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ORIGINAL ARTICLE

Different stressors uniquely affect the expression of endocannabinoid-metabolizing enzymes in the central ring ganglia of *Lymnaea stagnalis*

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Abstract

The endocannabinoid system (ECS) plays an important role in neuroprotection, neuroplasticity, energy balance, modulation of stress, and inflammatory responses, acting as a critical link between the brain and the body's peripheral regions, while also offering promising potential for novel therapeutic strategies. Unfortunately, in humans, pharmacological inhibitors of different ECS enzymes have led to mixed results in both preclinical and clinical studies. As the ECS has been highly conserved throughout the eukaryotic lineage, the use of invertebrate model organisms like the pond snail *Lymnaea stagnalis* may provide a flexible tool to unravel unexplored functions of the ECS at the cellular, synaptic, and behavioral levels. In this study, starting from the available genome and transcriptome of *L. stagnalis*, we first identified putative transcripts of all ECS enzymes containing an open reading frame. Each predicted protein possessed a high degree of sequence conservation to known orthologues of other invertebrate and vertebrate organisms. Sequences were confirmed by qualitative PCR and sequencing. Then, we investigated the transcriptional effects induced by different stress conditions (i.e., bacterial LPS injection, predator scent, food deprivation, and acute heat shock) on the expression levels of the enzymes of the ECS in *Lymnaea*'s central ring ganglia. Our results suggest that in *Lymnaea* as in rodents, the ECS is involved in mediating inflammatory and anxiety-like responses, promoting energy balance, and responding to acute stressors. To our knowledge, this study offers the most comprehensive analysis so far of the ECS in an invertebrate model organism.

KEYWORDS
anxiety, endocannabinoids, pond snails, food deprivation, inflammation, heat shock

1 | INTRODUCTION

Mounting research underscores the endocannabinoid system (ECS) as a pivotal mediator that connects our perception of both external and internal signals with specific neurophysiological and behavioral responses, such as fear, anxiety, and stress reactions (Lutz et al., 2015).

This sophisticated system enables organisms to adjust their behaviors in ways that are crucial for survival, maintaining balance within the body, and building resilience (Morena et al., 2016). This

Abbreviations: 2-AG, 2-arachidonoyl glycerol; AEA, *N*-arachidonoyl-ethanolamine; CE, crayfish effluent; CNS, central nervous system; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; LPS, lipopolysaccharide; MAGL, monoacylglycerol lipase; NAPE-PLD, *N*-acyl-phosphatidylethanolamine-specific phospholipase D; PEA, *N*-palmitoylethanolamide.

is supported by findings from several studies, which highlight the ECS's integral role in a broad spectrum of physiological functions, including brain activity, eating habits, immune responses, and hormone regulation (Lukowiak et al., 2014). Furthermore, disruptions in endocannabinoid signaling have been linked to various health issues, including stress-related conditions, neurological disorders, inflammation, and metabolic imbalances, indicating its significant impact on health and disease (Barrie & Manolios, 2017; Crowe et al., 2014; Morena et al., 2016; Viveros et al., 2005). Endogenous cannabinoids are produced in response to stress and generally function in opposition to the stress response (Morena et al., 2016). The ECS includes the cannabinoid receptor types 1 and 2 (CB1 and CB2), their endogenous lipid ligands [the most-studied of which are 2-arachidonoyl glycerol (2-AG)], and *N*-arachidonoylethanolamine (AEA; also known as anandamide), and their synthesizing and degrading enzymes (Lutz et al., 2015).

Unlike classical neurotransmitters, endocannabinoids are stored in the membrane as phospholipid precursors and are enzymatically released "on demand" by the increase of intracellular Ca^{2+}, membrane depolarization, or stimulation of metabotropic receptors (Donvito et al., 2018).

The first endocannabinoid molecules identified and characterized have been AEA and 2-AG (Devane et al., 1992; Mechoulam et al., 1995), which are biosynthesized from different membrane phospholipid families, both esterified by arachidonic acid (Mechoulam et al., 1995).

AEA is primarily generated through the hydrolysis of the membrane phospholipid precursor *N*-arachidonoyl phosphatidylethanolamine (NAPE) by *N*-arachidonoyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD), although alternative biosynthetic pathways have been suggested. Specific phospholipases A1/A2 (PLA1/2) and phospholipase C were proposed as alternative enzymes to yield AEA by converting NAPE; moreover, the sequential deacylation of NAPE catalyzed by α,β-hydrolase 4 (Abhd4) generates the precursor glycerophospho-arachidonoyl ethanolamide that is then converted to AEA (Hussain et al., 2017; Leishman et al., 2016; Liu et al., 2006, 2008; Simon & Cravatt, 2006; Sun et al., 2004).

So far, NAPE-PLD is the sole enzyme catalyzing this reaction in mammal tissues, while the expression of PLA2 was very low. To date, evidence on the presence of putative enzymes involved in the biosynthesis of AEA in the invertebrates only concern NAPE-PLD, and emerge in *Hydra vulgaris* polyps (De Petrocellis et al., 1999), *Paracentrotus lividus* (Bisogno et al., 1997), *Caenorhabditis elegans* (Harrison et al., 2014), and *Ciona intestinalis* (Matias et al., 2005).

While the synthesis of AEA occurs via multiple pathways, metabolic degradation appears to be predominantly mediated by the fatty acid amide hydrolase (FAAH), which metabolizes it into arachidonic acid and ethanolamine (Okamoto et al., 2004). Besides serving as the major catabolic enzyme of AEA, FAAH hydrolyzes other bioactive lipids, such as N-palmitoylethanolamide (PEA) which functions as an endogenous ligand for peroxisome proliferator receptor-α, which in turn, exerts anti-inflammatory effects (Egertová et al., 2003; Guo et al., 2016, 2022; Locci & Pinna, 2019; Paterniti et al., 2013; Ueda, 2002). On the other hand, the synthesis of 2-AG is primarily mediated by diacylglycerol lipases (DAGL-α and DAGL-β) (Tanimura et al., 2010), whereas monoacylglycerol lipase (MAGL) catalyzes its degradation into arachidonic acid and glycerol (Tanimura et al., 2010). Nevertheless, 2-AG can be generated by an alternative pathway in which phosphatidylinositol 4,5-bisphosphate is dephosphorylated by PIP_2 phosphatase producing an *sn*1-ester intermediate; the latter is hydrolyzed via phospholipase A_1 generating 2-arachidonoyl-lysophosphatidylinositol, which is subsequently dephosphorylated by lysophospholipase C to produce 2-AG (Higgs & Glomset, 1994). Unfortunately, to date, pharmacological inhibitors and regulators of the different ECS enzymes have led to mixed results in both preclinical and clinical studies (Farrell et al., 2021; Gunduz-Cinar, 2021; Simon et al., 2022). Thus, further research is needed to fully understand the complex roles of the ECS and to unlock its real potential as a source of therapeutic targets. Although the ECS is highly conserved throughout the eukaryotic lineage, the most common organisms used to investigate the functions and the pharmacological regulation of the ECS have been rodents (Egertová et al., 2003; Elphick, 2012). However, the use of invertebrate models in preclinical research has proven to be highly advantageous in terms of experimental efficiency (Ottaviani & Franceschi, 1996; Ottaviani et al., 2013; Tascedda et al., 2015).

Thus, a full characterization of the ECS in one of these models may be beneficial to unraveling the complexity of the ECS and allowing those insights to be translated into mammals (Clarke et al., 2021; Rivi et al., 2020; Rivi, Batabyal, Benatti, et al., 2021).

This is further supported by a growing number of studies supporting the existence of an endocannabinoid-like system in invertebrates (Bailone et al., 2022; Clarke et al., 2021; Esdin et al., 2010; Estrada-Valencia et al., 2023).

The pond snail, *Lymnaea stagnalis* (Linnaeus, 1758), serves as a great model system for enhancing our comprehension of the basic and conserved mechanisms underlying central nervous system (CNS) physiology and pathology (Fodor et al., 2020, 2021; Rivi, Batabyal, Benatti, Tascedda, et al., 2023). *L. stagnalis* belongs to the *phylum Mollusca* class *Gastropoda* and has been widely used to investigate genetic and epigenetic processes, aging, learning, and memory, as well as in ecotoxicological studies (Amorim et al., 2019; Batabyal et al., 2022; Benatti et al., 2020; Rivi, Batabyal, Juego, et al., 2021; Rivi, Benatti, Actis et al., 2022; Rivi, Batabyal, Wiley et al., 2022; Rivi, Batabyal, Lukowiak, Benatti, et al., 2023; Rivi, Batabyal, Benatti, Tascedda, et al., 2023). The CNS of *L. stagnalis* consists of approximately 25000 neurons organized in a ring of interconnected ganglia, offering a relatively large amount of biological material that can be molecularly, physiologically, and morphologically analyzed (Feng et al., 2009; Straub et al., 2004; Syed et al., 1990; Yeoman et al., 1996).

Recently, research by Sunada et al. (2017) identified two genes encoding G-protein-coupled receptors in the CNS of *L. stagnalis*. These genes produce proteins akin to the well-studied cannabinoid receptors in vertebrates, providing the first evidence of the ECS presence in this invertebrate model (Sunada et al., 2017).

In this study, our initial goal was to test the hypothesis that endocannabinoid metabolic enzymes are present in the *L. stagnalis* genome. Next, given that endocannabinoid signaling is involved in preserving health, and that perturbation by stress-related conditions compromises health and survival (Crowe et al., 2014; Elphick et al., 2003; Gunduz-Cinar, 2021), we tested whether their expression changed in the snails' central ring ganglia under various stress conditions known to trigger universally conserved behavioral responses (Lukowiak et al., 2008).

In mammals, ECS has been demonstrated to play a key role in inflammation and immune regulation (Crowe et al., 2014; Dos Santos et al., 2023). Systemic administration of bacterial LPS is commonly used in rodents to induce inflammation-associated behavioral changes, akin to symptoms of depression in humans (Dantzer &

TABLE 1 Putative genes coding for the enzymes of the ECS in *Lymnaea stagnalis*.

Lymnaea stagnalis					Biomphalaria glabrata		
Enzyme	Contig	Match on	ORF	Aa size	Accession	Aa size	E value
LymDAGL-like diacylglycerol lipase	FCFB01005499.1	FX181219.1	MLRADILNQIKNSSDSKWKIITQGLMCCGRNSA ETLPKEMLERDVTAHPSNSAIGLSAHLPLYP PGQMIHVVRSHLTDKKSRGYCDDSDFIYQAIWAN NGDFDEVLVSPTMLNDHMADRVLEALQKVVLKVAPE KPVHTLTEEQRREMLNHPSVSSLATPSDVMLSNPINT PDITRGSEDSNLGPNGEVFLEEVDDSMLFGMPNIE AEELVRAPLASPETLSLVSGVAVFSPSSSIVGRDSLK QSLQRCAGKSVNNPHEVPLENESATVNRGPRSASQ KSNPAHKPGFDGNSKNGSSGSR DLEAGHGP SQEPDYQVKLRSGNMISVKAEVVNEMIKQNN KAPSPARSQ DKDGSRSSAVLEPSSHTHFVSPSHHKLAHADSTEETST LQPHPSVNEYHNPTFEIDKPESIGLIVGWGSRGG EDGQDNVSGLLDDYARHSPSESNQHEYGHPNVYYSQGG IGYPHNAFHPPLRFNSFPGDEAINDL CSNDTENTHICGD SINQTLVNSAEVDAEDEISTPKTAQKKPFTHAESE SNLLAKSKHLPRMRRSAEQGDIAHLCDALEVFE TEEDKARLSHSSLTLLEETTEDLLVAQRAEEEKGIPPLSPPCT GKNKVNKQSVSFGGSVIIPTHQGASDQASPDNHSKDLA SVMKVSSVDDTTSSETDNVSLSNPNDESSADHVHNA NPNESSLSTKLLQDNQIHVSNLTNVFLDQQNPSLQETHL	721	XP_013079604	906	0
LymMAGL-like monoglyceride lipase	FCFB01082919.1	FX189644.1	MTASNDSSESFFTNERGKKIYCK YWNKDIKSPRALAFISHGAGEHCLWYTELAHQLADKGLYVF SHDHEGHGQSEGSRMHITDFRHYINDVFQHTDTVSKQ FPNVPIFIVGHSMGGAVSIVAALDRPD YFTGVVLIAPCVTPEQDTAGPIKIFFGKLA SRIMPQCPVLWLDDKYISRDQAIR QKYKDDPLVYHGGMKAKWAISLL QALQEVESKLSTIQWPFLVLHGDSDKIVNSKGSEALY KQAASVDKTIKILKDCFHQLHNEPEPDGSKVKEEIVGWIVQRLP	286	XP_013060524.2	286	3.00E-160
LymNAPE-PLD like N-acyl-phosphatidy-lethanolamine-hydrolyzing phospholipase D	FCFB01080182.1	FX191515.1	MSKERVDNIHTEECTQPTLMGN GRYQNPWETWQAPKLNKLLKFI FMRGDANVPKEAELDKTLPVRKP DLSQFETSPQSGVRHMWIGHASSLVQLDGLTILTDPIFSDR CSPFQWFGTKRYRPPPCTVDQLPK VDCVVISHNHYDHLDYGSVVSLN QRFGENLKWYVPKGLKSWMN DSGCKNVVELSWWEEHIHSEASG VKIVNTPCQHWCKRSLNDDNKVLWSSWCVLGPKHSFFFAGDT GYCEGFKQIGQRYGPFTLSTIPIGAYCPRWFLGPQHVDPAEAV DIHNDLGSKTSIGIHWGTFVLSKEPYLEPRDKLKEELEKRGMK PSSFITVDHGDVIVIDNQ	344	XP_013089040.2	345	0
LymFAAH-like fatty acid amide hydrolase	FCFB01020578.1	FX195089.1	MIPPYTMKEVVTFVYGHPRTAPVLLSL LGCFAVQKLIYTLWKKKSVRNK RSLRRAKAEKRRKIIEGRLAKVSPPSEEILNKSASELVEALHSGDL SAVEVLYAYQRRALSLTKEINCITEFIPEAEAQAKRLDECPTKT GLLHGMPISLKENIAVSGYDTTAG MEINIDKNATDDCVAVKVLKLHGAIPFARTNIPQTMLSYCCEN PIYGETLNPQDKTRCPGGSSGGEGAIIGGGGSMMGIGT DIGGSARIPAEFCGIFSLKTTRGRISSKNYYTPVRGNL AIQGSIGPLARNFDALLLLSQALMSPEMYELDRSLPPLPFDKQK YEKKGPLRIGFYTFDGTFECLPPV	368	XP_055899291.1	584	4E-38

Note: Homology between the ORF of the putative enzymes of the ECS in *L. stagnalis* with orthologues from different organisms. For each contig, are reported the ID, the contig length (bp), the FX_ value corresponding to *L. stagnalis* TSA, and the RefSeq protein ID identified in *B.glabrata*, *A. californica*, *M. musculus*, and *H. sapiens* with the corresponding gene definition.

Abbreviations: ECS, endocannabinoid system; ORF, Open Reading Frame.

Kelley, 2007; Yarar, 2021). We recently demonstrated that snails respond to LPS (Rivi et al., 2022b).

This immune challenge induces a significant upregulation of immune- and stress-related genes in snails' central ring ganglia (Rivi, Batabyal, Benatti, et al., 2022; Rivi, Batabyal, Benatti, Tascedda, et al., 2023; Rivi, Benatti, Rigillo, & Blom, 2023), peaking at 2–6 h post-injection and returning to basal levels after 24 h (Rivi et al., under review) similar to what observed in mice (Alboni et al., 2020; Benatti et al., 2019; Tortoriello et al., 2021).

Accumulating evidence also reports the involvement of the ECS in anxiety and fear enabling organisms to adapt to their changing environments (Lutz et al., 2015). Humans are not the only species that experience such emotional states but currently, there have been studies to show that all species, from vertebrates to invertebrates, might

	Aplysia californica				Mus musculus				Homo sapiens			
% identities	Accession	Aa size	E value	% identities	Accession	Aa size	E value	% identities	Accession	Aa size	E value	% identities
59.62%	XP_012940156.1	1249	0	47.94%	NP_932782.2	1044	1.00E-15	36.09%	NP_006124.1	1042	2.00E-15	35.61%
71.13%	XP_005090870.1	294	7.00E-140	65.95%	NP_035974.1	303	2.00E-82	42.34%	NP_009214.1	313	1.00E-76	41.39%
73.15%	XP_005109823.1	340	0	70.87%	NP_001346893.1	404	4.00E-126	52.20%	NP_001116310.1	393	3E-130	52.45%
44.76%	XP_005108181.3	284	1E-17	62.50%	NP_034303.3	579	1.00E-72	42.61%	NP_001432.2	532	8.00E-70	41.88%

experience central emotional states such as anxiety, arousal, and fear among others, that have associated behavioral and physiological responses (Anderson & Adolphs, 2014; Berridge & Kringelbach, 2013; Zanette & Clinchy, 2020). Although most organisms might not be able to consciously be aware of such states which is still a matter of debate, we can at least show evolutionary similarities between the expression and function of such states across invertebrates to humans (Bateson et al., 2011; Zhu et al., 2011). The current focus of this study is thus on characterizing such a conserved physiological system in humans that connects such emotional states and might also help untangle the cause-effect debate about behavioral responses and physiological correlates of the brain that lead to such responses. Laboratory-inbred snails—that have never experienced a natural predator—show innate anti-predatory behaviors in the presence of the effluent of a historically sympatric predator [i.e., crayfish effluent (CE)] suggestive of stress-induced anxiety-like behaviors (Kita et al., 2011). When snails detect CE, they increase aerial respiration, exploratory/searching phase, and sensitivity to the shadow-elicited full-body withdrawal response (Forest et al., 2016; Orr et al., 2007), while decreasing both their righting response time when dislodged from the substratum and their basal cutaneous oxygen consumption (Batabyal et al., 2021, 2022; Dalesman et al., 2007; Il-Han et al., 2010; Orr et al., 2007; Orr & Lukowiak, 2010). Similarly, we found that the predator scent can be used to induce configural learning, a form of higher-order associative learning, in *Lymnaea* (Batabyal et al., 2021; Kagan & Lukowiak, 2019; Rivi, Batabyal, Benatti, Blom, et al., 2023; Rivi, Benatti, Lukowiak, et al., 2021; Swinton et al., 2019).

Another emerging role for ECS is its involvement in food intake (Aguilera Vasquez & Nielsen, 2022; Galles et al., 2018). Different lengths of food deprivation differently affect snails' behavior and learning and memory abilities (Ito et al., 2015; Kagan et al., 2022, 2023; Ito et al., 2017; Rivi, Benatti, Actis et al., 2022; Totani et al., 2019). In particular, animals food-deprived for 1 day before training are better learners than the ad libitum-fed ones; while 5 days of food deprivation impairs the expression of the formed memory (Aonuma et al., 2018; Rivi, Benatti, Actis et al., 2022; Totani et al., 2020).

Finally, because studies from mammals indicate that the ECS acts as both a regulator and an effector of the acute stress response (Longaretti et al., 2020; Lutz et al., 2015), we investigated the transcriptional effects induced by acute stress, as a heat shock, would affect the expression levels of the ECS enzymes.

Exposure to 30°C for 1 h is perceived by snails as a significant stressor (Fernell et al., 2021; Foster et al., 2015; Rivi, Batabyal, Benatti, Blom, et al., 2023; Teskey et al., 2012), that induces a time-related up-regulation of the mRNA levels of heat-shock protein HSP40 and HSP70 in snails' central ring ganglia peaking 2 and 4 h later (Foster et al., 2015; Teskey et al., 2012).

Against this background, we decided to evaluate the expression levels of enzymes of the ECS in the central ring ganglia of *L. stagnalis* experiencing a series of stressors: (1) systemic injection with 25 μg of *Escherichia coli*-derived lipopolysaccharide (LPS), (2) exposure to predator scent (crayfish effluent, 45 min), (3) varying periods of food deprivation (1 and 5 days), and a (4) acute heat shock (30°C for 1 h).

2 | MATERIALS AND METHODS

2.1 | Snails and animal maintenance

In this study, we used a laboratory-inbred strain (i.e., W-strain) of *Lymnaea stagnalis* (Linnaeus 1758) housed at the University of Calgary Biology Department. This strain originated from an inbred stock maintained at the Vrije University of Amsterdam and was originally bred from animals collected in the 1950s in polders near Utrecht, The Netherlands. The snails were housed in artificial pond water, which consisted of deionized water supplemented with Instant Ocean (Spectrum Brands, Madison, WI, USA) at a concentration of 0.25 g/L. The snails were maintained under controlled conditions at a temperature of 20 ± 1°C, following a light–dark cycle of 16 h of light and 8 h of darkness. Six-month-old snails having shell lengths of 20–25 mm were used in these experiments.

Ethical approval is not required for research work with *L. stagnalis*; however, every effort was made to ameliorate the suffering of animals, ensuring adequate food, clean oxygenated water, and low-density housing conditions. The LPS treatment and exposures to various stressors (i.e., predator effluent, heat shock, and food deprivation) have proven to have no long-term effects on snails (personal observations).

2.2 | Identification of putative endocannabinoid pathway genes in the *L. stagnalis* genome

To identify putative ECS enzymes in *L. stagnalis*'s central ring ganglia, we made use of the contig annotation table generated by (Cristina et al., 2022). To identify the exact sequence of transcripts annotated to the endocannabinoid pathway, we manually extracted putative exonic sequences visualizing RNA-seq reads on the *L. stagnalis* genome with Integrative Genomics Viewer (IGV, http://www.broadinstitute.org/igv/). Then, we aligned these sequences to the transcriptome shotgun assembly of *L. stagnalis* using BLASTn to retrieve the precise mRNA sequence (Table 1).

2.3 | Qualitative PCR analysis and sequencing

To confirm whether these transcripts were expressed in the *L. stagnalis* CNS, we performed qualitative PCR on each transcript using the set of primers listed in Table 2. The primer sequences were designed by NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesized by Merck KGaA (Darmstadt, Germany). Qualitative PCR was performed using DreamTaq DNA polymerase (Thermo Scientific, Waltham, Massachusetts, United States) under the general 3-step amplification of 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s; 72°C for 30 s, and final extension of 72°C for 7 min.

TABLE 2 Validated primers for sequencing.

Match on	Predicted enzyme	Position on transcript		Primer sequence
FX181219.1	*LymDAGL-like* diacylglycerol lipase	FW	2119	CACCATTATCCCCACCGTGT
		RV	2895	TTTGTGCAGCCACGGAAAAC
		FW	2623	CCATGTGACCCCACAATGGA
		RV	3414	GGAACAGTGAGCTCTGCATT
		FW	3111	GCTACGTGATTACAAGTGCTCC
		RV	3955	TGACACAAGGCACAGATGTC
		FW	3396	ATGCAGAGCTCACTGTTCCA
		RV	4527	CTGAGCTGTGGATCTTCCCC
FX189644.1	*LymMAGL-like* monoglyceride lipase	FW	303	CGACCAGGGCTGTACACTTA
		RV	1151	CCAGAATCATGCCCCAGTGT
		FW	1076	TTCTGACGGATTGCCTGGTC
		RV	1406	GTGAAGGTTCTCGCATGCAC
		FW	1393	GCGAGAACCTTCACTTTGTCC
		RV	1777	TTTACATACACCGTCGGCCA
FX191515.1	*LymNAPE-PLD like* N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D	FW	352	AAGCCCCAATCGGAATGGTT
		RV	1082	TGGAAGGTACCAGAACCCCT
		FW	866	CAGCTGGACAAGGGATGAGG
		RV	1364	GGCGGGCTAAGCTAAGTTGT
FX195089.1	*LymFAAH-like* fatty acid amide hydrolase	FW	20	CACTTTGAACCCCCTTCGTC
		RV	707	GCAACACAGTCATCAGTGGC
		FW	435	AGAAGCCCTTCACTCTGGTG
		RV	900	AATGCCCATCATGGAACCCC
		FW	726	ACATGGGGCCATTCCCTTTG
		RV	1126	GCAGTGATCGATCCAGCTCAT

Note: For each predicted enzyme of the endocannabinoid system are reported the relative transcript FX_, the forward (FW), and reverse (RV) primers' sequences, with the corresponding position on the gene.

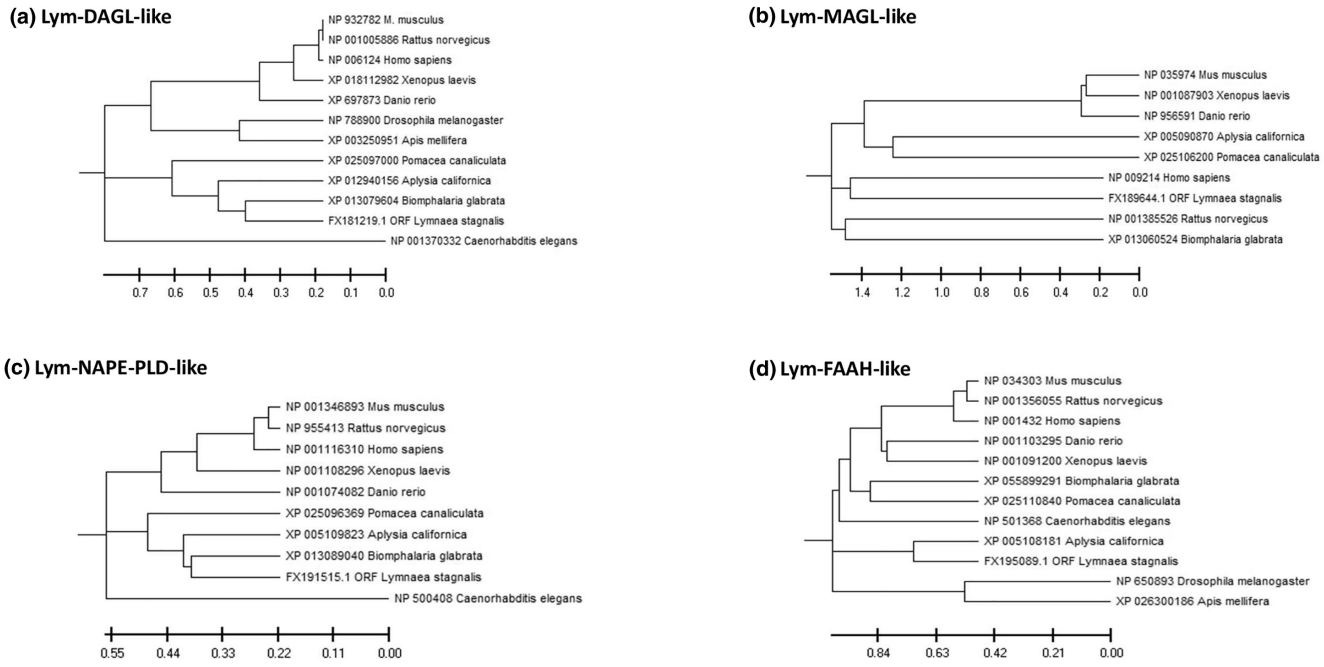

FIGURE 1 Molecular phylogenetic tree of the putative enzymes of the ECS in *Lymnaea stagnalis*. (a) LymDAGL-like, (b) LymMAGL-like, (c) LymNAPE-PLD-like, and (d) LymFAAH-like. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA X. The GenBank accession numbers of proteins used are indicated and listed in Table S1.

Amplicons were electrophoresed on agarose gel (2%), and DNA fragments were visualized by UV illumination to confirm the correct amplicon size. PCR products (600–800 bp) were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics Corporation, USA) following the manufacturer's instructions and were directly sequenced using the Sanger sequencing method. Sequencing was performed using ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, California, USA) and BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies Corporation, Massachusetts, USA). The sequence analysis of the PCR fragments was performed using Sequence Scanner Software 2.0 (Applied Biosystems, California, USA), and sequences were compared with the contigs of *L. stagnalis* using the online version of BLASTn.

2.4 | Sequence and phylogenetic analysis

We predicted the amino acid sequences using the Open Reading Frame (ORF) Finder tool of NCBI (https://www.ncbi.nlm.nih.gov/orffinder/) (Table 1). To determine sequence evolutionary organization and distribution, as well as to provide further evidence of the presence of putative proteins in *L. stagnalis*, we performed a phylogenetic analysis using the UPGMA method 54 of Molecular Evolutionary Genetic Analysis (MEGA)-X tool (https://www.megasoftware.net/). Specifically, we used the amino acid sequences of *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio*, *Aplysia californica*, *Biomphalaria glabrata*, *Pomacea canaliculata*, *Xenopus laevis*, *Drosophila melanogaster*, *Apis mellifera*, and *Caenorhabditis elegans* obtained from NCBI (Table S1). The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site (Figure 1). The predicted amino acid sequences of *L. stagnalis* KP enzymes were aligned with those of *H. sapiens*, *M. musculus*, *R. norvegicus*, *D. rerio*, *A. californica*, *B. glabrata*, *P. canaliculata*, *X. laevis*, *D. melanogaster*, *A. mellifera*, and *C. elegans* using T-Coffee tool and visualized with BoxShade as previously reported (Cristina et al., 2022).

2.5 | Design, validation, and optimization of primers for quantitative RT-PCR analysis

Candidate primers for quantitative Real-Time PCR (qRT-PCR) were designed with NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Merck KGaA (Darmstadt, Germany). Primers were designed to have a length of 20 nucleotides, a melting temperature between 58 and 62°C, a GC content between 40% and 60%, and generate an amplicon between 168 and 312 bp (Table 3).

Primer specificity was assessed by qualitative PCR as previously described (Kagan et al., 2023; Rivi, Batabyal, Wiley et al., 2022). Each experiment contained two biological replicates of cDNA from the central ring ganglia of *L. stagnalis*, minus reverse transcription

TABLE 3 Validated primers for gene expression analysis.

Target	Transcript	Primer FW sequence	Primer RV sequence	Size (bp)	Efficiency	R^2	Ct value 20 ng
LymDAGL-like Diacylglycerol Lipase	FX181219.1	CCATGTGACCCCACAATGGA	TTTGTGCAGCCACGGAAAAC	272 bp (2623–2895)	104%	0.98	27.27
LymMAGL-like Monoglyceride Lipase	FX189644.1	TTCTGACGGATTGCCTGGTC	ACTCAATGGGAGGTGCAGTG	216 bp (1076–1292)	99.04%	0.99	25.29
LymNAPE-PLD-Like N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D	FX191515.1	CCATGTTTCCCAGGGGTTCT	GGCGGGCTAAGCTAAGTTGT	312 bp (1052–1364)	96.32%	0.99	28.57
LymFAAH-like Fatty Acid Amide Hydrolase	FX195089.1	CCCTCAAGACAAGACGCGAT	CTAATGCGCCCCCTAGTTGT	168 bp (815–983)	118.47%	0.99	28.42
LymβTUB Snail mRNA fragment for β-tubulin	X15542.1	GAAATAGCACCGCCATCC	CGCCTCTGTGAACTCCATCT	127 bp (92–219)	99.88%	0.99	18.9
LymEF1α Elongation factor 1α	DQ278441.1	GTGTAAGCAGCCCTCGAACT	TTCGCTCATCAATACCACCA	150 bp (7–157)	99.97%	0.99	17.19

Note: For each predicted enzyme of the endocannabinoid system and the housekeeping genes elongation factor 1-α, LymEF1α, and β-tubulin, LymTUB are reported the relative transcript FX_, the forward (FW) and reverse (RV) primers' sequences, with the corresponding size (bp), efficiency, R^2 score resulting from the validation experiments, and the Ct value obtained with 20 ng of cDNA.

controls to assess the genomic DNA and non-template controls. PCR products were run as previously described. Then primer efficiency was evaluated by Bio-Rad CFX Connect. Curves were generated from four-fold serial dilutions (1:4 from 40 to 0.625) of cDNA run in triplicate. Amplification of all genes was detected with SyBR Green dye which generates fluorescence based on the synthesis of double-stranded DNA. The reactions contained 5 μL of cDNA with 10 μL of Bio-Rad SsoAdvanced Universal SyBR Mix, 300 nM forward and reverse primer concentration, and topped to 20 μL with double distilled water. The qPCR reactions were performed using a Bio-Rad CFX Connect thermocycler. The custom qPCR program consisted of 95°C for 30 s, 40 cycles of 95°C for 15 s, and 60°C for 30 s.

2.6 | Transcriptional regulation of the expression levels of ECS enzymes

Once we identified and characterized ECS putative enzymes in *Lymnaea*'s central ring ganglia, we then investigated their transcriptional effects induced by different stressful stimuli: bacterial LPS-induced immune response, predator cue (i.e., crayfish effluent), moderate and severe food deprivation (i.e., 1 and 5 days, respectively), and acute heat shock exposure.

2.6.1 | LPS treatment

Forty-five adult snails were divided into two groups receiving either: *Lymnaea* saline (41.15 mM NaCl; 0.54 mM KCl; 3.55 mM $CaCl_2$; 2.61 mM $MgCl_2$; 5 mM Tris; pH 7.5; Benatti, 2017); or 25 μg (estimated concentration 6.25 μg/mL) of *Escherichia coli*-derived LPS serotype O127:B8 (L3129) dissolved in *Lymnaea* saline (Sigma-Aldrich, USA) (Rivi, Batabyal, Benatti, Tascedda, et al., 2023).

The calculated volume of hemolymph in a snail with a 20-mm shell length was 400 μL (Murakami et al., 2013), a single injection of 40 μL was performed intramuscularly in the foot of the snail using a 31G syringe. We know from previous studies that this LPS dose is sufficient to induce a sickness-like behavior (Rivi et al., 2022b; Rivi, Batabyal, Benatti, Blom, et al., 2023; Rivi, Batabyal, Benatti, Tascedda, et al., 2023). Thirty snails were injected with LPS, and 15 snails were injected with snail saline. Ten LPS-injected snails and their relative five sham-injected controls were killed 2 h after the injection, 10 LPS-injected snails and their relative five sham-injected controls were killed 6 h after the injection, while the remaining snails were killed after 24 h. A group of five uninjected snails was killed as controls.

2.6.2 | Predator cue exposure

Crayfish are natural predators of *Lymnaea* and the laboratory-bred W-strain of *L. stagnalis* used in this study detects and innately recognizes crayfish as predators, responding to them with multiple anti-predator behaviors (Dalesman et al., 2007; Orr et al., 2007). Here we used the crayfish effluent (CE, i.e., pond water from the crayfish aquarium) as our predation cue. We used a freshly collected 7.5 cm crayfish (*Faxonius virilis*; Hagen 1870) from Whitesand Lake (a pond in Saskatchewan, Canada) and housed it in a 70-L aquarium containing artificial pond water. The crayfish was fed a diet of lettuce and snails and was maintained in the aquarium for over 6 months before the current study commenced. Eight snails were moved from their home aquaria and placed into a 1 L beaker containing 500 mL of crayfish effluent. 45 min later, animals returned to their home aquaria for 3 h, before being killed. Control snails were placed into a 1 L beaker containing 500 mL of clean pond water for 1 h.

2.6.3 | Short- and long-term food deprivation

Here, seven naïve snails had ad libitum access to food, eight snails were food-deprived for 1 day, and eight snails were food-deprived for 5 days before being killed. All these fasting regimens have been previously successfully used (Sugai et al., 2006, 2007). Thus, we defined snails food deprived for 1 day as "moderately hungry," whereas those fasting for 5 days as "severely hungry snails" (Kagan et al., 2023; Rivi, Benatti, Actis et al., 2022).

2.6.4 | Acute heat shock stress

Seven naïve snails were placed in a 1-L beaker filled with 500 mL of artificial pond water which was previously heated to 30°C. The beaker containing *Lymnaea* ($n = 7$) was then maintained in a water bath at 30°C for 1 h. We know from previous studies that the exposure of snails to 30°C for 1 h represents severe acute stress (Batabyal et al., 2024; Foster et al., 2015; Rivi, Batabyal, Benatti, Blom, et al., 2023; Rivi, Batabyal, Benatti, et al., 2022; Rivi et al., 2022a). Control snails ($N = 7$) were placed in a 1-L beaker filled with 500 mL of 20°C (i.e., room temperature) artificial pond water. After that, animals returned to their home aquaria for 3 h, before being killed.

2.7 | Total RNA extraction, reverse transcription, and real-time PCR

Snails were killed following the standard procedure that has been extensively used before: first animals were fully anesthetized by placing them on ice until they do not respond to external stimuli and do not release hemolymph as a defensive behavior (usually for 10 min), then the central ring ganglia (buccal ganglia were excluded) were quickly dissected and stored at −80°C before analysis. Total RNA extraction and DNAse treatment were performed using GenEluteTM Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Merck Millipore) as previously described (Rivi, Batabyal, Benatti, Tascedda, et al., 2023). For each snail, the central ring ganglia were collected and used for total RNA extraction. Seven samples were analyzed for each group. A 200-ng sample of total RNA was reverse

transcribed with a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). Quantitative RT-PCR was carried out on 20 ng of cDNA using a Bio-Rad® CFX ConnectTM Real-Time PCR Detection System with SYBR Green Master Mix (Bio-Rad). The cycling parameters were 95°C for 2 min and 95°C for 10 s, 60°C for 30 s for 40 cycles, and a dissociation curve analysis followed the amplification. Cycle threshold (Ct) values were determined by CFX MaestroTM Software (Bio-Rad). Dissociation curve analysis and electrophoresis in 2% agarose gel excluded the presence of different PCR products or primer dimers. We used two reference genes (elongation factor 1-α, LymEF1α, and β-tubulin, LymTUB), and their stability was assessed using Normfinder® (https://moma.dk/normfinder-software), taking into account intra and intergroup variation. LymTUB was the most stable gene across groups and was used for gene normalization as a calibrator. The endogenous control mRNA levels were not affected by any procedure [one-way analysis of variance (ANOVA)] and the amplification efficiency of the target genes and the endogenous control gene was approximately equal. The comparative $2^{-\Delta\Delta Ct}$ method was performed using as a calibrator the average levels of expression of control animals according to each experiment.

2.8 | Statistical analyses

Firstly, the sample size for the experiments performed in this study is $n = 7$–10. The calculated effect size for this experiment is 0.77. We calculated this using the formula for Friedman's test: $W = X^2/N(K-1)$; where W is Kendall's W value; X^2 is the Friedman test statistic value; N is the sample size and K is the number of repeats. Moreover, G*power software, allowed us to determine statistical power with alpha = 0.05, power = 0.80 and a medium effect size ($d = 0.5$). Our data were then analyzed for normality assumption using Kolmogorov–Smirnov one-sample test for normality (K-S distance and P): all targets displayed a normal distribution. To study the transcriptional effects of LPS on the expression levels of the enzymes of the ECS in snails' central ring ganglia, we performed a two-way ANOVA for the main effects of the treatment (saline or LPS injection), time (2, 6, and 24 h), or interaction between the two factors. To follow up on significant interactions we performed planned pairwise comparisons by Tukey's post-hoc tests. To study the transcriptional effects on each target in ad libitum-*fed* snails and those food-deprived for 1 or 5 days we performed one-way ANOVA followed by Tukey's post-hoc tests. Transcriptional effects induced by the exposure to the predator effluent (Figure 3) or to a heat shock stressor (Figure 5) were examined by Student's *t*-test. No exclusion criteria were pre-determined, and no animals were excluded from the study. Extreme outliers were excluded before statistical analysis using the boxplot tool in SPSS (instances more than three times the interquartile range outside the end of the interquartile box). All statistical analyses were performed using SPSS software ver. 26.0 (IBM Corp., Armonk, NY, USA), whereas graphs were created using GraphPad Prism ver. 9.00e for Windows® (GraphPad Software, Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | Identification and characterization of putative transcripts of the endocannabinoids' metabolic enzymes in *L. stagnalis*

Using the contig annotation table generated by (Cristina et al., 2022), we identified four *L. stagnalis* contigs annotated to the four genes of the endocannabinoids' pathway in *B. glabrata*. To identify transcripts in these contigs, we first manually extracted the putative exonic sequences visualizing the RNA-seq reads with the Integrative Genomics Viewer. Then, we retrieved the precise mRNA sequence aligning these putative exonic sequences to the transcriptome shotgun assembly of *L. stagnalis*. All transcripts resulted in being expressed (TPM > 0) in the publicly available *L. stagnalis* CNS transcriptome. To confirm whether these transcripts were expressed in the *L. stagnalis* CNS, we performed qualitative PCR on each transcript using the set of primers listed in Table 3. The PCR products were purified, sequenced, and aligned to FX181219.1 (LymDAGL-like), FX189644.1 (LymMAGL-like), FX191515.1 (LymNAPE-PLD-like), and FX195089.1 (LymFAAH-like). All sequences uniquely matched their respective templates. All transcripts contained at least one ORF (Table 1), whose identity was further confirmed by blasting each sequence with the amino acid sequences of the corresponding enzyme from *H. sapiens*, *M. musculus*, *A. californica*, and *B. glabrata*. In *H. sapiens*, we identified four orthologs that had an average homology of about 40% with the corresponding sequence of *L. stagnalis* (ranging from 35.61% for LymDAGL-like to 52.45%, for LymNAPE-PLD-like). A similar degree of homology was found with *M. musculus* sequences (ranging from 36.09% for LymDAGL-like to 52.20%, for LymNAPE-PLD-like), while we observed a higher degree of homology for LymNAPE-PLD-like and LymMAGL-like with mollusk sequences (i.e., 70.87% and 65.95% for *A. californica* and 73.15% and 71.13% for *B. glabrata*, respectively) (Table 1). Phylogenetic analyses revealed a predictable pattern in the relatedness of *L. stagnalis* sequences of LymDAGL-like, LymFAAH-like, and LymNAPE-PLD-like to those of Mollusca (Figure 1). Interestingly we found that LymMAGL-like aminoacidic sequences were closely related to those of humans. This conservation pattern was also confirmed using multiple sequence alignment between each putative enzyme identified in *L. stagnalis* and orthologues from other species, mainly used as model organisms in translational neuroscience (Figure S1).

3.2 | Primer specificity, efficiency, and validation

Real-time PCR was used to evaluate the expression levels of the identified *L. stagnalis* ECS-enzymes. All gene-specific primers for the analysis are shown in Table 3. All primers produced consistent results without amplifying off-target products or generating primer dimers. Following amplification, each primer pair produced amplicons that yielded single bands at the correct size after electrophoresis in 2% agarose gels. Primer specificity was also checked by melt curve analysis. A single sharp peak with no primer-dimer was observed for

all used primer pairs (data not shown). Our results showed that the PCR efficiency was between 96% and 118%, and the R^2 of primers was greater than 0.94. In addition, the mean Cq of all targets ranged from 25.29 to 29.82 for 20 ng of cDNA with moderately abundant mRNA levels in the ganglia of *L. stagnalis* (Table 3).

3.3 | Transcriptional effects of LPS on the expression levels of ECS enzymes in snails' central ring ganglia

First, the mRNA levels of the ECS enzymes were analyzed at 2, 6, and 24 h post-treatment with 25 μg of LPS (Figure 2). No significant differences were found between saline-exposed animals killed at different times after injection and control uninjected animals for any of the considered targets. A two-way ANOVA showed a significant main effect of LPS for all the enzymes analyzed [LymDAGL-like: $F(1, 13) = 14.07$, $p = 0.002$ (Figure 2a); LymMAGL-like: $F(1, 13) = 42.25$, $p < 0.0001$ (Figure 2b); LymNAPE-PLD-like: $F(1, 13) = 10.28$, $p = 0.007$ (Figure 2c) and LymFAAH-like: $F(1, 13) = 12.86$; $p = 0.007$ (Figure 2d)]. Moreover, a main effect of the time after injection was present for LymDAGL-like [$F(2, 26) = 3.13$, $p = 0.005$], LymFAAH-like [$F(2, 26) = 17.02$; $p = 0.0002$], and LymNAPE-PLD-like [$F(2, 26) = 5.49$; $p = 0.007$].

Interaction among the main factors emerged only for LymMAGL-like [$F(2, 26) = 3.74$, $p = 0.004$] and LymNAPE-PLD-like [$F(2, 26) = 8.06$; $p = 0.002$]. Post-hoc analyses showed that LPS significantly reduced the expression of all ECS enzymes at both 2 and 6 h post-LPS injection (LymDAGL-like: Sal 2 h vs. LPS 2 h: $p = 0.0005$,

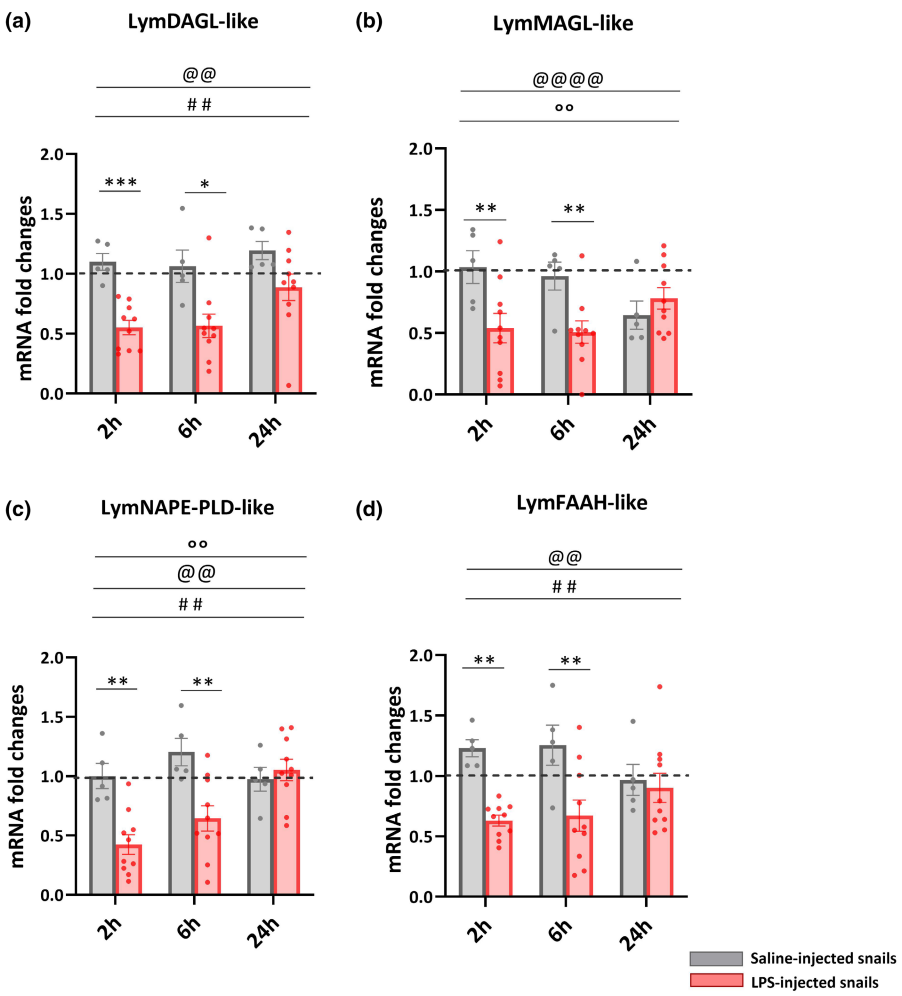

FIGURE 2 Transcriptional effects induced by an immune challenge on the expression levels of the putative enzymes of the endocannabinoid system in the central nervous system of *L. stagnalis*. Adult snails were injected with either snail saline (black bars) or 25 μg of LPS (red bars) and killed 2, 6, or 24 h later. RNA from the central ring ganglia was extracted and retrotranscribed. (a) LymDAGL-like (i.e., the enzyme that catalyzes the synthesis of 2-AG), (b) Lym MAGL-like (i.e., the enzyme that catalyzes 2-AG degradation into arachidonic acid and glycerol), (c) LymNAPE-PLD-like (i.e., the enzyme that catalyzes the synthesis of AEA), and (d) LymFAAH-like (i.e., the enzyme that catalyzes AEA degradation into arachidonic acid and ethanolamine) mRNA expression in the ganglia, with LymTUB as endogenous control, were measured by qRT-PCR. *n*, number of snails = 8–10. The dashed line represents the control group (untreated snails) value set to 1. Data are represented as means ± SEM and were analyzed with Two-way ANOVA Mixed effects followed by Bonferroni post-hoc: main effect of LPS-treatment ($^{@@}p < 0.01$; $^{@@@@}p < 0.0001$); main effect of Time ($^{\#\#}p < 0.01$; $^{\#\#\#\#}p < 0.0001$); interaction LPSxTime ($^{\circ\circ}p < 0.01$). Post-hoc: $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

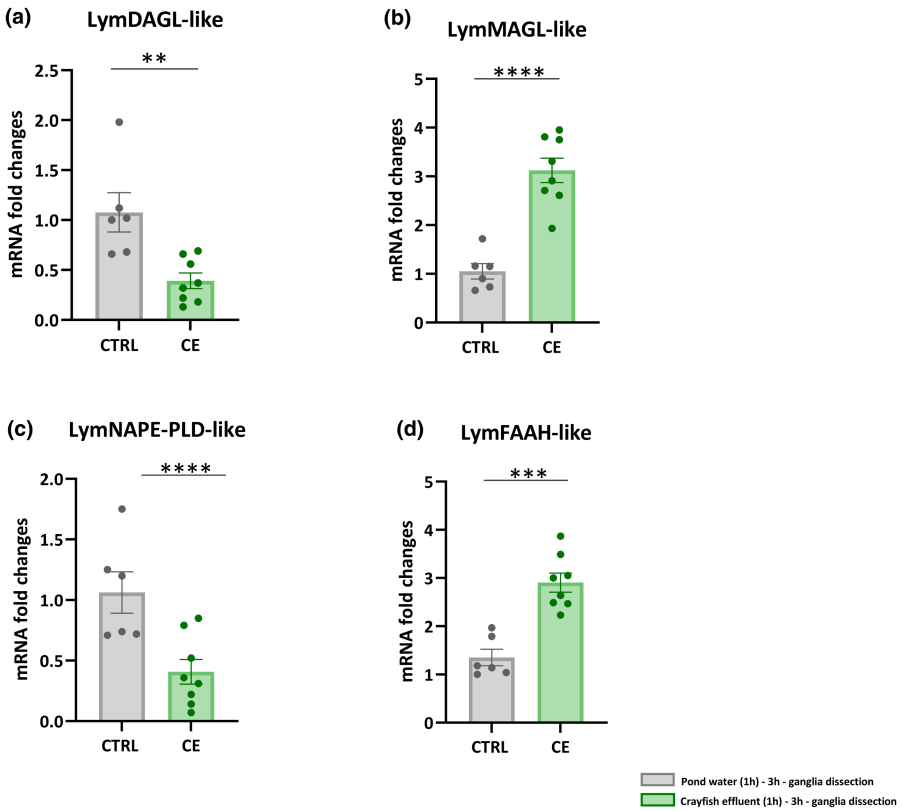

FIGURE 3 Transcriptional effects induced by the exposure to the predator effluent (i.e., crayfish scent) on the expression levels of the putative enzymes of the endocannabinoid system in the central nervous system of *L. stagnalis*. Eight naïve snails were exposed to the crayfish effluent (CE—green bars) for 45 min, whereas their unexposed counterparts (i.e., control (CTRL) snails; *n*, number of snails = 8) were maintained in artificial pond water. Three hours after the exposure, snails were killed, and RNA from the central ring ganglia was extracted and retrotranscribed. (a) LymDAGL-like (i.e., the enzyme that catalyzes the synthesis of 2-AG), (b) Lym MAGL-like (i.e., the enzyme that catalyzes 2-AG degradation into arachidonic acid and glycerol), (c) LymNAPE-PLD-like (i.e., the enzyme that catalyzes the synthesis of AEA), and (d) LymFAAH-like (i.e., the enzyme that catalyzes AEA degradation into arachidonic acid and ethanolamine) mRNA expression in the ganglia, with Lym TUB as endogenous control, were measured by qRT-PCR. Data are represented as means ± SEM and were analyzed with unpaired student *t*-test. $^{****}p < 0.0001$; $^{***}p < 0.001$ and $^{**}p < 0.01$.

Sal 6 h vs. LPS 6 h: $p = 0.05$; LymMAGL-like: Sal 2 h vs. LPS 2 h: $p = 0.001$, Sal 6 h vs. LPS 6 h: $p = 0.003$; LymNAPE-PLD-like: Sal 2 h vs. LPS 2 h: $p = 0.002$; Sal 6 h vs. LPS 6 h: $p = 0.002$; LymFAAH-like: Sal 2 h vs. LPS 2 h: $p = 0.003$, Sal 6 h vs. LPS 6 h: $p = 0.005$). At 24 h post-LPS injection, no differences emerged in gene expression of all targets considered between LPS-injected snails and their saline counterparts.

3.4 | Transcriptional effects of CE on the expression levels of ECS enzymes

Next, the effects of exposure to the predator scent (i.e., CE) on the transcriptional levels of ECS enzymes were evaluated (Figure 3). We found a significant upregulation of mRNA levels of LymMAGL-like (unpaired *t*-test: $t = 6.45$, df = 12, $p < 0.0001$; Figure 3b) and LymFAAH-like (unpaired *t*-test: $t = 5.69$, df = 12, $p = 0.0001$; Figure 3d), and significant downregulation of LymDAGL-like (unpaired *t*-test: $t = 3.59$, df = 12, $p = 0.004$; Figure 3a) and LymNAPE-PLD-like (unpaired *t*-test: $t = 3.47$, df = 12, $p = 0.005$; Figure 3c) in the central ring ganglia of snails exposed to the crayfish effluent for 45 min compared to their unexposed counterparts.

3.5 | Transcriptional effects induced by food deprivation on ECS enzymes expression levels

Further, we investigated the effects of 1-day or 5-day food deprivation on the transcriptional levels of ECS enzymes (Figure 4). A main effect of the feeding regimens was observed for all the targets examined [LymDAGL-like: $F(2, 20) = 29.30$, $p < 0.0001$ (Figure 4a); LymMAGL-like: $F(2, 20) = 12.84$, $p = 0.0003$ (Figure 4b); LymNAPE-PLD-like: $F(2, 20) = 15.25$; $p < 0.0001$ (Figure 4c) and LymFAAH-like: $F(2, 20) = 15.51$; $p < 0.0001$ (Figure 4d)]. Tukey's multiple comparisons tests revealed a significant downregulation of the mRNA levels of LymMAGL-like and LymFAAH-like in both modestly and severely food-deprived snails (LymMAGL-like: ad libitum vs. Day1: $p = 0.013$ and ad libitum vs. Day5: $p = 0.0002$; LymFAAH-like: ad libitum vs. Day1: $p = 0.0006$ and ad libitum vs. Day1: $p = 0.0002$). However, severe food deprivation induced a significant upregulation of the

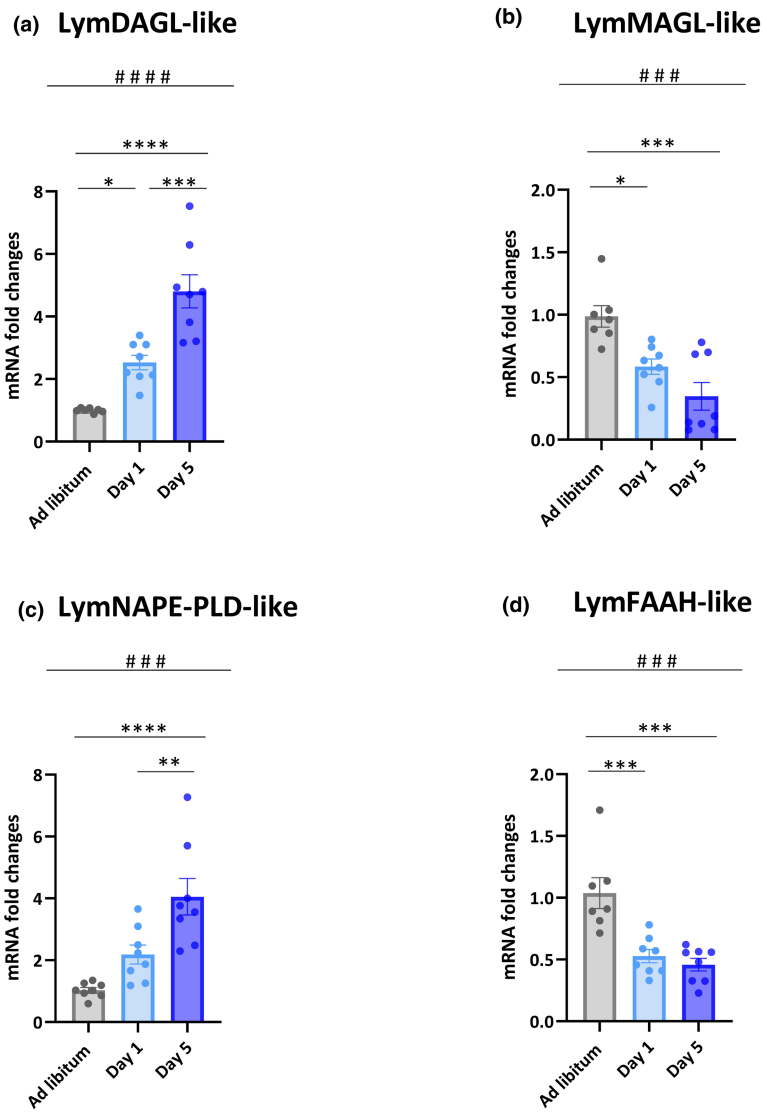

FIGURE 4 Transcriptional effects induced by different lengths of food deprivation on the expression levels of the putative enzymes of the endocannabinoid system in the central nervous system of *L. stagnalis*. The expression levels of (a) LymDAGL-like (i.e., the enzyme that catalyzes the synthesis of 2-AG), (b) Lym MAGL-like (i.e., the enzyme that catalyzes 2-AG degradation into arachidonic acid and glycerol), (c) LymNAPE-PLD-like (i.e., the enzyme that catalyzes the synthesis of AEA), and (d) LymFAAH-like (i.e., the enzyme that catalyzes AEA degradation into arachidonic acid and ethanolamine) have been measured in the central ring ganglia of snails fed ad libitum (gray bars; *n*, number of snails = 7), food-deprived for 1 day (Day 1—light blue bars—*n* = 8), or food-deprived for 5 days (Day 5—blue bars—*n* = 8). Data are represented as means ± SEM and were analyzed with one-way ANOVA followed by Tukey post-hoc analyses: main effect of feeding regimen ($^{###}p < 0.001$; $^{####}p < 0.0001$). Post-hoc: $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

mRNA levels of LymNAPE-PLD-like compared to the ad libitum fed and 1-day food-deprived counterparts ($p < 0.0001$ and $p = 0.007$, respectively). Also, LymDAGL-like was significantly up-regulated following food deprivation: the effect was present in both 1- and 5-day food-deprived snails compared to the ad libitum-*fed* ones ($p = 0.02$ and $p < 0.0001$, respectively), moreover, severely food-deprived snails showed higher levels of this target with respect to their moderately food-deprived counterparts ($p = 0.0004$).

3.6 | Transcriptional effects induced by an acute heat shock on ECS enzymes expression levels

Finally, we studied the transcriptional effects induced by the exposure to 30°C for 1 h on the mRNA levels of ECS enzymes (Figure 5). Statistical analysis revealed a significant upregulation of LymDAGL-like (unpaired *t*-test: $t = 5.09$, df = 12, $p = 0.0003$; Figure 5a) and LymFAAH-like mRNA levels (unpaired *t*-test: $t = 3.18$, df = 12, $p = 0.008$; Figure 5d), while a significant downregulation was detected for LymMAGL-like (unpaired *t*-test: $t = 2.55$, df = 12, $p = 0.0252$; Figure 5b) and LymNAPE-PLD-like mRNA levels (unpaired *t*-test: $t = 3.37$, df = 12, $p = 0.005$; Figure 5c) in the central ring ganglia of snails exposed to the heat shock compared to the unexposed ones.

4 | DISCUSSION

The identification and analysis of ECS-related enzymes in *L. stagnalis* mark an important advancement in our understanding of the ECS across the evolutionary spectrum. This research underscores the evolutionary conservation of the ECS from invertebrates to vertebrates, a demonstration of its fundamental role in various physiological processes including stress responses, energy balance, and immune response to an inflammatory challenge (Hill, McLaughlin, et al., 2010; Hill, Patel, et al., 2010; Lutz et al., 2015). The implications of such conservation are profound, suggesting the potential of the ECS as a therapeutic target for a myriad of conditions (Clarke et al., 2021; Crowe et al., 2014; Katona & Freund, 2012; Viveros et al., 2005).

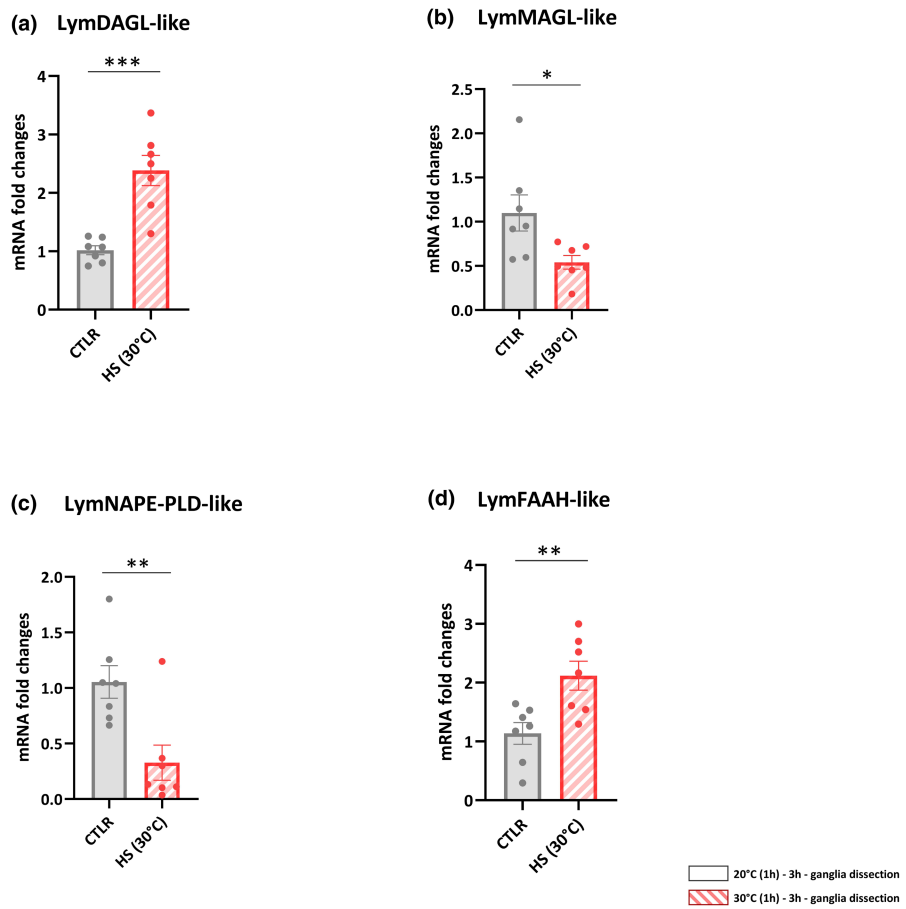

FIGURE 5 Transcriptional effects induced by the exposure to a heat shock stressor (i.e., 30°C for 1 h) on the expression levels of the putative enzymes of the endocannabinoid system in the central nervous system of *L. stagnalis*. Seven naïve snails were exposed to 30°C for 1 h (heat shock [HS]—diagonal red bars), whereas their unexposed counterparts (i.e., control (CTRL) snails; *n*, number of snails = 7) were maintained at room temperature (20°C) in artificial pond water. Three hours after the exposure, snails were killed, and RNA from the central ring ganglia was extracted and retrotranscribed. (a) LymDAGL-like (i.e., the enzyme that catalyzes the synthesis of 2-AG), (b) Lym MAGL-like (i.e., the enzyme that catalyzes 2-AG degradation into arachidonic acid and glycerol), (c) LymNAPE-PLD-like (i.e., the enzyme that catalyzes the synthesis of AEA), and (d) LymFAAH-like (i.e., the enzyme that catalyzes AEA degradation into arachidonic acid and ethanolamine) mRNA expression in the ganglia, with Lym TUB as endogenous control, were measured by qRT-PCR. Data are represented as means ± SEM and were analyzed with unpaired student *t*-test. $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

The ECS encompasses cannabinoid receptors, endogenous metabolites, and enzymes that regulate functions across the nervous, endocrine, and immune systems. The system's imbalance is linked to various disorders, emphasizing the importance of its study for health-related interventions (Mir et al., 2023; Seghetti et al., 2022).

The characterization of putative CB1 and CB2 receptors in *L. stagnalis* by Sunada et al. (2017) and their proven involvement in learning and memory processes further underscores the system's complexity and functional significance (Sunada et al., 2017). To address the challenge of limited functional and pharmacological understanding of conserved genes and transcripts within this model organism (Feng et al., 2009; Murakami et al., 2013; Yang et al., 2021), we utilized the dataset provided by Benatti et al. (2022) to identify *L. stagnalis* contigs that correspond to genes encoding ECS enzymes in the annotated genome of *B. glabrata* (Cristina et al., 2022). Each enzyme was distinctly identified, with contigs precisely aligning with the predicted transcripts in the CNS of *L. stagnalis*. These identified transcripts all contained ORFs, with the encoded proteins sharing significant amino acid similarity with those of humans and orthologues in widely used preclinical model organisms, such as *M. musculus*, *A. californica*, and *B. glabrata*. Phylogenetic analysis and multiple alignments demonstrated a high level of sequence conservation between *L. stagnalis* and other gastropods and model organisms (Elphick, 2007; McPartland et al., 2006), further validated through Sanger sequencing of the snails' central ring ganglia.

Acknowledging the pivotal role of the ECS in mediating the interaction between the perception of external and internal stimuli and various neurophysiological and behavioral outcomes, such as inflammation, fear, anxiety, and stress responses, we explored how different stressors (Cavener et al., 2018; Gunduz-Cinar, 2021; Lutz et al., 2015; Ruehle et al., 2012)—LPS, predator cues, food deprivation, and heat shock—affect the transcriptional expression of ECS enzymes in the central ring ganglia of *L. stagnalis*.

The response to each stressor was unique, indicating that the expression of ECS enzymes is specifically modulated according to the nature of the stress encountered. These findings not only underscore the vital role of the ECS in environmental adaptation and stress physiology but also illuminate the intricate workings of this biochemical pathway to affect central emotional states. With mounting evidence supporting the role of endocannabinoids in modulating fear and stress through their dynamic synthesis and breakdown (Ahmed et al., 2022), we explored how the expression of ECS enzymes responds to fear and anxiety stimuli by subjecting snails to CE. This exposure led to a significant increase in LymFAAH mRNA levels in snails' central ring ganglia, indicating a crucial role for FAAH, the enzyme responsible for breaking down AEA, in adapting to anxiety and stress (Spagnolo et al., 2016). Consistently, FAAH-deficient transgenic mice exhibit less anxiety-like behavior (Moreira et al., 2008; Spagnolo et al., 2016). As gene expression changes do not directly imply shifts in enzyme activity or protein abundance, further research is required to determine if predator-induced stress and anxiety could decrease AEA levels through enhanced LymFAAH activity.

Furthermore, we found a significant upregulation of LymMAGL in the central ring ganglia of snails exposed to CE, hinting at the increased degradation of 2-AG in CNS. Our results are supported by data from rodents reporting that the impairment of 2-AG signaling in hippocampal glutamatergic neurons by viral overexpression of MAGL also decreased 2-AG levels and increased anxiety-like behavior (Guggenhuber et al., 2015). Conversely, pharmacological inhibition of MAGL produces anxiolytic effects under basal conditions, and increased aversive conditions (Manduca et al., 2015; Pavón et al., 2021). Moreover, it has recently been reported that a deficiency of DAGLα or NAPE-PLD caused a marked decrease in 2-AG brain levels and increased anxiety-like behavior in mice (Lutz et al., 2015; Shonesy et al., 2018). The observed upregulation of LymMAGL, alongside the reduction of LymDAGL and LymNAPE-PLD expression in snails exposed to predator scent, suggests a potential increase in 2-AG degradation within the CNS. This phenomenon in mammals correlates with heightened anxiety-like behaviors (Lutz et al., 2015; Shonesy et al., 2018).

Growing evidence is emerging on the role of the ECS in modulating stress responses, with impaired ECS signaling implicated in stress-related disorders like anxiety (Bambico et al., 2009; Cavener et al., 2018; Griebel et al., 2005; Haj-Dahmane & Shen, 2011). The modulation of stress-related behaviors by the ECS is believed to occur, at least in part, through the regulation of the serotoninergic system (Bambico et al., 2009; Cavener et al., 2018; Griebel et al., 2005; Haj-Dahmane & Shen, 2011). While the interplay between the ECS signaling and the serotoninergic system influencing mood, anxiety, and other stress-related behaviors is intricate and multifaceted, it seems to be highly conserved (Stampanoni Bassi et al., 2017). Data emerging from this study are consistent with our previous data showing that the exposure of snails to the predator scent induces a significant upregulation of key targets for the serotoninergic system, as well as HSP70, in snails central ring ganglia (Batabyal & Lukowiak, 2021; Rivi, Batabyal, Wiley et al., 2022). Thus, the serotoninergic and endocannabinoid systems may represent conserved mechanisms involved in the modulation of stress-induced arousal and vigilance behaviors in snails as well as in mammals (Batabyal et al., 2024; Benatti et al., 2017; Il-Han et al., 2010).

The role of the ECS in regulating metabolic balance, influencing hunger, feeding behaviors, and nutrient assimilation in mammals, has become increasingly evident (Aguilera Vasquez & Nielsen, 2022; Jager & Witkamp, 2014; Li et al., 2011; Watkins & Kim, 2014). In particular, the levels of 2-AG and AEA have proven to be closely linked with the stimulation of hunger and food intake (Aguilera Vasquez & Nielsen, 2022; Jager & Witkamp, 2014; Watkins & Kim, 2014). Furthermore, an overactive ECS is suggested to play a role in obesity, with studies highlighting a correlation between elevated endogenous cannabinoid levels and obesity (Matheson et al., 2021; Richey & Woolcott, 2017). In *L. stagnalis*, experiencing food deprivation led to a notable increase in the mRNA levels of LymDAGL and LymNAPE-PLD and a decrease in LymMAGL and LymFAAH within the central ring ganglia of the snails.

Considering the catabolic functions of MAGL and FAAH and synthetic functions of DAGL and NAPE-PLD of 2-AG and AEA respectively, our data suggest that food deprivation may lead to a rise in the production of both 2-AG and AEA. This hypothesis aligns with rodent studies where food deprivation was shown to elevate endocannabinoids levels in the nucleus accumbens and hypothalamus (Kirkham et al., 2002), and food consumption was found to be influenced by FAAH-mediated AEA signaling (Kirkham et al., 2002) and that food intake is regulated by FAAH-mediated AEA signaling (Pucci et al., 2022). The transcriptional effects induced by different lengths of food deprivation suggest that extended periods of fasting may be linked with the snails' efforts to maintain energy equilibrium.

Exploring the transcriptional effects induced by various stressors on the expression of ECS enzymes in *L. stagnalis* central ring ganglia sheds light on the ECS's critical role in environmental adaptation and stress physiology (Lutz et al., 2015). The specific patterns of regulation observed under different stress conditions reflect the complexity of the ECS pathway and its involvement in stress response mechanisms. Exposure to acute heat shock—which is perceived as severe stress by lab-inbred snails (Rivi et al., 2022a; Rivi, Batabyal, Benatti, 2022)—significantly increased the expression levels of LymDAGL and LymFAAH, while notably decreasing LymMAGL and LymNAPE-PLD mRNA levels in snails' central ring ganglia. In rodents, similar acute stress conditions lead to lowered AEA levels in the hippocampus, primarily through enhanced hydrolysis mediated by FAAH (Gunduz-Cinar, 2021; Spagnolo et al., 2016), along with a reduction in MAGL activity (Morena et al., 2016). Although future research is necessary to ascertain if these transcriptional changes in *L. stagnalis* correlate with decreased AEA signaling and a potential increase in 2-AG signaling, our findings remark on the importance of endocannabinoid signaling at multiple levels as a regulator of stress

response and adaptation and underscore the conserved role of the ECS in the neurobiology of stress.

Finally, in previous studies we found that administering 6.25 mg/kg of LPS has been shown to trigger an inflammatory response in *L. stagnalis*, affecting both immune-related targets and enzymes involved in the kynurenine pathway (Rivi, Benatti, Rigillo, & Blom, 2023; Rivi et al., under review). Here, we found a significant decrease in the expression of LymMAGL, LymDAGL, LymNAPE-PLD, and Lym-FAAH within the snail ganglia following LPS exposure, effects that persist for up to 6 h but not beyond 24 h.

These findings align with previous research conducted on an in vitro microglial model, where a similar transcriptional suppression of DAGL, FAAH, and NAPE-PLD enzymes was observed after 2 h of LPS exposure (Borgonetti et al., 2022). Moreover, studies from mammals demonstrated that LPS promotes inflammation by suppressing NAPE-PLD expression (Zhu et al., 2011) and highlighted that the DAGL-MAGL axis is responsible to a large extent for the free arachidonic acid pool in the brain, which in turn, plays a key role in the regulation of neuro-inflammatory responses (Grabner et al., 2017; Mulvihill & Nomura, 2013). Furthermore, the genetic deletion or pharmacological inhibition of DAGL has proven to exert a protective effect against LPS-induced inflammatory responses in rodents, preventing the DAGL-induced synthesis of arachidonic acid and downstream prostanoids that mediate inflammatory macrophage responses (Zhang et al., 2020). The LPS-induced downregulation of LymFAAH in snails' central ring ganglia is consistent with studies from rodents, showing that inhibiting the degradation of AEA through the administration of FAAH inhibitors results in anti-inflammatory effects (Vecchiarelli et al., 2021) and decreased responses to LPS administration (Naidu et al., 2010). On the contrary, here we found that LPS injection induces a significant downregulation of LymNAPE-PLD in the central ring ganglia of LPS-injected snails. Again, these results are consistent with previous studies from mammals demonstrating that LPS promotes inflammation, by suppressing NAPE-PLD expression (Zhu et al., 2011). Overall, our results underscore that in *Lymnaea*, as in mammals, the systemic LPS administration triggers the activation of the ECS-mediated inflammatory response, which lasts for at least 6 h but not 24 h, suggesting an immunomodulatory function of the ECS and the critical role that its molecular signaling may have in controlling neuroinflammation.

Together, our study addresses the limited functional and pharmacological characterization of conserved genes and transcripts in this model, leveraging datasets to identify contigs of *L. stagnalis* matching genes coding for ECS enzymes. The univocal identification of enzymes and the high degree of sequence conservation revealed by phylogenetic analyses validate the significance of these findings and their potential implications. This opens up opportunities in pharmaceutics and pharmacology for exploring novel therapeutic targets, especially for neurological and psychiatric disorders associated with ECS dysregulation (Vered et al., 2023). However, further analysis to measure endocannabinoid levels in *L. stagnalis* will be needed to fully understand ECS signaling, as does the caution required in directly applying findings from snails to humans because of differences in nervous system complexity.

5 | LIMITATIONS AND FINAL REMARKS

While the study provides valuable insights, the direct applicability of findings from *L. stagnalis* to humans requires cautious interpretation because of the vast differences in nervous system complexity. Furthermore, the research focuses on specific stressors, indicating that a broader examination of environmental and physiological challenges could offer more comprehensive insights into the ECS's roles. Finally, grounded in the empirical evidence and theoretical frameworks provided, our study highlights the significance of *L. stagnalis* in ECS research. It underscores the potential therapeutic implications of the ECS, offering a promising direction for future studies aimed at unraveling the complexities of ECS signaling and its roles in health and disease. Our study provides the most thorough characterization to date of the ECS metabolic pathway in an invertebrate model system, adding to the value of *L. stagnalis* for future functional studies of this pathway at the cellular, synaptic, and behavioral levels.

Although evolutionarily quite distant from humans, *L. stagnalis* shows molecular and behavioral properties that make it a versatile platform to study in depth the role of the ECS in CNS physiology and pathology. The use of this snail model may make it possible to reduce the number of studies involving mammals and allow the use of mammalian models only for the validation of the results obtained from invertebrates. This will reduce by several orders of magnitude the costs of numerous studies. Thus, *L. stagnalis* as a model system provides an important experimental tool to efficiently characterize molecular pathways and offers a translational approach that may help gain important knowledge and comprehension in the fields of neuroscience and pharmacology.

AUTHOR CONTRIBUTIONS

Veronica Rivi: Conceptualization; data curation; methodology; validation; investigation; formal analysis; writing – original draft. **Giovanna Rigillo:** Conceptualization; supervision; writing – review and editing. **Anuradha Batabyal:** Investigation; writing – review and editing. **Ken Lukowiak:** Writing – review and editing; supervision; resources. **Luca Pani:** Writing – review and editing; funding acquisition. **Fabio Tascedda:** Supervision; writing – review and editing; resources; funding acquisition. **Cristina Benatti:** Supervision; resources; funding acquisition; writing – review and editing. **Johanna M. C. Blom:** Supervision; writing – review and editing; resources.

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CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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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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