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Dietary DL-α-Tocopheryl Acetate Enhances Thermal Tolerance and Modulates Oxidative Stress Responses in Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

Rising water temperatures necessitate nutritional strategies to enhance resilience in rainbow trout (*Oncorhynchus mykiss*). This study evaluated the effects of dietary vitamin E on thermal tolerance and physiological, antioxidant, and hepatic molecular responses to gradual warming. Juvenile trout (22.44 ± 0.03 g; mean ± SD) were fed CTRL, LDvitE, and HDvitE diets with analyzed α-tocopherol levels of 206.5, 265.6, and 608.1 mg/kg, respectively, for 60 days. Thermal tolerance was assessed using critical thermal maximum (CTMax), and thermal stress responses were evaluated during gradual warming from 17 to 24 °C (1 °C/day). At the end of the feeding period (Final), compared with CTRL, LDvitE and HDvitE showed lower hepatic TBARS levels, higher CAT activity, and higher expression of *hepcidin* and *α-ttp*. HDvitE also had lower serum cortisol and AST and a significantly higher CTMax than CTRL. Following thermal stress (Post-stress), serum cortisol and AST remained lower in HDvitE than in CTRL, with the lowest serum glucose level in HDvitE. Hepatic *hsp90* and *defb3* expression levels were higher in HDvitE than in CTRL. Overall, HDvitE improved thermal tolerance and was associated with favorable physiological and molecular responses before and after thermal stress, supporting its potential to enhance thermal resilience in rainbow trout.

Keywords: vitamin E; critical thermal maximum; redox homeostasis; molecular stress response; innate immunity

Key Contribution: This study demonstrates that dietary vitamin E supplementation enhances upper thermal tolerance and supports physiological resilience to gradual warming in rainbow trout. The findings provide an integrated physiological and molecular basis for using vitamin E as a nutritional strategy against thermal stress in cold-water aquaculture.

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1. Introduction

Increasing water temperatures associated with global climate change represent a growing concern for aquaculture, particularly for stenothermal cold-water fish species [1,2]. Water temperature is a major ecophysiological factor in fish, influencing metabolic rate, oxygen demand, energy allocation, growth, behavior, and survival [3]. When water temperature exceeds the optimal range, increased metabolic and respiratory demands may

enhance reactive oxygen species production and disrupt redox homeostasis, leading to oxidative damage, cellular stress responses, and activation of endocrine and heat shock protein-mediated defense mechanisms [3–5]. If these compensatory mechanisms are overwhelmed, thermal stress can impair physiological performance, health status, and survival, with negative consequences for aquaculture productivity. In this context, rainbow trout (*Oncorhynchus mykiss*) represents a particularly relevant species for investigating thermal stress, as it is one of the most widely cultured and economically important salmonids, with an optimal temperature range generally considered to be 12–18 °C, while temperatures approaching or exceeding 25 °C can pose a substantial threat to survival [6]. Because water temperature cannot always be fully regulated under farming conditions, feed-based nutritional strategies may provide a practical approach to support fish resilience during periods of elevated temperature, particularly through functional nutrients and dietary additives with antioxidant or immunomodulatory properties [7,8].

Among such functional nutrients, vitamin E has received considerable attention in aquaculture because of its central role in antioxidant protection, membrane stability, and fish health. It comprises a group of fat-soluble compounds, including tocopherols and tocotrienols, among which α-tocopherol is generally regarded as the most biologically active form in fish [9,10]. Within cellular membranes, vitamin E exerts its antioxidant effect by neutralizing peroxyl radicals before they attack polyunsaturated fatty acids, thereby helping to protect membrane lipids from oxidative damage [10,11]. Consistent with these functions, adequate vitamin E supply is associated with cellular integrity, antioxidant status, immune competence, growth performance, and fillet quality in farmed fish [9,10]. Because fish cannot synthesize vitamin E de novo, they rely on dietary supply to meet their physiological requirements [9,12]. Therefore, the dietary inclusion level is important for maintaining adequate antioxidant defenses, particularly under conditions that increase oxidative challenges. Recent evidence suggests that vitamin E levels above the NRC minimum requirement for salmonids, generally reported as 50–60 mg kg^{-1} diet [13], may be beneficial in aquafeeds, particularly under intensive or stressful farming conditions. Based on recent studies and current industry practices, dietary vitamin E levels of approximately 200–600 mg kg^{-1} have been proposed as a broader functional supplementation range for salmonid feeds under intensive or stressful production conditions, rather than as a defined nutritional optimum [12]. A recent review also supports the potential benefits of vitamin E levels above minimum requirements under challenging production conditions [14].

These antioxidant and membrane-protective properties may be particularly relevant during thermal stress, when increased metabolic activity can promote the generation of reactive oxygen species (ROS) and heighten the demand for antioxidant and immune defenses. In spotted seabass (*Lateolabrax maculatus*) reared under different temperature conditions, dietary vitamin E has been shown to enhance antioxidant capacity, including increased SOD activity and reduced MDA levels, while also supporting immune-related responses [15]. More recently, α-tocopherol acetate supplementation was reported to alleviate heat stress-related physiological disturbances, improve antioxidant enzyme responses, reduce lipid peroxidation, and support innate immune parameters in Nile tilapia (*Oreochromis niloticus*) [16]. In juvenile golden shiner (*Notemigonus crysoleucas*), α-tocopherol supplementation improved survival following a high-temperature challenge [17]. In rainbow trout, vitamin E has been evaluated under other stress models, including crowding-related stress [18–20] and sublethal ammonia exposure as part of combined antioxidant supplementation [21]. Additional vitamin E supplementation above basal dietary levels was shown to improve antioxidant status and physiological and immune responses in triploid rainbow trout exposed to high stocking density [22]. At the molecular level, vitamin E has been shown to modulate antioxidant gene expression associated with the nuclear factor

erythroid 2-related factor 2/Kelch-like ECH-associated protein 1 (Nrf2/Keap1) signaling pathway in other fish species [23], while Mahmoudi et al. [22] highlighted the need for further investigation of Nrf2-regulated antioxidant genes and cytokine-related responses in rainbow trout. However, direct evidence linking dietary vitamin E supplementation to thermal resilience in rainbow trout remains scarce, particularly regarding its effects on transcriptional responses related to antioxidant defense, heat shock, and immunity during progressive warming.

In this regard, we hypothesized that additional dietary vitamin E supplementation would enhance upper thermal tolerance and modulate physiological, antioxidant, and molecular responses to progressive warming. The present study was therefore designed to evaluate whether supplementing a commercial rainbow trout diet with vitamin E could provide a practical nutritional strategy for enhancing resilience to warming-related stress.

2. Materials and Methods

2.1. Fish Maintenance, Experimental Design and Diets

Juvenile rainbow trout weighing approximately 2 g were acquired from a nearby fish farm in Aras, Kahramanmaraş, Türkiye. The fish were then transported to the Faculty of Fisheries at Çukurova University in Adana, Türkiye. During the pre-experimental rearing and acclimation period, the fish were kept in 1000 L tanks connected to a recirculating aquaculture system (RAS) and fed a commercial diet (Optiline, Skretting, Stavanger, Norway) for 14 weeks. Then, a total of 225 rainbow trout (22.44 ± 0.03 g; mean ± SD) were distributed into nine 190-L fiberglass tanks connected to a temperature-controlled recirculating aquaculture system (RAS), with 25 fish per tank and three replicate tanks per dietary treatment. During the 60-day feeding trial, water temperature was maintained at 17 °C using a water-chilling system. The temperature, dissolved oxygen, and pH were measured daily using a multiparameter YSI professional-plus probe (Yellow Spring Instrument Co., Yellow Spring, OH, USA), and the photoperiod was set to 12L:12D. The concentration of dissolved oxygen exceeded 7.0 mg/L, whereas the pH ranged from 7.0 to 8.5. The levels of ammonium, nitrite, and nitrate were maintained below critical thresholds (NH_4^+ < 0.1 mg/L, NO_2^- < 0.1 mg/L, NO_3^- < 0.5 mg/L).

The experimental diets consisted of a control diet based on a commercial trout feed (Skretting Optiline) and two vitamin E-supplemented diets. The basal feed formulation was not otherwise altered. ROVIMIX® E-50 Adsorbate ((DSM Nutritional Products, Heerlen, The Netherlands)), containing a minimum of 50% DL-α-tocopheryl acetate, was incorporated at 200 and 1000 mg/kg diet for the LDvitE and HDvitE treatments, respectively, corresponding to approximately 100 and 500 mg/kg supplemental DL-α-tocopheryl acetate. The ROVIMIX® inclusion levels refer to the amounts of commercial product added per kilogram of final diet. Based on the product's minimum 50% DL-α-tocopheryl acetate content, the corresponding supplemental amounts are theoretical values rather than analytically determined concentrations. The analyzed values reported below represent the total α-tocopherol concentrations measured in the finished diets, including α-tocopherol originating from the commercial basal feed, and were used to represent the actual dietary exposure of the fish. The supplementation levels were selected to provide a lower and a higher vitamin E addition above the vitamin E already present in the commercial basal diet, based on levels previously evaluated in rainbow trout [20,24]. To prepare the experimental diets, the commercial trout feed was ground and thoroughly mixed with the required amount of ROVIMIX® E-50 Adsorbate. Water was then added to form a homogeneous dough. The dough was processed using a laboratory pellet mill (La Monferrina-Dolly (Imperia & Monferrina S.p.A., Castell'Alfero, Italy)) equipped with a 3 mm die. The resulting pellets were dried at room temperature in an air-conditioned environment and stored at

4 °C until use. The CTRL diet was prepared using the same procedure without ROVIMIX® E-50 supplementation. The analyzed proximate composition of the experimental diets (n = 3) was similar across groups. Crude protein contents were 44.80 ± 0.36, 44.89 ± 0.16, and 45.34 ± 0.07%; crude lipid contents were 20.10 ± 0.30, 19.99 ± 0.56, and 20.20 ± 0.43%; dry matter contents were 92.65 ± 0.55, 93.04 ± 0.07, and 92.97 ± 0.15%; and crude ash contents were 8.74 ± 0.14, 8.66 ± 0.05, and 8.71 ± 0.10% for CTRL, LDvitE, and HDvitE, respectively. Accordingly, all diets were considered isonitrogenous and isolipidic. The fatty acid composition of the experimental diets is presented in Table S1. The measured α-tocopherol concentrations in the experimental diets were 206.5 ± 0.71, 265.6 ± 3.13, and 608.1 ± 2.96 mg/kg for the CTRL, LDvitE, and HDvitE diets, respectively.

Fish were hand-fed the three experimental diets twice daily (09:00 and 16:00) to apparent satiation for 60 days. Feed was offered gradually in small portions, and consumption was monitored throughout each feeding to ensure that the supplied feed was consumed. No visible uneaten pellets remained in the tanks; therefore, no correction for uneaten feed was required. The amount of feed supplied to each tank was weighed and recorded daily to determine feed intake. At the end of the 60-day feeding period, the effects of vitamin E-enriched diets on critical thermal maximum (CTMax) were evaluated (Section 2.5). In the second phase of the experiment (thermal stress challenge), water temperature in the experimental tanks was increased by 1 °C per day, from 17 to 24 °C over a 7-day period, using a heater (Gemaş, Fairland PHC25V, İstanbul, Türkiye), following a gradual warming protocol similar to that described by Li et al. [25]. Post-stress sampling was conducted at the end of this seven-day warming period.

2.2. Sampling

Sampling was conducted at two time points: the end of the 60-day feeding period (Final) and after the thermal stress protocol (Post-stress). At the final sampling, all fish in each tank were weighed to calculate the growth parameters reported below. At each sampling point, two fish were randomly selected from each tank (six fish per treatment group; n = 3 tank replicates) and anesthetized with MS-222 (150 mg/L). Blood samples were collected from the caudal vasculature. A portion of whole blood was transferred into microhematocrit capillary tubes and centrifuged at 3500× *g* for 15 min to determine hematocrit (HCT) values. The remaining blood samples were allowed to clot at 4 °C and were subsequently centrifuged at 5000 rpm for 5 min. Serum was separated and stored at −80 °C until biochemical analyses. Following blood collection, liver tissues were excised, immediately frozen in liquid nitrogen for gene expression and antioxidant enzyme analyses, and stored at −80 °C until analysis. Fillets collected for proximate composition and fatty acid analyses were stored at −20 °C until analysis; α-tocopherol was additionally determined in samples collected at the end of the feeding period.

The growth and feed utilization parameters were determined using the subsequent equations:

$$\text{Weight gain (WG, \%)} = [(\text{final weight} - \text{initial weight})/\text{initial weight}] \times 100$$

$$\text{Specific growth rate (SGR, \% day-1)} = [\ln(\text{final weight}) - \ln(\text{initial weight})]/\text{experimental duration (days)} \times 100$$

$$\text{Feed conversion ratio (FCR)} = \text{dry feed intake (g)}/\text{live weight gain (g)}$$

$$\text{Protein efficiency ratio (PER)} = \text{live weight gain (g)}/\text{dietary protein intake (g)}$$

$$\text{Feed intake} = \text{total dry feed consumed per tank during the feeding period (g)}/\text{number of fish in the tank}$$

$$\text{Hepatosomatic index (HSI, \%)} = 100 \times [\text{liver weight (g)}/\text{total weight (g)}]$$

$$\text{Viscerosomatic index (VSI, \%)} = 100 \times [\text{visceral weight (g)}/\text{total weight (g)}]$$

2.3. Proximate Composition, Fatty Acid, and α-Tocopherol Analyses

The proximate composition (crude protein, crude lipid, dry matter and ash) of the feeds and fillets of fish was analyzed according to AOAC [26]. Lipids were extracted following Folch et al. [27]. The extracted lipids were transmethylated to fatty acid methyl esters (FAME) according to Metcalfe and Schmitz [28], with modifications described by Czesny and Dabrowski [29]. FAMEs were analyzed by gas chromatography (Agilent Technologies 6820A, Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a flame ionization detector. Hydrogen was used as the carrier gas at a flow rate of 2 mL min^{-1} with a split ratio of 1:30. Fatty acids were separated using a capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent, Agilent Technologies Inc., Santa Clara, CA, USA). The oven temperature was held at 190 °C for 35 min, then increased to 220 °C at 30 °C min^{-1}, and maintained for 5 min. Fatty acids were identified by comparing retention times with those of a Supelco 37 FAME standard mixture. Fatty acid composition was expressed as area percentage (%).

α-Tocopherol concentrations were determined in the experimental diets (n = 3 per dietary treatment) and in fillet samples collected at the end of the 60-day feeding period (n = 3 per dietary treatment). Analyses were performed by TÜBİTAK Marmara Research Center (TÜBİTAK Marmara Research Center (MAM, Gebze, Türkiye)), using its in-house method D.05.G84, which is based on high-performance liquid chromatography with fluorescence detection (HPLC-FLD). Results were expressed in mg/kg.

2.4. Serum Biochemical Analysis

Serum iron, ferritin, total iron-binding capacity (TIBC), total protein, albumin, AST, glucose, cortisol, and total immunoglobulin levels were determined using commercially available diagnostic kits, according to the manufacturer's instructions, on an auto-analyzer (Abbott ARCHITECT c8000,Abbott Laboratories, Abbott Park, IL, USA). All serum biochemical analyses were performed at an accredited veterinary diagnostic laboratory.

2.5. CTMax

After 60 days of feeding, fish were fasted for 24 h before the CTMax test. The test was conducted using three replicate tanks per dietary group, with three fish randomly selected from each tank (n = 3 replicate tanks; N = 9 fish per group). Fish were transferred directly, without anesthesia, to a glass aquarium maintained at the acclimation temperature (17 °C) to avoid potential anesthetic-related effects on thermal tolerance and behavioral endpoints. After a brief observation period, during which regular swimming behavior was restored, water temperature was gradually increased by adding heated water at a rate of 0.3 °C min^{-1} [30]. During heating, the water was gently mixed to ensure uniform temperature distribution. Throughout the test, dissolved oxygen was maintained above 6.5 mg L^{-1} by continuous aeration; water temperature and dissolved oxygen were continuously monitored with an oxygen meter (YSI 550A, YSI Incorporated, Yellow Springs, OH, USAUSA). The endpoint was defined as loss of equilibrium, indicated by failure to maintain normal dorsoventral orientation for at least 1 min [31]. The water temperature at this endpoint was recorded individually as the CTMax value. For statistical analysis, the three individual CTMax values obtained from fish originating from the same tank were averaged to generate a single tank-level value. Fish used for CTMax testing were not used in any subsequent experimental procedures.

2.6. Hepatic Antioxidant Enzyme Activities and Lipid Peroxidation

Liver samples were homogenized at a ratio of 1:10 (*w*/*v*) in a buffer containing 100 mM potassium phosphate (pH 7.4), 100 mM KCl, and 1 mM EDTA, using a homogenizer at 9500 rpm for 1.5 min. Homogenates were centrifuged at 10,000× *g* for 30 min at 4 °C

(Thermo Fisher Scientific Inc., Waltham, MA, USA), and the supernatants were collected for subsequent analyses.

Total protein concentration was determined spectrophotometrically at 750 nm using the Lowry assay [32], with bovine serum albumin used as the calibration standard. Protein concentrations were expressed as mg/mL.

Lipid peroxidation was evaluated using the thiobarbituric acid reactive substances (TBARS) assay by incubating supernatants with thiobarbituric acid at 100 °C under aerobic conditions. The formation of the pink chromogen was measured at 532 nm, and TBARS concentrations were calculated using an external standard curve prepared with 1,1′,3,3′-tetramethoxypropane and expressed as nmol/mg protein [33].

Catalase (CAT) activity was assayed according to Lartillot et al. [34]. The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 25 mM H_2O_2, and 20 µL of supernatant. The decrease in absorbance at 240 nm was monitored for 1 min, and CAT activity was reported as µmol H_2O_2 decomposed per mg protein per minute.

Superoxide dismutase (SOD) activity was measured using an indirect method based on the inhibition of cytochrome *c* reduction at 550 nm, as described by McCord and Fridovich [35]. The reaction mixture (final volume of 1 mL) consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 µM cytochrome *c*, 0.05 mM hypoxanthine, and 10 µL of supernatant; the reaction was initiated by the addition of xanthine oxidase (1.88 mU/mL). One unit of SOD activity was defined as the amount of enzyme required to inhibit cytochrome *c* reduction by 50%, and SOD activity was expressed as U/mg protein. All assays were performed in duplicate.

2.7. mRNA Expression Analysis

Total RNA was isolated from fish liver tissue using TRIzol™ reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration was assessed fluorometrically using a Qubit 2.0 system (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using the OneScript® Plus cDNA Synthesis Kit (Applied Biological Materials Inc., Richmond, BC, Canada). Reverse transcription was carried out in a final volume of 20 µL containing 4 µL of 5× RT buffer, 1 µL of dNTP mix, 1 µL of random primers, 1 µL of reverse transcriptase, 1 µg of total RNA, and nuclease-free water to complete the reaction volume. The reaction mixture was incubated at 25 °C for 10 min, followed by 55 °C for 15 min and 85 °C for 5 min. The resulting cDNA was stored at −20 °C until further use.

Quantitative real-time PCR (qRT-PCR) analyses were performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Each reaction was prepared in a total volume of 25 µL, comprising 12.5 µL of SYBR Green Master Mix, 1.25 µL of forward primer (12.5 pmol), 1.25 µL of reverse primer (12.5 pmol), 2 µL of cDNA, and 8 µL of nuclease-free water. Amplifications were carried out on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems). The cycling program included initial steps at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 15 s and 60 °C for 1 min.

β-actin was selected as the internal reference gene based on its stability under the experimental conditions, including temperature stress. The stability of *β-actin*, *gapdh*, and 18S was evaluated using the NormFinder Excel (version 0.953; Excel add-in, Department of Molecular Medicine, Aarhus University Hospital, Aarhus, Denmark) add-in across the heat stress × vitamin E treatment combinations, and *β-actin* was identified as the most stable individual reference gene, with a stability value of 1.290. In addition, the amplification efficiency of the *β-actin* primers was evaluated using a serial dilution of the template, yielding an R^2 value of 0.9937 and an amplification efficiency of 99.8%. Primer

sequences are provided in Table 1. Relative gene expression levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method based on threshold cycle (Ct) values obtained from replicate measurements [36]. All qRT-PCR analyses were performed in technical triplicate.

Table 1. Sequences of primers used for real-time PCR analysis.

Gene	Forward (5′–3′)	Reverse (5′–3′)	Amplicon (bp)	Tm (°C)	Accession No.
nrf2	TTTTGTCCCTTCCTGAGCTG	AGGTTGGGCAATGGGTAGAA	139	60	XM_021597223.2
sod	ACCAAACTAGGCGAAGGTCTAC	TGTGGATGGCTTTTGACTGATG	139	60	XM_021619043.2
cat	TGGCTTTGCAGTCAAGTTCTAC	TGGATGAATGATGGGAACAGC	108	60	XM_021557350.2
gpx	AATCAGCAGTCGCACATTTTC	ATCTTGCACAGGAGAGCAAAG	139	60	XM_021569971.2
hsp70	GCAGGACGCTGACAAATACAA	CGCTGATCTTGCCTTTCATGT	134	60	NM_001124228.1
hsp90	AAGCACAACGATGATGAGCA	ACCTTAGTTCCACGCAACAT	101	60	NM_001124231.1
tlr4	CATTTCTCCCATTCAACTGTGC	ATGATGACTTTGCGGCTACC	108	60	XM_036959900.1
myd88	TGAAGCTGTGTGTGTTTGACC	CGACCATCCTTTTACACCTCT	96	60	NM_001124421.1
il1β	ACAAGGACCTGCTCAACTTCT	ACTGTGATGTACTGCTGAACCC	117	60	NM_001124347.2
tnfa	ACTCCATCGGGGTTAATGCT	CTTCGCCAATTTCGGACTCA	90	60	NM_001124357.1
il8	TGAGACGGAAAGCAGACGAA	TCAATGACCCTCTTGACCCA	159	60	NM_001124362.1
il10	CGCCATGAACAACAGAACACA	TTCCTGCATTGGACGATCTCT	105	60	NM_001245099.1
il4/13	CGGCAGCATAGAAGTCACAAT	TGGTCGCAGTCAAATGTGTT	127	60	NM_001246341.1
defb3	TGGCTCTTTTAGTCATTGCTTG	CAAGGCACAAACGAAGGTTTC	102	60	NM_001195183.2
hepcidin	ACAGTCCAGTTGGGGAACAT	AATTTGCAGCAGAAGCCACA	148	60	XM_036947245.1
α-ttp	AGTGCGAGGAATCCATCTGA	TCTCTTTGAAGGTGCTGCCA	125	60	XM_021589663.2
cyp3a27	AGTCGCTTTGTTTCCCTTCAC	AAAAGAAGTCTGTCACCGCAG	83	60	XM_036948688.1
β-actin	AGATGAAATCGCCGCACT	TCCCACGTAGCTGTCTTTCT	160	60	NM_001124235.1

Gene symbols: *nrf2*, nuclear factor erythroid 2-related factor 2; *sod*, superoxide dismutase; *cat*, catalase; *gpx*, glutathione peroxidase; *hsp70*, heat shock protein 70; *hsp90*, heat shock protein 90; *tlr4*, Toll-like receptor 4; *myd88*, myeloid differentiation primary response 88; *il1β*, interleukin-1 beta; *tnfa*, tumor necrosis factor alpha; *il8*, interleukin-8; *il10*, interleukin-10; *il4/13*, interleukin-4/13A; *defb3*, beta-defensin 3; *hepcidin*, hepcidin antimicrobial peptide; *α-ttp*, alpha-tocopherol transfer protein; *cyp3a27*, cytochrome P450 family 3 subfamily A member 27; *β-actin*, beta-actin.

2.8. Statistical Analysis

All variables measured at the end of the feeding period and after thermal stress, with the exception of growth performance and CTMax, were analyzed by two-way ANOVA to assess the effects of diet, thermal stress, and their interaction (Diet × Stress). One-way ANOVA was also used to evaluate differences among dietary treatments within each sampling period; for growth performance and CTMax, only one-way ANOVA was used. For variables measured in individual fish, values obtained from the two fish sampled from the same tank were first averaged to obtain a single tank-level value. These tank means were used as the experimental units for all statistical analyses (n = 3 tanks per treatment). When significant differences were detected, Tukey's post hoc test was applied for multiple comparisons. Differences between the end of the feeding period and the post-stress period within each dietary group were evaluated using an independent-samples t-test on tank-level means. Homogeneity of variances was assessed using Levene's test. All analyses were performed using SPSS Statistics version 20.0 (IBM Corp., Armonk, NY, USA). Data are presented as mean ± SD, and statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Fish Performance Parameters

No significant differences were observed among the dietary groups in growth performance parameters, including FW, SGR, FCR, PER, and FI ($p > 0.05$). Somatic indices (HSI and VSI) also did not differ significantly among dietary groups ($p > 0.05$). All diets were readily accepted by the fish, and no mortality occurred during the feeding trial (Table 2).

Table 2. Growth performance and feed utilization parameters of rainbow trout fed the different experimental diets for 60 days.

	Experimental Groups		
	CTRL	**LDvitE**	**HDvitE**
Initial weight (g)	22.41 ± 0.11	22.44 ± 0.04	22.47 ± 0.09
Final weight (g)	193.71 ± 19.10	203.60 ± 15.66	202.57 ± 5.71
WG (%) [1]	764.43 ± 83.88	807.25 ± 68.72	801.63 ± 21.92
SGR (% day-1) [2]	3.59 ± 0.17	3.67 ± 0.13	3.66 ± 0.04
FCR [3]	0.89 ± 0.02	0.87 ± 0.04	0.87 ± 0.03
PER [4]	2.52 ± 0.04	2.57 ± 0.12	2.55 ± 0.10
FI [5] (g/fish)	151.87 ± 18.73	156.64 ± 12.69	155.80 ± 9.35
HSI (%)	0.85 ± 0.03	0.95 ± 0.11	0.90 ± 0.03
VSI (%)	3.66 ± 0.06	4.14 ± 0.80	3.90 ± 0.64

Values are presented as mean ± SD of three tank replicates (n = 3). No significant differences were detected among dietary treatments for any parameter (one-way ANOVA, $p > 0.05$). [1] WG: weight gain. [2] SGR: specific growth rate. [3] FCR: feed conversion ratio. [4] PER: protein efficiency ratio. [5] FI: feed intake.

3.2. Fillet α-Tocopherol, Proximate and Fatty Acid Composition

At the end of the 60-day feeding period, fillet α-tocopherol concentration was significantly higher in the HDvitE group (42.4 ± 1.63 mg/kg) than in the LDvitE (29.97 ± 2.06 mg/kg) and CTRL (27.9 ± 1.80 mg/kg) groups ($p < 0.05$). The fillet proximate composition of rainbow trout fed different experimental diets, measured before and after thermal stress, is presented in Table 3. At either sampling point, no significant differences among dietary groups were detected in fillet protein, lipid, dry matter, or ash content (one-way ANOVA, $p > 0.05$). However, within-diet comparisons between sampling points showed that ash content decreased in LDvitE and increased in HDvitE after thermal stress (independent-samples *t*-test, $p < 0.05$), whereas protein, lipid, and dry matter contents remained unchanged within all dietary treatments.

Table 3. Proximate composition (% wet weight) of fillets of rainbow trout at the end of the 60-day feeding period and following thermal stress.

	Final			**Post Stress**			**Two-Way ANOVA *p* Value**		
	CTRL	**LDvitE**	**HDvitE**	**CTRL**	**LDvitE**	**HDvitE**	**Diet**	**Stress**	**Diet x Stress**
Protein	20.53 ± 0.70	20.78 ± 0.13	20.40 ± 0.73	20.48 ± 0.71	21.82 ± 1.12	21.55 ± 0.98	0.258	0.079	0.381
Lipid	4.40 ± 1.01	4.89 ± 1.57	4.49 ± 0.57	4.69 ± 0.81	4.00 ± 0.33	3.86 ± 0.23	0.753	0.343	0.490
Dry matter	26.03 ± 0.52	25.73 ± 1.26	25.43 ± 0.80	26.89 ± 1.22	25.60 ± 0.59	26.22 ± 0.95	0.330	0.272	0.597
Ash	1.60 ± 0.19	1.62 ± 0.05 *	1.41 ± 0.01	1.52 ± 0.18	1.42 ± 0.09	1.59 ± 0.04 *	0.695	0.551	0.074

Values are presented as mean ± SD of three tank replicates (n= 3). No significant differences were detected among dietary treatments for any parameter (one-way ANOVA, $p > 0.05$). Asterisks (*) indicate the higher value within a treatment when a significant difference was detected between the end of the feeding period and post-stress periods (independent-samples *t*-test, $p < 0.05$).

The fatty acid composition of rainbow trout fillets at the end of the feeding period and following thermal stress is presented in Table 4. The fatty acid profile was dominated by palmitic acid (16:0), oleic acid (18:1n−9), linoleic acid (18:2n−6), and docosahexaenoic acid (22:6n−3). At the end of the feeding period, neither individual fatty acids nor summed fatty acid classes differed significantly among dietary treatments ($p > 0.05$). Two-way ANOVA revealed significant dietary effects on 14:0, 16:0, 18:0, 24:1n−9, and total SFA ($p < 0.05$). Significant stress effects were observed for 14:0 and 24:1n−9 ($p < 0.05$), whereas significant diet x stress interactions were detected for 18:0 and 24:1n−9 ($p < 0.05$). Following thermal stress, significant differences among dietary treatments were observed for 14:0, 16:0,

16:1n−7, 18:0, 24:1n−9, and total SFA ($p < 0.05$). Total MUFA was not significantly affected by diet, stress, or their interaction ($p > 0.05$), although a within-treatment comparison showed a significant decrease in the HDvitE group after thermal stress ($p < 0.05$). Total PUFA, n−3, n−6, and the n−3/n−6 ratio were not significantly affected by diet, stress, or their interaction ($p > 0.05$).

Table 4. Fatty acid composition (area %) of rainbow trout fillets at the end of the 60-day feeding period and following thermal stress.

	Final			Post Stress			Two-Way ANOVA *p* Value		
	CTRL	LDvitE	HDvitE	CTRL	LDvitE	HDvitE	Diet	Stress	Diet x Stress
14:0	2.51 ± 0.02	2.54 ± 0.05	2.44 ± 0.09	2.47 ± 0.04 ^{a}	2.37 ± 0.03 ^{b}	2.38 ± 0.02 ^{b}	0.036	0.002	0.068
16:0	18.48 ± 0.38	18.06 ± 0.24	17.96 ± 0.32	18.37 ± 0.28 ^{a}	18.44 ± 0.27 ^{a}	17.66 ± 0.21 ^{b}	0.009	0.951	0.160
16:1n−7	3.47 ± 0.24	3.51 ± 0.27	3.40 ± 0.08	3.41 ± 0.14 ^{a}	3.40 ± 0.05 ^{a}	3.12 ± 0.03 ^{b}	0.111	0.082	0.504
18:0	5.27 ± 0.08	5.19 ± 0.07	5.23 ± 0.08	5.23 ± 0.04 ^{b}	5.40 ± 0.06 ^{a}	5.10 ± 0.05 ^{c}	0.013	0.634	0.002
18:1n−9	22.02 ± 0.93	22.67 ± 1.46	21.89 ± 0.37	22.18 ± 1.30	22.12 ± 0.58	21.19 ± 0.15	0.303	0.420	0.696
18:1n−7	2.19 ± 0.10	2.21 ± 0.08	2.14 ± 0.03	2.20 ± 0.09	2.16 ± 0.01	2.12 ± 0.02	0.228	0.565	0.732
18:2n−6	14.20 ± 0.23	14.28 ± 0.50	14.31 ± 0.34	14.26 ± 0.22	14.07 ± 0.73	14.59 ± 0.26	0.512	0.818	0.619
20:4n−6	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.00	0.07 ± 0.01	0.08 ± 0.02	0.07 ± 0.01	0.602	0.204	0.508
20:5n−3	3.02 ± 0.08	2.84 ± 0.29	2.96 ± 0.01	2.79 ± 0.34	2.84 ± 0.12	2.96 ± 0.08	0.586	0.431	0.518
22:6n−3	15.50 ± 1.02	15.41 ± 1.60	15.59 ± 0.63	15.73 ± 0.91	15.52 ± 1.01	16.29 ± 0.28	0.706	0.471	0.864
24:1n−9	0.43 ± 0.01	0.42 ± 0.01	0.44 ± 0.02	0.43 ± 0.01 ^{a}	0.41 ± 0.01 ^{ab}	0.30 ± 0.08 ^{b}	0.022	0.009	0.007
SFA	27.47 ± 0.40	26.98 ± 0.34	26.83 ± 0.44	27.28 ± 0.3 ^{a}	27.35 ± 0.28 ^{a}	26.34 ± 0.15 ^{b}	0.005	0.531	0.128
MUFA	29.92 ± 1.24	30.76 ± 2.07	29.80 ± 0.51 *	30.23 ± 1.78	29.77 ± 0.62	28.80 ± 0.28	0.406	0.369	0.604
PUFA	38.72 ± 1.13	38.46 ± 1.54	38.83 ± 0.52	38.75 ± 1.08	38.43 ± 0.49	39.57 ± 0.55	0.426	0.602	0.751
n−3	22.54 ± 1.16	22.21 ± 1.99	22.60 ± 0.61	22.55 ± 1.36	22.39 ± 1.19	23.03 ± 0.28	0.772	0.729	0.958
n−6	16.19 ± 0.25	16.25 ± 0.56	16.23 ± 0.34	16.20 ± 0.28	16.04 ± 0.73	16.54 ± 0.33	0.641	0.854	0.622
n−3/n−6	1.39 ± 0.08	1.37 ± 0.16	1.39 ± 0.06	1.39 ± 0.11	1.40 ± 0.14	1.39 ± 0.02	0.989	0.851	0.964

Values are presented as mean ± SD (n = 3). Different lowercase superscripts within the same row indicate significant differences among experimental groups (Tukey's post hoc test, $p < 0.05$). Asterisks (*) indicate the higher value within a treatment when a significant difference was detected between the end of the feeding period and post-stress periods (independent-samples *t*-test, $p < 0.05$).

3.3. Critical Thermal Maximum (CTMax)

CTMax values of fish fed the experimental diets are shown in Figure 1. CTMax values (mean ± SD) were 26.41 ± 0.20 °C, 27.02 ± 0.43 °C, and 27.83 ± 0.43 °C in the CTRL, LDvitE, and HDvitE groups, respectively. CTMax was significantly higher in the HDvitE group than in the CTRL group, while the LDvitE group showed intermediate values and did not differ significantly from either group (one-way ANOVA with Tukey's post hoc test, $p < 0.05$).

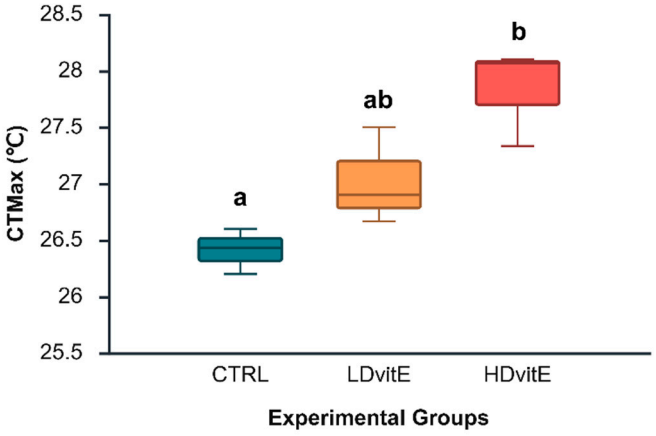


Figure 1. Critical thermal maximum (CTMax) values of rainbow trout fed the experimental diets for 60 days. Boxplots show the distribution of tank-based CTMax values (n = 3), with whiskers representing the minimum and maximum values. Different letters indicate significant differences among groups (one-way ANOVA followed by Tukey's test, $p < 0.05$).

3.4. Hematocrit and Serum Biochemical Analyses

Hematocrit and serum biochemical parameters are presented in Figure 2A–J. Hematocrit was significantly affected by thermal stress ($p = 0.001$); specifically, levels in the LDvitE group decreased significantly following thermal stress relative to those at the end of the feeding period ($p < 0.05$).

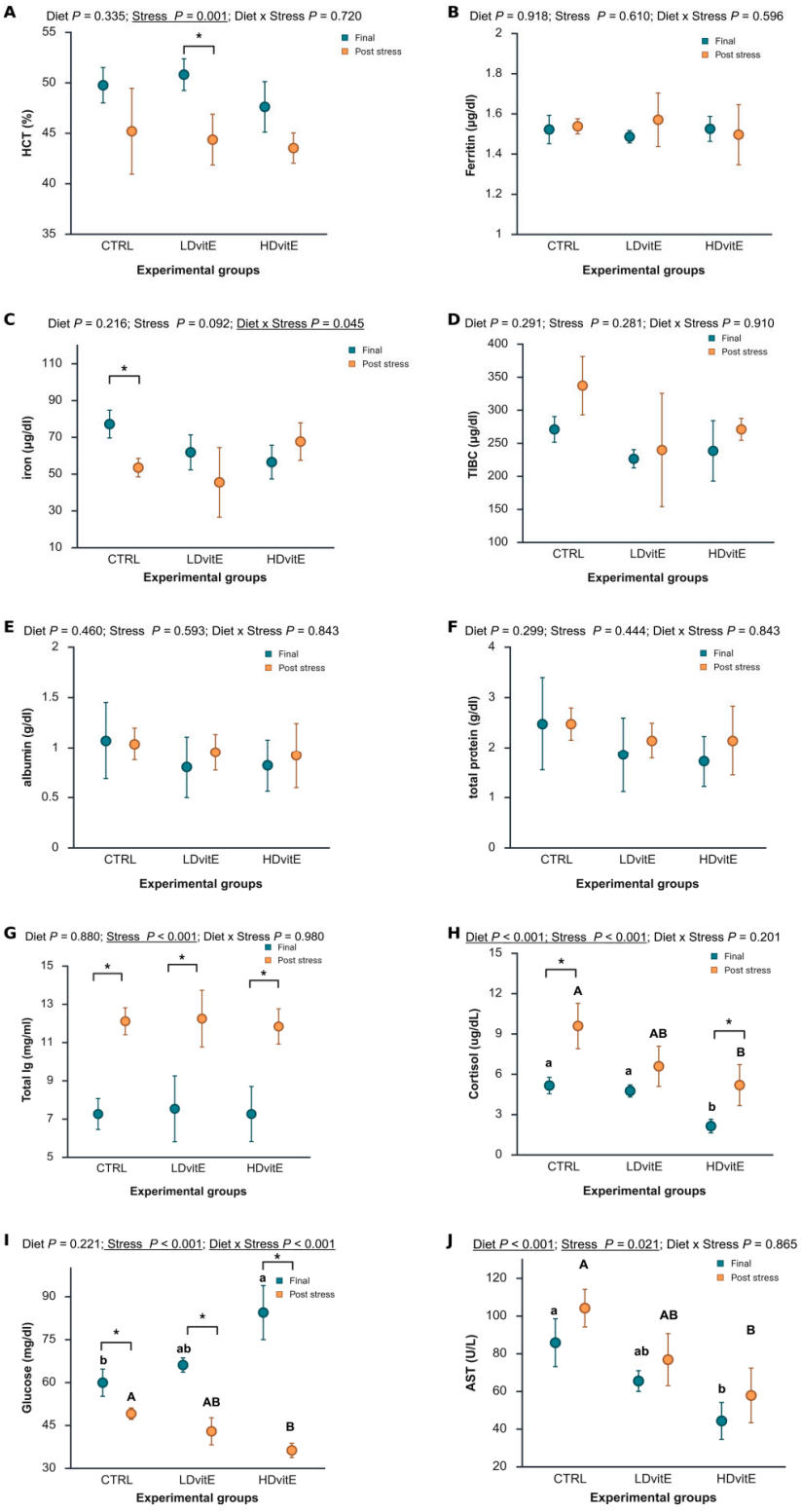


Figure 2. Hematocrit and serum biochemical parameters of juvenile rainbow trout fed different experimental diets before and after thermal stress: (**A**) hematocrit (HCT), (**B**) ferritin, (**C**) iron, (**D**) total

iron-binding capacity (TIBC), (**E**) albumin, (**F**) total protein, (**G**) total immunoglobulin (Total Ig), (**H**) cortisol, (**I**) glucose, and (**J**) aspartate aminotransferase (AST). Data are presented as mean ± SD of replicate tank means (n = 3). Within each sampling point, different lowercase letters indicate significant differences among dietary treatments at the end of the feeding period, whereas different uppercase letters indicate significant differences among dietary treatments after thermal stress (one-way ANOVA followed by Tukey's test, $p < 0.05$). Asterisks (*) indicate significant differences between the end of the feeding period and post-stress values within the same treatment (independent-samples *t*-test, $p < 0.05$). Two-way ANOVA *p*-values for Diet, Stress, and Diet x Stress interaction are shown in each panel.

Among the iron-related serum parameters, ferritin and TIBC were not significantly affected by diet, thermal stress, or their interaction ($p > 0.05$). Serum iron was not significantly affected by the main effects of diet or thermal stress; however, a significant diet x stress interaction was detected ($p = 0.045$), with the CTRL group exhibiting a significant decrease in serum iron following thermal stress ($p < 0.05$).

Albumin and total protein levels were not significantly affected by diet, thermal stress, or their interaction ($p > 0.05$). Total Ig levels were significantly affected by thermal stress ($p < 0.001$), whereas no significant dietary effect or diet x stress interaction was observed. Although total Ig levels did not differ among dietary groups at either sampling point, all groups showed a significant increase following thermal stress ($p < 0.05$).

Cortisol levels were significantly affected by diet and thermal stress ($p < 0.001$). At the end of the feeding period, the HDvitE group had significantly lower cortisol levels than CTRL and LDvitE ($p < 0.05$). Following thermal stress, cortisol levels remained significantly lower in the HDvitE group than in CTRL, with LDvitE showing intermediate values ($p < 0.05$). Within-group comparisons revealed a significant post-stress increase in cortisol in both the CTRL and HDvitE groups ($p < 0.05$).

Glucose levels were significantly affected by thermal stress and the diet x stress interaction ($p < 0.001$). At the end of the feeding period, glucose concentrations were significantly higher in HDvitE than in CTRL, with LDvitE showing intermediate values ($p < 0.05$). Following thermal stress, the lowest glucose level was observed in HDvitE, the highest in CTRL, with LDvitE remaining intermediate ($p < 0.05$). Glucose levels decreased significantly following thermal stress in all groups ($p < 0.05$).

AST activity was significantly affected by diet ($p < 0.001$) and thermal stress ($p = 0.021$), whereas the diet x stress interaction was not significant ($p = 0.865$). The significant stress effect reflected an overall post-stress increase in AST activity across treatments, although no significant within-group differences were detected. At both sampling points, AST activity was significantly lower in the HDvitE group than in the CTRL group, with the LDvitE group showing intermediate values ($p < 0.05$).

3.5. Hepatic Antioxidant and Oxidative Stress Markers

Hepatic antioxidant responses in juvenile rainbow trout fed the experimental diets are shown in Figure 3A–D. Hepatic protein levels were significantly affected by diet ($p = 0.001$), thermal stress ($p = 0.010$), and the diet x stress interaction ($p = 0.001$). No significant differences among dietary groups were observed at the end of the feeding period (Figure 3A). After thermal stress, protein levels were significantly lower in the LDvitE group than in the CTRL and HDvitE groups ($p < 0.05$). Within-group comparisons showed that thermal stress significantly increased hepatic protein levels in both the CTRL and HDvitE groups ($p < 0.05$).

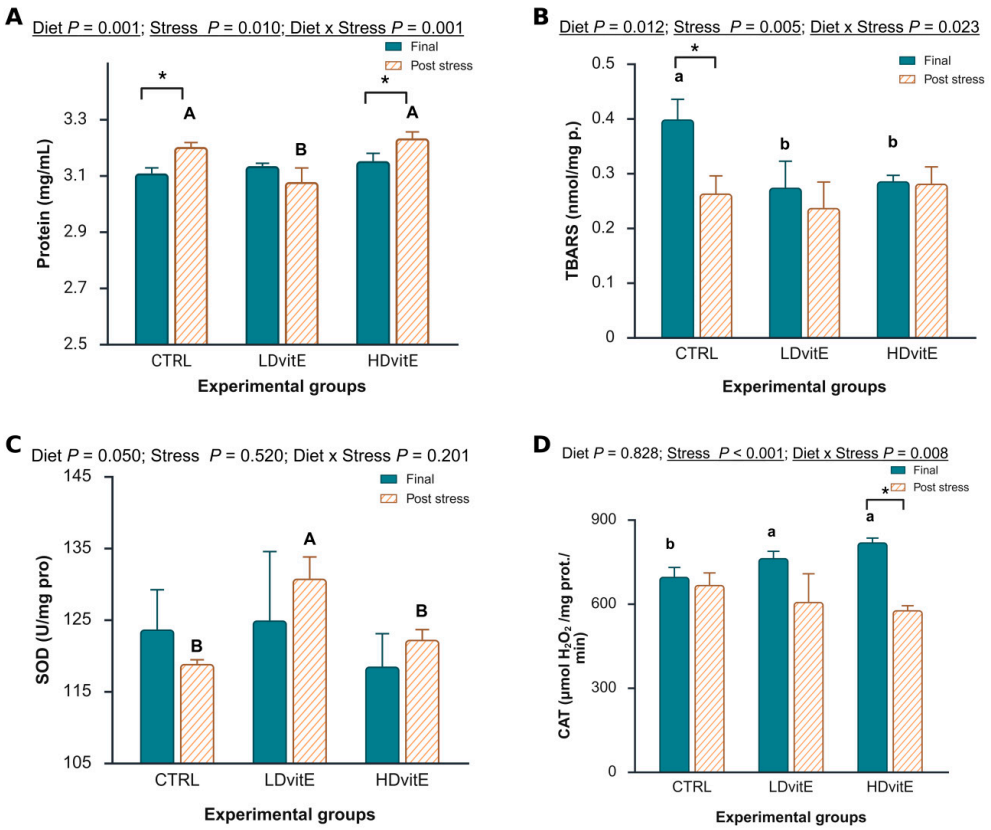


Figure 3. Hepatic antioxidant responses of juvenile rainbow trout fed different experimental diets before and after thermal stress: (**A**) protein levels, (**B**) TBARS levels, (**C**) SOD activity, and (**D**) CAT activity. Bars represent mean ± SD of replicate tank means (n = 3). Within each sampling point, different lowercase letters indicate significant differences among dietary treatments at the end of the feeding period, whereas different uppercase letters indicate significant differences among dietary treatments after thermal stress (one-way ANOVA followed by Tukey's test, $p < 0.05$). Asterisks (*) indicate significant differences between the end of the feeding period and post-stress values within the same treatment (independent-samples *t*-test, $p < 0.05$). Two-way ANOVA *p*-values for Diet, Stress, and Diet x Stress interaction are shown in each panel.

TBARS levels were significantly affected by diet ($p = 0.012$), thermal stress ($p = 0.005$), and the diet x stress interaction ($p = 0.023$). At the end of the feeding period, TBARS levels were significantly lower in the LDvitE and HDvitE groups than in the CTRL group ($p < 0.05$; Figure 3B). No significant differences among dietary groups were detected after thermal stress. A significant decrease between sampling periods was observed only in the CTRL group ($p < 0.05$).

SOD activity was not significantly affected by diet, thermal stress, or the diet x stress interaction. No significant differences among dietary groups were observed at the end of the feeding period; however, after thermal stress, SOD activity was significantly higher in the LDvitE group than in the CTRL and HDvitE groups ($p < 0.05$; Figure 3C).

CAT activity was significantly affected by thermal stress ($p < 0.001$) and the diet x stress interaction ($p = 0.008$), but not by dietary treatment alone. At the end of the feeding period, CAT activity was significantly higher in the LDvitE and HDvitE groups than in the CTRL group ($p < 0.05$; Figure 3D). After thermal stress, no significant differences among dietary groups were observed. Within-group comparisons showed that CAT activity decreased significantly after thermal stress only in the HDvitE group ($p < 0.05$).

3.6. Hepatic mRNA Expression of Selected Antioxidant-, Immune-, Stress-, and Metabolism-Related Genes

Relative mRNA expression levels of hepatic antioxidant- and stress-related genes are presented in Figure 4A–F. No significant effects of diet, thermal stress, or their interaction were detected in the expression of *nrf2*, *sod*, or *gpx* ($p > 0.05$). Among the antioxidant-related genes, *cat* expression was significantly affected by thermal stress ($p = 0.032$), whereas the effects of diet and the diet x stress interaction were not significant ($p > 0.05$). Within-group comparisons showed a significant post-stress increase in *cat* expression in the HDvitE group only ($p < 0.05$). Among heat shock protein genes, *hsp70* expression was significantly affected by thermal stress ($p = 0.022$) and by the diet x stress interaction ($p = 0.036$), while the diet effect was not significant ($p = 0.520$). In contrast, *hsp90* expression was significantly influenced by diet ($p = 0.008$) and thermal stress ($p = 0.003$), but not by their interaction ($p = 0.110$). Following thermal stress, *hsp90* expression was significantly higher in the HDvitE group than in the CTRL group, while the LDvitE group showed an intermediate response. In addition, *hsp90* expression increased significantly following thermal stress within the HDvitE group ($p < 0.05$).

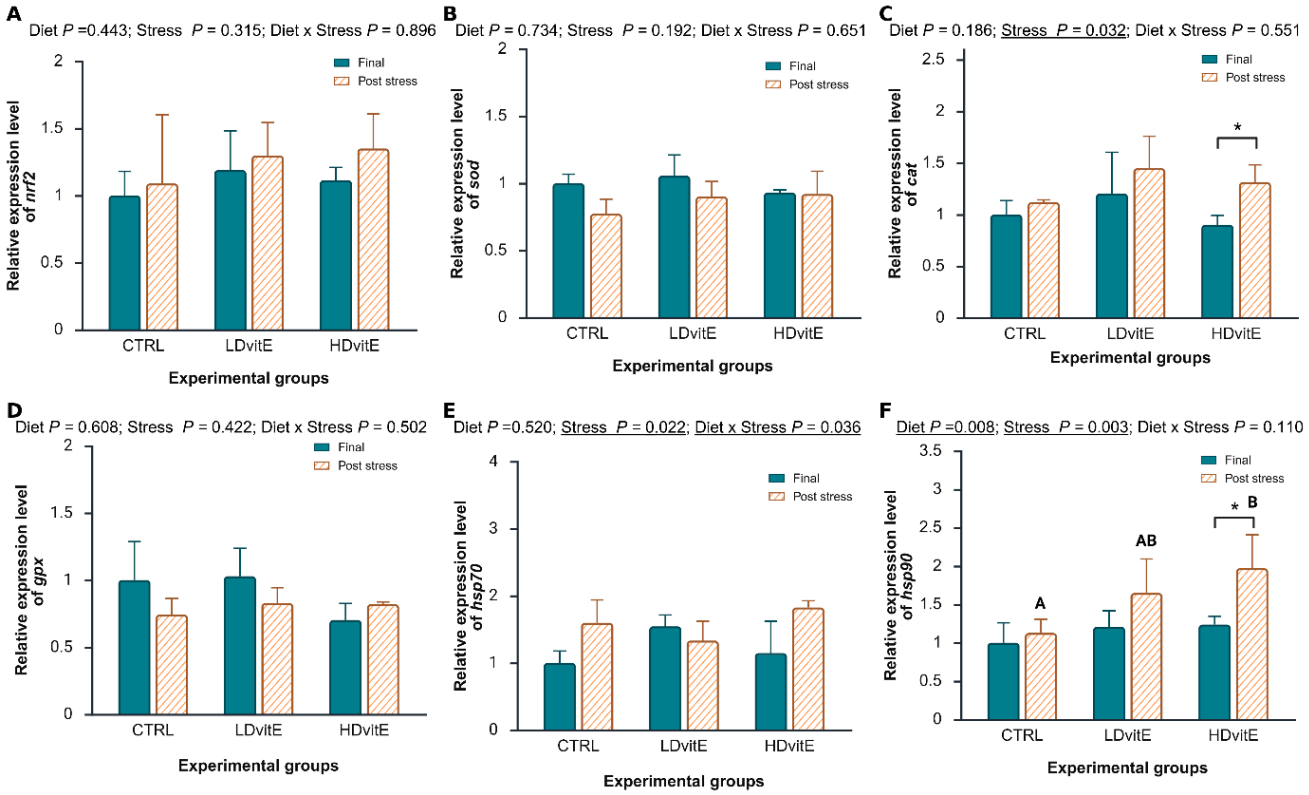


Figure 4. Relative mRNA expression levels of hepatic stress- and antioxidant-related genes in juvenile rainbow trout fed different experimental diets before and after thermal stress: (**A**) *nrf2*, (**B**) *sod*, (**C**) *cat*, (**D**) *gpx*, (**E**) *hsp70*, and (**F**) *hsp90*. Bars represent mean ± SD of replicate tank means (n = 3). Within each sampling point, different uppercase letters indicate significant differences among dietary treatments after thermal stress (one-way ANOVA followed by Tukey's test, $p < 0.05$). Asterisks (*) indicate significant differences between the end of the feeding period and post-stress values within the same treatment (independent-samples *t*-test, $p < 0.05$). Two-way ANOVA *p*-values for Diet, Stress, and Diet x Stress interaction are shown in each panel.

Hepatic expression of the immune-related genes is shown in Figure 5A–I. Diet significantly affected *myd88* ($p = 0.003$), *defb3* ($p = 0.030$), and *hepcidin* ($p = 0.001$), while thermal stress significantly affected *myd88* ($p = 0.028$), *il1β* ($p = 0.002$), *il8* ($p = 0.013$), *il4/13* ($p = 0.032$),

and *hepcidin* ($p = 0.001$). No significant diet x stress interaction was detected for any of the immune-related genes ($p > 0.05$), and *tlr4*, *tnfa*, and *il10* were unaffected by either factor. At the end of the feeding period, *myd88* expression was significantly lower in HDvitE than in CTRL, whereas *hepcidin* expression was significantly higher in both LDvitE and HDvitE than in CTRL ($p < 0.05$). Following thermal stress, *defb3* expression was significantly higher in HDvitE than in CTRL ($p < 0.05$). Within-group comparisons further indicated that *il1β* increased significantly and *il8* decreased significantly in HDvitE following thermal stress, while *hepcidin* increased significantly in all three dietary groups ($p < 0.05$).

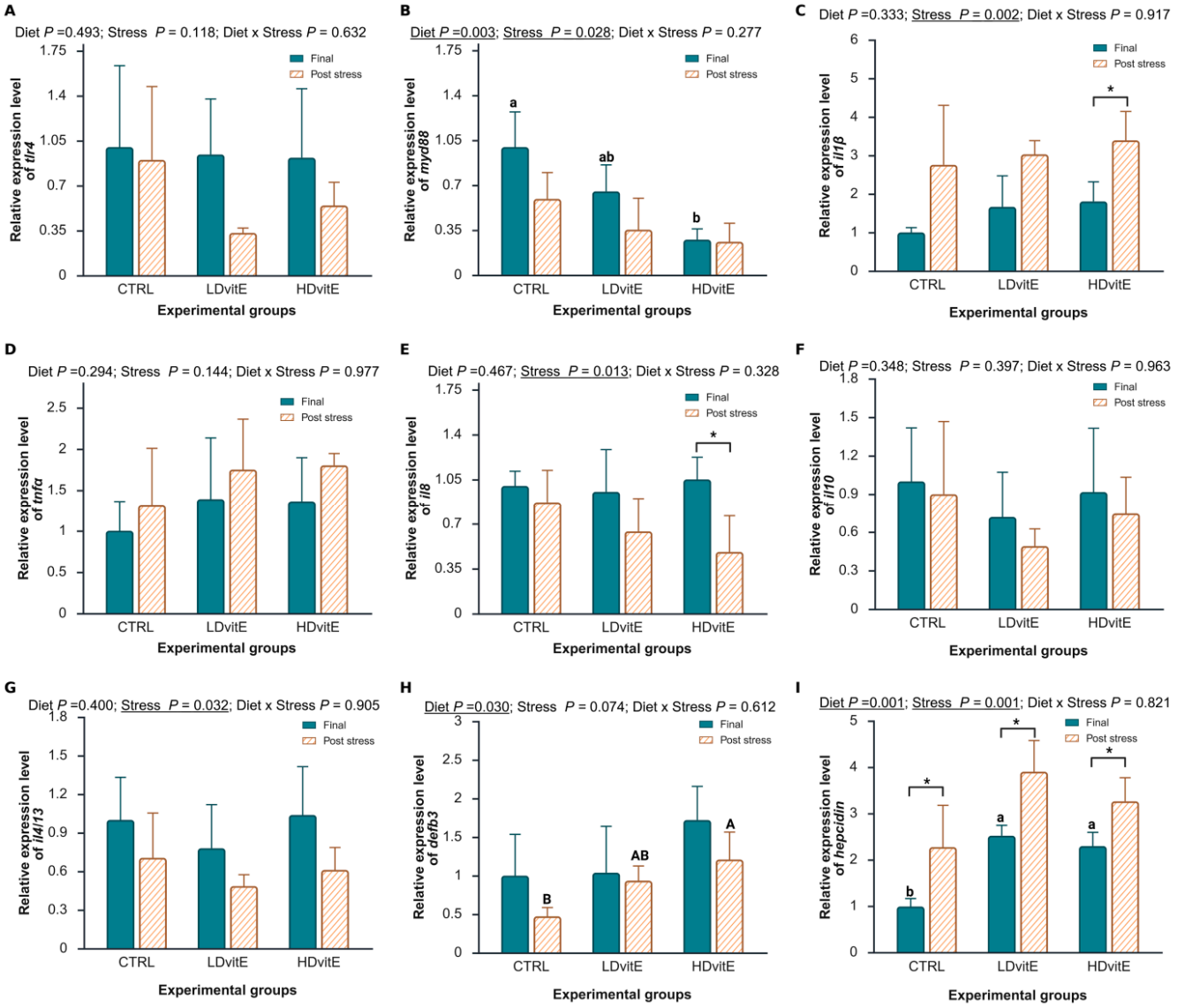


Figure 5. Relative mRNA expression levels of hepatic immune-related genes in juvenile rainbow trout fed different experimental diets before and after thermal stress: (**A**) *tlr4*, (**B**) *myd88*, (**C**) *il1β*, (**D**) *tnfa*, (**E**) *il8*, (**F**) *il10*, (**G**) *il4/13*, (**H**) *defb3*, and (**I**) *hepcidin*. Bars represent mean ± SD of replicate tank means (n = 3). Within each sampling point, different lowercase letters indicate significant differences among dietary treatments at the end of the feeding period, whereas different uppercase letters indicate significant differences among dietary treatments after thermal stress (one-way ANOVA followed by Tukey's test, $p < 0.05$). Asterisks (*) indicate significant differences between the end of the feeding period and post-stress values within the same treatment (independent-samples *t*-test, $p < 0.05$). Two-way ANOVA *p*-values for Diet, Stress, and Diet x Stress interaction are shown in each panel.

Hepatic expression levels of *α-ttp* and *cyp3a27* are presented in Figure 6A,B. Diet significantly affected *α-ttp* expression ($p = 0.006$), and there was a significant diet x stress interaction ($p < 0.001$). However, the overall effect of thermal stress was not significant ($p > 0.05$). At the end of the feeding period, *α-ttp* expression was significantly higher in both LDvitE and HDvitE than in CTRL ($p < 0.05$). After thermal stress, *α-ttp* expression increased in CTRL, decreased in LDvitE, and showed no significant change in HDvitE. In contrast, expression of *cyp3a27* was significantly affected only by diet ($p = 0.037$); there were no significant effects of thermal stress or the diet x stress interaction ($p > 0.05$). At the end of the feeding period, *cyp3a27* expression was significantly higher in HDvitE than in CTRL ($p < 0.05$).

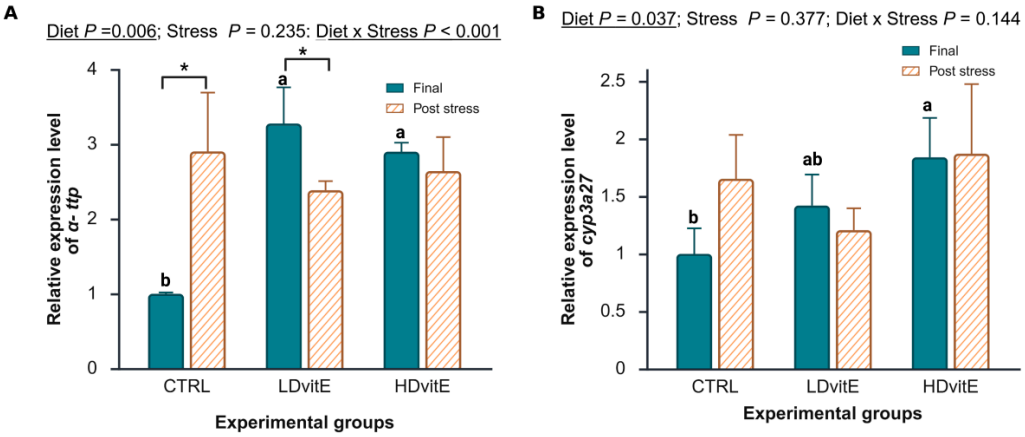


Figure 6. Relative mRNA expression levels of selected hepatic vitamin E transport and metabolic genes in juvenile rainbow trout fed different experimental diets before and after thermal stress: (**A**) *α-ttp* and (**B**) *cyp3a27*. Bars represent mean ± SD of replicate tank means (n = 3). Within each sampling point, different lowercase letters indicate significant differences among dietary treatments at the end of the feeding period (one-way ANOVA followed by Tukey's test, $p < 0.05$). Asterisks (*) indicate significant differences between the end of the feeding period and post-stress values within the same treatment (independent-samples *t*-test, $p < 0.05$). Two-way ANOVA *p*-values for Diet, Stress, and Diet x Stress interaction are shown in each panel.

4. Discussion

The main finding of the present study was that the HDvitE diet increased CTMax, while additional dietary vitamin E modulated hepatic antioxidant markers and several physiological and transcriptional responses before and after progressive warming. Across all dietary treatments, fish exhibited substantial growth, reaching approximately nine times their initial body weight, while growth performance and somatic indices did not differ among groups. This suggests that the vitamin E supplied by the practical CTRL diet was adequate to support growth under the present experimental conditions and that additional supplementation had no adverse effects on these parameters. Consistent with these findings, Faizan et al. [37] reported no significant differences in final weight or FCR in Atlantic salmon fed diets containing 22–310 mg/kg α-tocopherol. Similarly, Yıldız [38] reported no significant differences in final weight, BWG, SGR, HSI, or VSI in rainbow trout across dietary vitamin E concentrations of 130.2–580.9 mg/kg. Fillet proximate composition was largely unaffected by dietary vitamin E, thermal stress, or their interaction; protein, lipid, and dry matter contents remained stable, consistent with findings in rainbow trout fed graded or high vitamin E levels [38,39]. The absence of thermal effects on these major proximate constituents might be attributed to the gradual warming regime and the short exposure at the final temperature, as changes in energy allocation may not yet be evident in fillet proximate composition.

Fillet fatty acid composition was generally stable, with no significant changes in PUFA, n−3, n−6, EPA, or DHA, suggesting that additional vitamin E was not associated with marked changes in these nutritionally important fractions under the present conditions. Similarly, Faizan et al. [37] reported unchanged fillet EPA and DHA concentrations in Atlantic salmon following α-tocopherol supplementation. However, Zhang et al. [40] reported higher raw muscle DHA and n−3 PUFA contents at moderate dietary vitamin E levels than at lower or higher levels in golden pompano, indicating that responses may vary among species and experimental conditions. Despite this overall stability, selective differences emerged following thermal stress: total SFA was significantly lower in HDvitE than in CTRL and LDvitE, whereas total MUFA decreased significantly within HDvitE. In rainbow trout, heat stress has been shown to alter hepatic PUFA metabolism, with associated changes in fatty acid composition discussed in relation to thermal adaptation and membrane fluidity [41]. Although these responses were characterized in the liver, they demonstrate that thermal stress can modify fatty acid metabolism in rainbow trout. Selective fatty acid utilization has also been demonstrated in isolated rainbow trout red muscle mitochondria, where several SFA and MUFA were oxidized more rapidly than the essential C18 PUFAs, 18:2n−6 and 18:3n−3 [42]. Together, these findings provide a possible physiological context for the selective SFA and MUFA changes observed in HDvitE. However, as fatty acids were expressed only as relative proportions, absolute fatty acid mobilization or β-oxidation cannot be directly inferred.

Critical thermal maximum (CTMax) is widely used as a measure of acute upper thermal tolerance in fish [43]. Using an acclimation temperature of 17 °C and a heating rate of 0.3 °C min^{-1}, as applied in the present study, Pandey et al. [44] reported a mean CTMax of 27.6 °C, which fell within the range of CTMax values measured across the dietary treatments. Notably, CTMax was significantly higher in the HDvitE group than in the CTRL group, demonstrating increased acute upper thermal tolerance under the present test conditions. Although direct evidence linking dietary vitamin E supplementation to CTMax in fish remains limited, an antioxidant-supporting nutritional intervention has been shown to increase upper thermal tolerance: dietary selenium nanoparticles combined with vitamin B2 (riboflavin) increased CTMax and enhanced protection against oxidative and cellular stress in *Pangasianodon hypophthalmus* [45]. Further supporting a possible association between oxidative protection and upper thermal tolerance, Christen et al. [46] found that greater susceptibility of cardiac membrane lipids to peroxidation was associated with lower CTMax in salmonids. These studies provide a physiological basis for considering antioxidant and membrane protection as possible contributors to the observed CTMax response. However, this mechanistic explanation remains hypothetical because the present design did not directly establish a causal relationship between these processes and CTMax.

As a widely used indicator of physiological status in fish [47], HCT was significantly affected by thermal stress, although a post-stress decrease was significant only in the LDvitE group. This indicates that dietary vitamin E did not have a consistent effect on HCT under the present conditions, in agreement with Faizan et al. [37], who reported no significant differences in hematocrit among Atlantic salmon fed increasing levels of dietary α-tocopherol. Elevated temperatures increase metabolic and respiratory demands and may induce hematological adjustments; consequently, HCT responses to warming may depend on the intensity and duration of thermal exposure [3]. In rainbow trout, Rebl et al. [48] observed no significant change in hematocrit following a gradual temperature increase from 16 to 27 °C, whereas Alak et al. [49] reported decreased HCT with increasing water temperature. In this study, ferritin and TIBC remained unchanged, whereas serum iron decreased only in the CTRL group after thermal stress but remained stable in the vitamin E-supplemented groups. This pattern suggests that vitamin E may have helped

preserve circulating iron during the thermal challenge. However, the unchanged ferritin and TIBC levels indicate that this pattern alone does not provide clear evidence of altered iron storage or iron-binding capacity; the interplay among dietary vitamin E, thermal stress, and iron regulation therefore warrants further investigation. Total protein and albumin were likewise unchanged, consistent with previous findings showing no dietary vitamin E effect on circulating protein in largemouth bass [50] or serum albumin in juvenile grass carp [51].

Circulating immunoglobulins are major components of humoral immune defense in fish. In the present study, total Ig increased after thermal stress in all dietary groups, suggesting that this response was primarily associated with the warming challenge rather than vitamin E supplementation. A temperature-dependent increase in circulating IgM has also been reported in Nile tilapia, with plasma IgM increasing within a moderate thermal range before declining at higher temperatures [52]. These findings suggest that immunoglobulin responses to elevated temperature may vary with the thermal regime and exposure conditions.

Thermal stress activates the neuroendocrine stress response in fish, leading to the release of cortisol, the principal glucocorticoid coordinating physiological and metabolic adjustments to environmental challenges [3]. Serum cortisol was significantly influenced by both diet and thermal stress, with concentrations increasing overall after the thermal challenge and remaining lowest in the HDvitE group at both sampling points. A similar increase in circulating cortisol has been reported in triploid rainbow trout exposed to chronic thermal stress [53]. Consistent with the dietary pattern observed here, Albaqami and Monier [16] reported progressively lower plasma cortisol with increasing dietary α-tocopherol acetate, both before and after thermal stress in Nile tilapia. They suggested that this pattern may be related to the antioxidant action of α-tocopherol, which may limit the accumulation of reactive oxygen species and consequent oxidative stress. Although this mechanism provides a possible context for the lower cortisol concentrations observed in HDvitE, serum cortisol is not a direct indicator of antioxidant capacity, and this association cannot be established from the present data.

In contrast to cortisol, serum glucose decreased after thermal stress in all dietary groups and exhibited a significant diet x stress interaction, indicating that the glucose response to warming was influenced by diet. Notably, the HDvitE group showed the lowest post-stress glucose concentration, suggesting that high dietary vitamin E modulated the glucose response to the thermal challenge. Circulating glucose responses to thermal stress appear to depend on the exposure regime and sampling time. Pérez-Casanova et al. [54] observed transient increases in plasma glucose during gradual warming, whereas chronic thermal stress in triploid rainbow trout resulted in unchanged plasma glucose despite enhanced hepatic glycogenolysis [53]. Higher dietary α-tocopherol acetate was also associated with lower post-stress plasma glucose in Nile tilapia [16]. The lower post-stress glucose concentration in HDvitE may be associated with altered energy mobilization or glucose homeostasis during gradual warming [3]. Accordingly, these serum indicators are interpreted as diet-associated physiological responses to warming rather than as direct evidence of reduced stress or enhanced thermal tolerance.

Serum AST activity is commonly used to assess tissue integrity and hepatic function in fish because cellular injury and increased membrane permeability can release intracellular AST into the circulation [16]. In the present study, serum AST activity was significantly influenced by both diet and thermal stress, with values increasing overall after the thermal challenge but remaining significantly lower in HDvitE than in CTRL at both sampling points. Similarly, chronic thermal stress increased plasma AST in triploid rainbow trout, whereas higher dietary α-tocopherol acetate reduced post-stress AST activity in Nile

tilapia [16,53]. Taken together, these findings suggest that high dietary vitamin E may have contributed to better preservation of cellular integrity in the HDvitE group.

Vitamin E protects PUFA-rich cellular membranes from lipid oxidation by acting as a chain-breaking antioxidant and donating hydrogen to lipid peroxyl radicals [9–11]. Consistent with this function, hepatic TBARS levels were lower in both vitamin E-supplemented groups at the end of the feeding period, indicating reduced lipid peroxidation before the thermal challenge. Similar reductions in lipid peroxidation markers following vitamin E supplementation have been reported in grass carp, juvenile cobia, and Nile tilapia [51,55,56]. At the same sampling point, vitamin E supplementation increased hepatic CAT activity, whereas SOD activity remained unchanged, indicating a selective rather than uniform antioxidant enzyme response. Previous studies likewise indicate that vitamin E can reduce lipid peroxidation without uniformly increasing hepatic antioxidant enzyme activities and that these responses vary among species and vitamin E inclusion levels [37,40,57,58]. Following thermal stress, SOD activity was higher in LDvitE than in CTRL and HDvitE. Hepatic CAT activity did not differ among dietary groups, although it decreased significantly after thermal stress within HDvitE. Temperature-related changes in CAT activity vary among fish species [59], with reduced hepatic CAT activity reported in spotted seabass exposed to elevated temperature [15]. In Nile tilapia, thermal stress suppressed hepatic SOD, CAT, and GPx activities and increased lipid peroxidation, whereas dietary α-tocopherol acetate alleviated these effects [16]. In European seabass, lower antioxidant enzyme activities in antioxidant-supplemented fish during extreme warming were associated with lower oxidative damage rather than impaired antioxidant defense [60]. Accordingly, the decline in CAT activity in HDvitE should not necessarily be interpreted as impaired antioxidant defense, particularly because hepatic TBARS levels did not increase in this group. Overall, vitamin E supplementation was associated with reduced hepatic lipid peroxidation and increased CAT activity at the end of the feeding period, whereas antioxidant responses following thermal stress were more variable.

At the transcriptional level, thermal stress significantly affected hepatic *cat* expression; within this overall response, a significant post-stress increase was detected only in the HDvitE group, whereas *nrf2*, *sod*, and *gpx* expression showed no significant changes. This increase in *cat* transcript abundance was not reflected at the enzyme level: CAT activity decreased after thermal stress in HDvitE, indicating a divergence between transcript abundance and enzyme activity. Similar transcript–enzyme discrepancies have been reported in heatwave-exposed rainbow trout and vitamin E-fed juvenile Chinese sturgeon [57,61]. Huang et al. [23] attributed a similar lack of correspondence between antioxidant enzyme activity and mRNA levels to post-transcriptional regulation and qualitative enzyme modifications; enzyme turnover and differences in the timing of transcriptional and enzymatic responses may also have contributed to the discrepancy observed here.

Thermal stress can disrupt proteostasis and increase reliance on molecular chaperones [62,63], with HSP70 promoting the refolding and repair of denatured proteins and HSP90 supporting protein folding, degradation, and cellular signaling [64,65]. In the present study, hepatic *hsp70* showed a modest and variable transcriptional response to gradual warming, without significant pairwise differences among dietary groups; in contrast, *hsp90* showed a more distinct post-stress increase in HDvitE. A similar distinction between hepatic HSP responses was reported by Riesco et al. [61], who observed increased *hsp90ab1* expression without significant *hsp70a* induction in rainbow trout exposed to a gradual heatwave and noted that *hsp* transcriptional responses may differ between acute and prolonged exposures depending on stress magnitude and duration. Pandey et al. [44] likewise reported distinct temporal patterns among hepatic HSP transcripts, with non-significant changes in *hsp70a/b*, an early peak in *hsp90a*, and a more sustained *hsp90b* response. Sim-

ilarly, Hosseinpour et al. [8] showed transcript-specific HSP regulation in rainbow trout during heat shock, with *hsp70β* showing a more limited response than *hsp70α*, whereas *hsp90β* was more clearly induced and modulated by antioxidant-supplemented diets. Taken together, these findings suggest that the limited hepatic *hsp70* response at the post-stress sampling point may reflect the kinetics of *hsp70* expression and transcript-specific HSP regulation, rather than an absence of chaperone involvement. In contrast, the increase in *hsp90* expression in the HDvitE group may reflect selective transcriptional activation of the chaperone response associated with protein stabilization and cellular stress management during the thermal challenge.

Vitamin E can modulate inflammatory and innate immune responses in fish, although these effects appear to depend on vitamin E status rather than increase progressively with dietary supply. In Atlantic salmon, vitamin E deficiency impaired nonspecific immune function, whereas supplementation beyond adequate levels did not consistently provide additional immunostimulatory or baseline immunomodulatory effects [14]. In the present study, basal hepatic inflammatory gene expression was largely unaffected by additional vitamin E: *tlr4*, *il1β*, *tnfa*, *il8*, *il10*, and *il4/13* did not differ among dietary groups, whereas *myd88* expression was lower in HDvitE. Because MyD88 is a central adaptor linking TLR activation to downstream inflammatory signaling [66], its lower expression may indicate pathway-specific modulation; however, the absence of corresponding changes in the other inflammatory genes does not support broad suppression of basal inflammatory activity. A similarly gene-specific response was reported in longfin yellowtail, where high α-tocopherol supplementation did not affect hepatic *myd88* or *il10* expression but increased *il1β* expression [58]. In GIFT tilapia and spotted seabass, inflammatory gene responses also varied with vitamin E supply, without a uniform increase at higher dietary levels [15,67]. These findings suggest that vitamin E enrichment has limited effects on basal inflammatory gene expression when dietary supply is already adequate. Thermal stress altered hepatic expression of *myd88*, *il1β*, *il8*, and *il4/13*, whereas *tlr4*, *tnfa*, and *il10* remained unchanged. IL-1β contributes to pro-inflammatory signaling [68], IL-8 mediates leukocyte recruitment [69], and IL-10 and IL-4/13 have counter-regulatory roles [68,70]. Within HDvitE, the post-stress increase in *il1β* together with the decrease in *il8* therefore indicates differential rather than uniform regulation of inflammatory mediators, consistent with the variable cytokine responses reported across fish species and thermal exposure regimes [3].

β-Defensins and hepcidin are antimicrobial peptides involved in innate defense in fish [71–73]. In the present study, hepatic *hepcidin* expression was higher in both vitamin E-supplemented groups at the end of the feeding period. Dietary vitamin E has likewise been shown to alter *β-defensin* and *hepcidin* expression in grass carp, with responses varying among tissues and dietary levels [74]. Following thermal stress, *defb3* expression was higher in HDvitE than in CTRL, while *hepcidin* expression increased in all dietary groups. Thermal challenge has also been shown to alter skin *β-defensin 3* expression in rainbow trout [75]. Likewise, increased *hepcidin* expression has been reported in the liver of yellow catfish following acute thermal stress [76], in the skin of turbot during progressive warming [77], and in the gills of black cusk-eel following high-temperature exposure [78]. Together, these findings suggest that additional vitamin E selectively modulated basal *hepcidin* and post-stress *defb3* expression at the transcriptional level, whereas the general post-stress increase in *hepcidin* reflected a broader response to warming rather than a generalized enhancement of innate immune function.

α-TTP is involved in the hepatic transport and distribution of α-tocopherol [9]. In the present study, hepatic *α-ttp* expression was higher in both vitamin E-supplemented groups than in CTRL at the end of the feeding period, whereas fillet α-tocopherol concentration

was significantly higher only in HDvitE. Faizan et al. [37] similarly reported increasing α-tocopherol concentrations in Atlantic salmon fillets with increasing dietary supplementation. However, the higher *α-ttp* expression in LDvitE without a corresponding increase in fillet α-tocopherol indicates that hepatic *α-ttp* expression did not directly parallel muscle deposition. Consistent with this interpretation, Watt et al. [79] found that differences in hepatic α-tocopherol status in zebrafish were not accompanied by corresponding differences in *ttpa* expression. Following thermal stress, *α-ttp* expression increased in CTRL, decreased in LDvitE, and remained unchanged in HDvitE, resulting in a significant diet x stress interaction. The increase in CTRL may reflect a compensatory response to maintain α-tocopherol availability under thermal challenge, whereas the stable expression in HDvitE may indicate a lower need for transcriptional adjustment when dietary vitamin E supply is high. Overall, these contrasting responses suggest diet-dependent regulation of hepatic *α-ttp* during thermal stress, potentially contributing to α-tocopherol homeostasis. However, because α-tocopherol concentrations were not determined in the liver or after thermal stress, future studies should measure α-tocopherol in the liver and muscle before and after thermal challenge to clarify their relationship with hepatic *α-ttp* expression.

CYP3A27 is involved in hepatic biotransformation and responds to nutritional changes in rainbow trout [80]. In the present study, hepatic *cyp3a27* expression was higher in HDvitE than in CTRL at the end of the feeding period and was unaffected by thermal stress. Although mammalian studies suggest that α-tocopherol can influence CYP3A regulation [81], whether a similar relationship exists in fish remains unknown. Thus, the observed response appears to be associated with high dietary vitamin E supply rather than warming, although its biological significance requires further investigation.

More broadly, the findings of this study should be interpreted within the scope of the experimental design. Because only two levels of additional vitamin E supplementation above the practical CTRL diet were tested, the study does not define an optimal supplementation level. Moreover, responses were evaluated under a single progressive warming protocol and at one post-stress time point, so their temporal course and persistence, as well as their applicability to other thermal exposure scenarios, cannot be determined.

5. Conclusions

In conclusion, dietary vitamin E supplementation, particularly at the HDvitE level, improved thermal resilience in juvenile rainbow trout without adverse effects on growth performance or major fillet compositional traits. Fish fed the HDvitE diet showed the clearest overall response, characterized by higher CTMax, lower hepatic TBARS levels and higher CAT activity before thermal stress, lower serum cortisol and AST levels, and increased post-stress hepatic expression of *hsp90* and *defb3*. However, the study tested only two additional supplementation levels under a single progressive warming protocol and at one post-stress sampling point; therefore, it does not define an optimal supplementation level or establish whether the observed responses persist under other thermal scenarios. Future studies should refine the dose–response relationship and evaluate different warming intensities and recovery periods. Measuring α-tocopherol concentrations in the liver and muscle after thermal challenge, assessing Nrf2–Keap1 pathway activation, and evaluating humoral immune indicators such as lysozyme, complement, and serum bactericidal activities would further clarify the underlying redox mechanisms and the functional significance of the immune-related transcriptional responses. Collectively, the present findings indicate that dietary vitamin E supplementation may serve as a nutritional preconditioning strategy before predictable seasonal increases in water temperature under farming conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes11100600/s1, Table S1: Selected fatty acids and summed fatty acid classes (area %) of the experimental diets.

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Institutional Review Board Statement: The animal study protocol was approved by the Çukurova University Animal Experiments Local Ethics Committee at its fifth meeting on 28 May 2024. All procedures involving fish were conducted in accordance with the relevant national and institutional guidelines for the care and use of experimental animals.

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