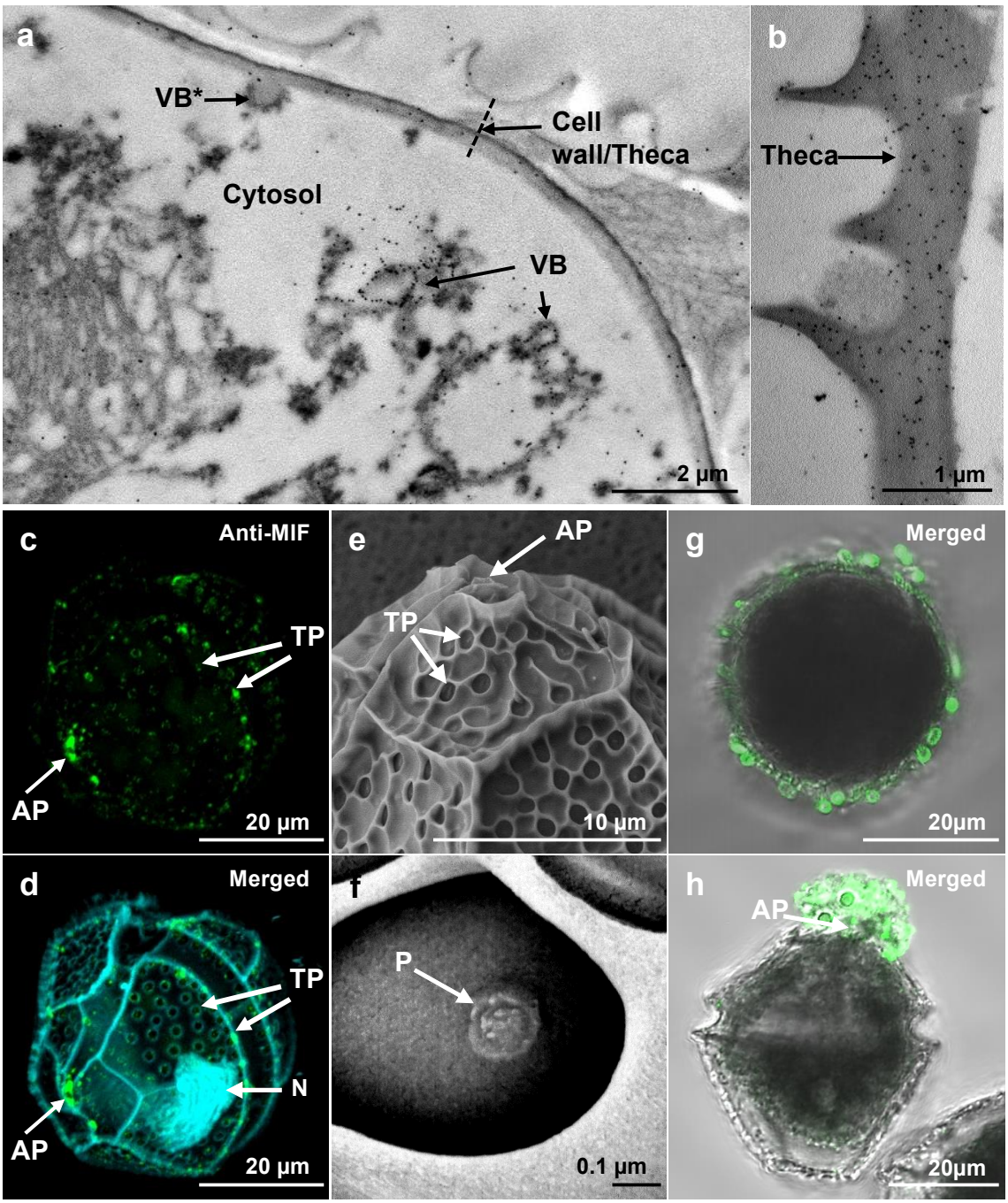

Figure 2: Subcellular localization of LpMIF. (**a,b**) TEM of immunogold-labeled *L. polyedra* cells showing the accumulation of gold particles (black dots) in membranes of the vesicular bodies (VB) and in the theca. Note that one of the vesicular bodies (indicated by a star) appears to be in a process of fusion with the cell wall. (**c,d**) Immunolocalization of LpMIF by confocal microscopy. LpMIF is labelled in green in (**c**) and (**d**), while the nucleus (N) appears in blue in (d) (DAPI staining). The overall structure of *L. polyedra* is visible under UV light due to autofluorescence in (d) and shows the shape of the thecal plates, the apical pore (AP) and the thecal pores (TP). Note the accumulation of LpMIF at the apical pore (AP) and the thecal pores (TP). (**e,f**) Scanning electron micrographs of *L. polyedra* showing the AP and TP. An enlargement of a TP in (**f**) shows the inner pore (P). (**g,h**). Representative pictures of *L. polyedra* cells that have been immunofluorescence-labelled under mild washing and fixing conditions revealing the accumulation of MIF-labelled extracellular vesicles. Here, an apical view of the cell shows labelled vesicles surrounding the cell (**g**) and a lateral view illustrate the commonly observed accumulation of labelled mucus at the apical pore (AP). Control samples exposed to pre-immune serum or no primary antibody did not show any fluorescent signal (not show). Please, see supplementary material for procedure description.

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release of MIF via exosomes [3]. While the trafficking processes of LpMIF clearly requires dedicated studies, the presence of this atypical transmembrane MIF in intra- and extracellular vesicles is reminiscent of the localization of vertebrate MIFs.

3.3. LpMIF is under-expressed during a stress response

Predicting putative functions for a transmembrane MIF in a free-living unicellular organism is particularly challenging. Previous studies on MIFs from unicellular species have been restricted to parasitic protists [12] such as *Plasmodium* species [13], *Leishmania* [14], *Giardia lamblia* [27] or *Entamoeba histolitica* [15]. MIFs from these species are secreted into the blood of the vertebrate host and participate in immune evasion, host invasion and pathogenesis [12,16]. Such functions are not relevant for a free-living marine dinoflagellate. Furthermore, the major function of MIFs as innate immune regulators, which has been reported in free-living metazoan species including invertebrates [10,16,28,29], is hardly applicable and measurable on non-model protists such as *L. polyedra*. We therefore hypothesized that LpMIF may be involved in a stress response, since functional links between immune and stress responses are well established in both animal and plant model species [30–34] and since MIFs from vertebrates are also involved in stress responses [2]. Under natural conditions, *L. polyedra* lives in phytoplankton communities that are exposed to grazing copepod predators. Copepods are known to induce defence responses, such as an increase in bioluminescence [35,36], a change of swimming behaviour [37] an increase of toxin production [38], and a decrease in photosynthetic activity [39,40]. It has been suggested that these induced defences may provide slow-growing *L. polyedra* the required advantage to co-exist with faster-growing competitors in phytoplankton communities [36]. Exposure of *L. polyedra* to the copepod *Sarsamphiascus cf. propinquus* for 24h correlated with a significant decrease in photosynthetic activity (Figure 3a), confirming that the copepod did induce defence responses in our experiment. This stress response is transient since the fluorescence recovered and increased above control levels seven days after copepod exposure (Figure 3a). A decrease in LpMIF transcript content (Figure 3b) and labelled LpMIF protein content (Figure 3c, d) occurred during this stress response. This response indicates that, in this context, the transmembrane MIF from *L. polyedra* is indeed a stress-responsive protein.

4. Conclusions

MIFs are enigmatic proteins with extremely complex trafficking processes, biological activities, functions and signalling pathways [3,11]. Here we provide evidence for a new dimension in the complexity of MIF structure and possible functions. The presence of LpMIF in the membranes of secreted extracellular vesicles evokes the fascinating possibility that LpMIF participates in intercellular communication and/or regulation in the marine environment. There are increasing evidences that phytoplankton communities, including a variety of protist and bacterial species, produce substantial amounts of extracellular vesicles that are involved in interspecies communication, nutrient acquisition and exchange, biofilm formation and cellular defence processes [41–43]. The multiple functions of typical MIF proteins such as the effects on cell migration, cell division, apoptosis and induction of other immune or hormonal regulators, may all have a role in

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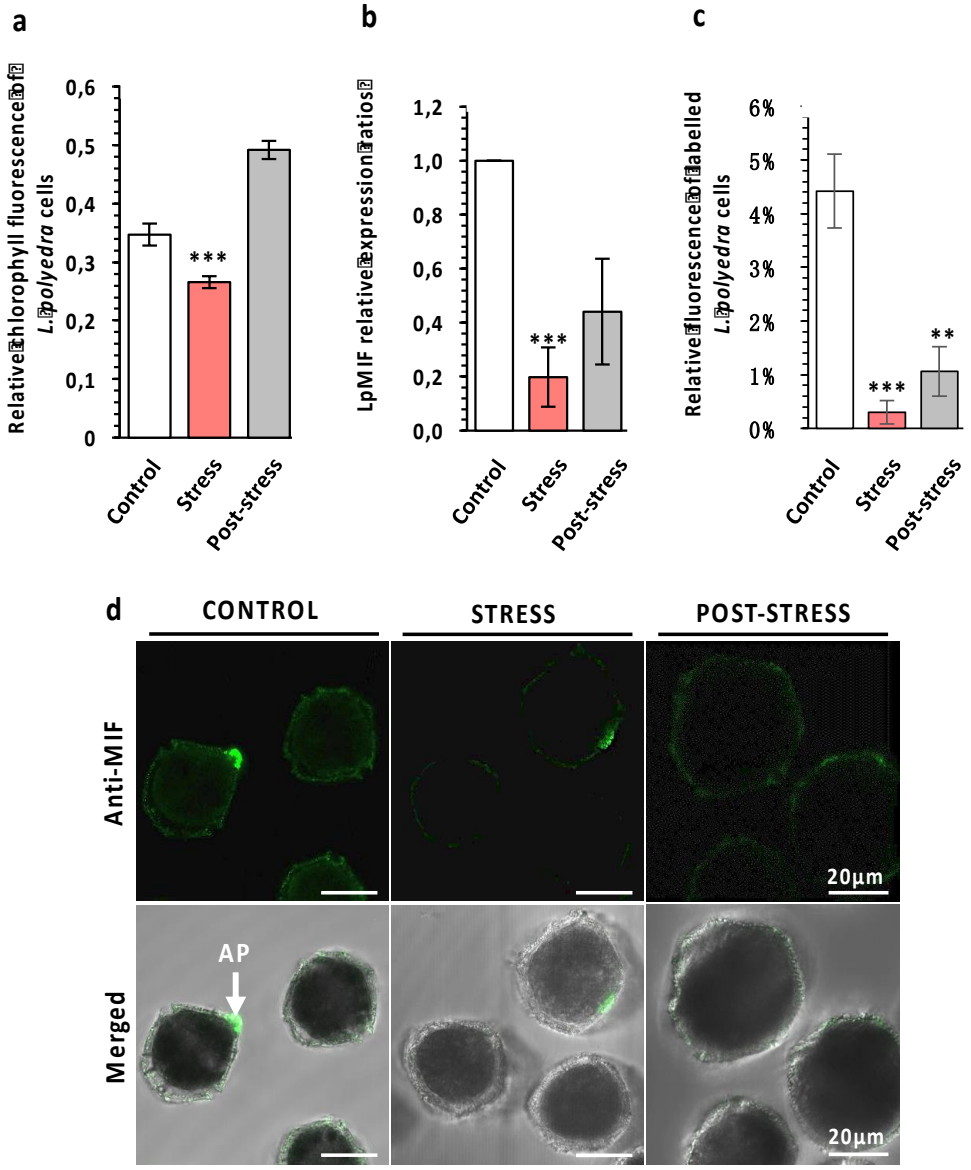

Figure 3: LpMIF expression during a stress response. (a) The exposure of *L. polyedrum* to copepods decreases photosynthetic activity as a stress response. Chlorophyll fluorescence parameters were measured with a Multi-Colour Pulse-Amplitude-Modulated fluorometer (Heinz Walz Gmbh, Effeltrich, Germany). Photosystem II activity was calculated from the ratio of variable fluorescence to maximum chlorophyll fluorescence (Fv/Fm). Values are means (+/- SEM) from 3 independent measurements/experiments. Asteriscs indicate statistical differences (*** p< 0,001), according to Kruskal-Wallis test. **(b)** Relative expression ratios (normalized to control) of LpMIF in *L. polyedra* cells before (control), during (stress) and after (post-stress) exposure to copepods. **(c)** Relative fluorescence of MIF-labelled *L. polyedra* cells expressed as the fluorescent area/total area, of cells before (control), during (stress) and after (post-stress) exposure to copepods (n=9 per sample; Kruskal-Wallis test:* = $p \leq 0,05$, ** = $p \leq 0,01$, *** = $p \leq 0,001$). **(d)** Representative pictures of MIF-labelled *L. polyedra* before (control), during (stress) and after (post-stress) exposure to copepods. Images in the upper row are fluorecence micrographs (Anti-MIF) that were merged in the lower row with transmission light micrographs (Merged).

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coordinating stress responses within a dinoflagellate colony. In addition, since MIF proteins are prone to interspecies and inter-kingdom interactions, as shown by their involvement in host-parasite interactions [16,44,45], they may also help regulate the interactions of multispecies planktonic communities. Such possibilities can now be explored by investigating LpMIF functions under biotic stress conditions.

Supplementary Materials: The following are available online, Material and methods; Figure S1: Immunogold-labelled electron micrographs of *L. polyedra*, Figure S2: Negative control samples of unstressed and stressed *L. polyedra*.

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