

Article

Effects of Dietary Microencapsulated Ctx(Ile²¹)-Ha on Egg Quality and Physiological Responses in Laying Hens Subjected to Heat Stress

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[†] In memoriam and honor of Prof. Dr. Danilo Florentino Pereira.

Abstract

This exploratory proof-of-concept study evaluated the physiological tolerability and performance of laying hens fed the microencapsulated antimicrobial peptide Ctx(Ile²¹)-Ha under severe heat stress (34.5 °C; THI = 85.3). Eighteen 40-week-old hens were evaluated in a 2 × 3 factorial design comprising two thermal environments (comfort: 26 °C; heat stress: 35 °C) and three peptide doses (0.0, 2.5, and 5.0 mg/kg feed). The peptide was synthesized (>95% purity) and microencapsulated by ionotropic gelation, exhibiting controlled *in vitro* intestinal release. Heat stress significantly reduced feed intake and egg weight ($p < 0.05$) while increasing rectal temperature ($p < 0.01$), consistent with a biologically relevant thermal challenge. No temperature × dose interactions were observed for feed intake, egg weight, feed conversion, Haugh unit, albumen height, or blood biochemical parameters ($p > 0.05$). However, significant interactions were detected for eggshell quality parameters ($p < 0.05$). In conclusion, while dietary Ctx(Ile²¹)-Ha did not mitigate performance losses induced by heat stress, the absence of blood biochemical alterations supports its preliminary physiological tolerability under these experimental conditions. Due to the limited sample size ($n = 3$), these exploratory findings require cautious interpretation.

Keywords: antimicrobial peptide; Ctx(Ile²¹)-Ha; heat stress; laying hens; microencapsulation; egg quality; physiological response; poultry



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1. Introduction

Antimicrobial peptides (AMPs) are naturally occurring components of innate immunity [1]. Their mechanisms of action are diverse: some AMPs are membranolytic and disrupt microbial membranes, whereas others can act through intracellular targets or other non-membranolytic pathways. Therefore, membrane disruption should not be generalized to all AMPs, and AMPs should not be considered inherently resistant to the development of antimicrobial resistance [2,3]. These biological properties have stimulated interest in the use of selected AMPs as feed additives in poultry production [4], particularly in laying hen farming to support animal health and egg quality [5].

Selected AMPs have shown potential to support bird health through antimicrobial, immunomodulatory, and anti-inflammatory activities [6,7]. Proposed benefits may involve modulation of the gastrointestinal microbiota, maintenance of intestinal barrier integrity,

regulation of inflammatory responses, and effects on nutrient utilization. These pathways could contribute indirectly to zootechnical performance, but their magnitude depends on peptide sequence, dose, delivery system, animal age, and physiological or health status. A recent meta-analysis confirmed that dietary AMPs significantly improve feed conversion, support immune organ development, and enhance small intestinal morphology by increasing villus height in broiler chickens [8]. Importantly, the intestinal, immunological, and nutritional mechanisms described here are proposed biological pathways and were not directly measured in the present study.

Heat stress is an important challenge in laying-hen production because elevated temperatures can reduce feed intake and alter egg production, egg weight, eggshell characteristics, and physiological homeostasis [9–12]. Acute and chronic heat stress may produce different responses: acute exposure can induce rapid behavioral and thermoregulatory changes, whereas prolonged exposure can produce sustained reductions in feed intake and production and may progressively affect eggshell and albumen quality [11]. In the present study, hens were exposed to a daytime temperature of 35 °C for the post-acclimatization experimental period, with relative humidity of approximately 56% during the heat-stress phase and approximately 68% under the 24–26 °C comfort conditions. This represented a sustained environmental challenge over the experimental period rather than an acute heat exposure.

Elevated temperature may also alter acid–base balance, electrolyte homeostasis, calcium utilization, intestinal function, and oxidative and inflammatory processes in poultry [9,11,13]. In chronic heat stress, increased intestinal permeability and disruption of the intestinal barrier have been proposed as contributors to systemic inflammatory burden. AMPs with antimicrobial, immunomodulatory, or barrier-stabilizing properties could theoretically influence these pathways by reducing pathogen pressure and supporting intestinal integrity. Under chronic thermal stress, laying hens experience increased intestinal permeability (commonly referred to as ‘leaky gut’) [14] and subsequent systemic endotoxemia, which triggers a chronic low-grade inflammatory response and disrupts critical metabolic pathways, including calcium transport. Beyond their direct membrane-disrupting antimicrobial activity, selected AMPs exhibit potent immunomodulatory, anti-inflammatory, and intestinal barrier-stabilizing properties.

We hypothesized that dietary supplementation with microencapsulated Ctx(Ile²¹)-Ha would stabilize the gut mucosal barrier, prevent pathogen translocation and reduce the systemic inflammatory burden, thereby indirectly supporting nutrient absorption and systemic homeostasis under thermal challenge [6,7,9]. However, gut permeability, microbiota composition, inflammatory cytokines, and oxidative-stress markers were not measured in the present study; therefore, these mechanisms remain hypothetical and should not be interpreted as demonstrated pathways. The biological rationale for the present experiment was consequently to test whether a peptide with previously reported antimicrobial activity and potential biological effects could alter selected measurable responses during thermal challenge.

Specifically, the AMP Ctx(Ile²¹)-Ha (GWLDVAKKIGKAAFNVAKNFI), isolated from the skin secretion of the frog *Boana albopunctata* [15], has previously been characterized for antimicrobial activity associated with interaction with bacterial membranes [16]. Microencapsulated Ctx(Ile²¹)-Ha has also been investigated in laying hens challenged with *Salmonella* Enteritidis, where reductions in bacterial burden and mortality-related outcomes were reported [17,18]. These infection studies provide supporting evidence for biological activity of the peptide in laying hens, but they do not reproduce the prolonged environmental heat-stress conditions evaluated here. Microencapsulation HPMCP coating was used to help protect the peptide during gastrointestinal transit and promote release under intestinal-like conditions, a process extensively documented for protecting active ingredients in poultry feeds [19].

The 2.5 and 5.0 mg/kg feed levels were selected based on previous *in vivo* evidence with Ctx(Ile²¹)-Ha and related antimicrobial-peptide studies in poultry. The 2.5 mg/kg level was supported by previous evidence of activity against *Salmonella Enteritidis* in laying hens [17]. The 5.0 mg/kg level was included to evaluate the response to a higher peptide dose under the environmental conditions of the present study. The experiment was not designed as a formal dose–response or safety-margin study; therefore, differences between the two doses should be interpreted as exploratory.

We hypothesized that the response to Ctx(Ile²¹)-Ha could differ according to the thermal environment because heat stress may alter intestinal function, microbial balance, nutrient utilization, and systemic homeostasis. Thus, the temperature × peptide-dose interaction was considered a biological hypothesis rather than an established effect. The study addressed two related questions: first, whether Ctx(Ile²¹)-Ha could mitigate selected physiological and productive effects associated with heat stress; and second, whether administration was associated with detectable adverse changes in the measured blood and productive variables. Accordingly, the objective was to evaluate body condition, egg production and quality, and selected hematological, biochemical, and physiological responses following prophylactic/preconditioning administration of microencapsulated Ctx(Ile²¹)-Ha under thermoneutral and heat-stress conditions.

2. Materials and Methods

2.1. Synthesis, Microencapsulation, and Release Assay of the Ctx(Ile²¹)-Ha Peptide

2.1.1. Synthesis, Purification and Characterization of the Ctx(Ile²¹)-Ha Peptide

The antimicrobial peptide Ctx(Ile²¹)-Ha was synthesized manually using the solid-phase peptide synthesis (SPPS) technique [20] shown in Figure 1. Rink Amide resin and the Fmoc strategy were used. Coupling was performed using DIC/HOBT and (DIEA)/HBTU for recoupling steps, with a reaction time of two hours. The base-labile Fmoc group was removed with 10% (*w/v*) piperazine in DMF/ethanol (9:1, *v/v*) for 20 min, followed by washing with DMF and DCM.

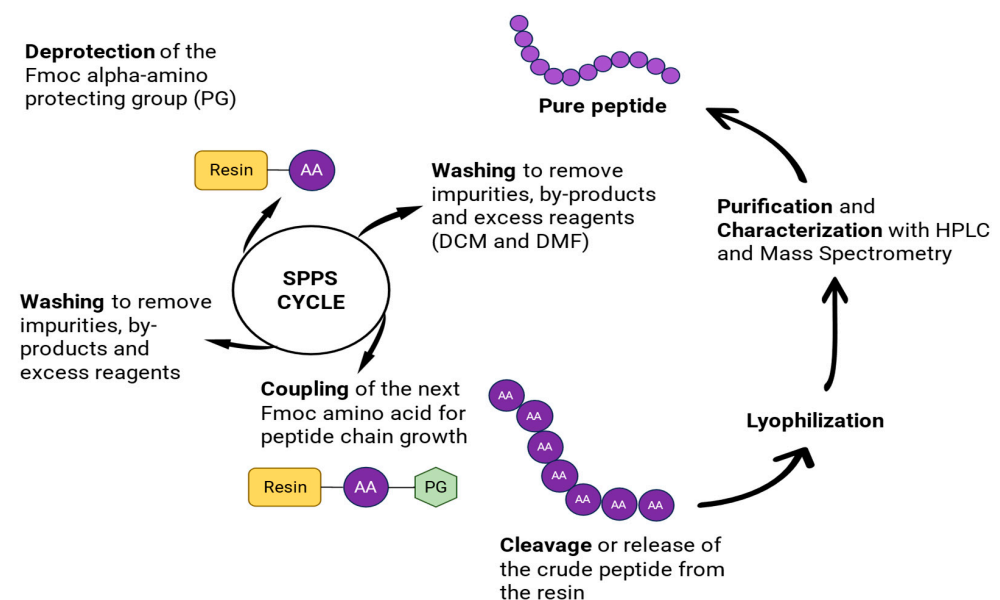


Figure 1. Solid-phase peptide synthesis (SPPS) technique flowchart employed in this work. [Enhanced Schematic Parameters: Rink Amide resin (1368 mg initial → 3500 mg peptidyl-resin); DIC/HOBT coupling for 2 h; 10% piperazine in DMF/ethanol (9:1, *v:v*) for 20 min; TFA/water/TIS cleavage solution (95:2.5:2.5, *v:v:v*); crude peptide yielded 2700 mg (77.14% efficiency)]. Legend: AA, amino acid and PG, protecting group.

After cleavage from the resin using TFA, ultrapure water, and TIS, the crude material was lyophilized. Purification was performed by reversed-phase HPLC using a C₁₈ analytical column (Shimadzu Prominence) with solvent A (0.045% TFA in ultrapure water) and solvent B (0.036% TFA in ACN), using a 5–95% B gradient over 30 min. The peptide was used in subsequent experiments only when analytical purity exceeded 95%. The synthesis yielded 2700 mg of crude peptide, corresponding to an overall synthesis efficiency of 77.14%. A separate purification-yield value was not calculated from the reported mass data.

Peptide identity was evaluated by mass spectrometry using a triple-quadrupole Shimadzu (Kyoto, Japan) spectrometer, model LCMS-8045 with ESI and direct injection coupled to a Shimadzu (Kyoto, Japan) Nexera X3 UHPLC system in scan mode. The theoretical molecular mass of Ctx(Ile²¹)-Ha was 2289.72 g mol^{−1}. The observed *m/z* signals (764.0 for *z* = +3, 573.3 for *z* = +4, and 459.0 for *z* = +5) were consistent with the expected multiply charged ions and were used together with the chromatographic purity profile to confirm peptide identity.

2.1.2. Microencapsulation and Coating of the Ctx(Ile²¹)-Ha Peptide

Two microencapsulation preparations were produced using initial peptide amounts of 100 mg and 200 mg. These amounts represent different peptide loads in the same ionotropic-gelation formulation approach rather than different coating materials. The corresponding encapsulation efficiencies were 79.84% and 58.47%, respectively. The two preparations were used as sources of peptides for dietary administration; no assumptions were made regarding particle size, as this parameter was not measured in the present study. (Figure 2).

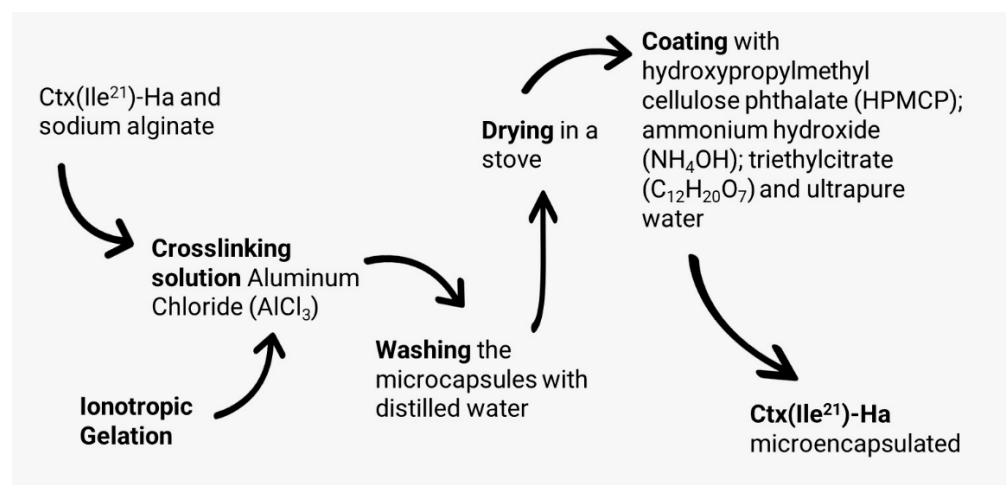


Figure 2. Microencapsulation technique flowchart employed in this study. [Enhanced Schematic Parameters: 10 g sodium alginate in 500 mL water; crosslinking in 5% AlCl₃ at 1 mL/min drip rate; oven drying at 35 °C for 24 h; HPMCP enterocoating (10% HPMCP, 25% NH₄OH, 2.5% triethyl citrate); Encapsulation efficiency: 79.84% (100 mg load) and 58.47% (200 mg load)].

Briefly, the peptide was mixed with sodium alginate at 10 g in 500 mL of water and homogenized with an Ultra-Turrax until a homogeneous alginate–peptide dispersion was obtained.

The crosslinking solution consisted of 5% AlCl₃. The alginate–peptide dispersion was added through a peristaltic pump coupled to a 200 µL pipette tip at 1 mL/min into the stirred crosslinking solution. The microparticles were recovered using a Büchner funnel, washed with 40 mL of ultrapure water, and dried in a continuous-flow oven at 35 °C for 24 h.

For coating, the microencapsulated particles were immersed in a 250 mL solution containing 10% (v/v) HPMCP, 25% (v/v) NH_4OH , 2.5% (v/v) triethyl citrate, and 62.5% (v/v) ultrapure water. These percentages refer to the final coating formulation.

The HPMCP coating was intended to reduce release under gastric-like conditions and favor release under intestinal-like pH; this *in vitro* behavior should not be interpreted as proof of *in vivo* intestinal delivery.

Microencapsulation efficiency was estimated using a Shimadzu UV1800 spectrophotometer by measuring absorbance at 280 nm after extraction of the microcapsules in a 1:1 (v/v) ultrapure-water/ACN solution for four hours. Because 280 nm is not peptide-specific, corresponding blank/reference measurements without peptide were used to minimize contributions from alginate, coating components, and other formulation constituents. The measurement was treated as an indirect spectrophotometric estimate based on the tryptophan residue in the Ctx(Ile²¹)-Ha sequence, and UV absorbance alone was not considered sufficient to confirm peptide identity or integrity.

A total of 50 mg of microcapsules was placed in contact with each simulated fluid at 42 °C. Samples were collected at 1, 2, 5, 10, 15, and 20 min for SGF and at 1, 2, 5, 10, 15, 20, 30, 45, and 60 min for SIF. Absorbance was measured at 280 nm as an indirect estimate of released peptide concentration using the Lambert–Beer equation (Equation (1)). The release values reported in Section 3.1.2 represent fractional (instantaneous) release at each sampling interval rather than cumulative release. Three analytical replicates ($n = 3$) were used for the release measurements. HPLC and/or mass spectrometry would be required to confirm the molecular integrity and identity of peptide recovered after release.

$$A = \epsilon lc \quad (1)$$

where A is the absorbance; ϵ represents the molar absorption coefficient, c is the molar concentration, and l corresponds to the optical path length (in cm) of the cuvette used.

2.1.3. Peptide Release Assays in Simulated Solutions of Avian Intestinal and Stomach Fluids

The SGF consisted of 10 mL of 0.1 mol/L HCl adjusted to pH 3.5. The SIF consisted of 10 mL of 0.1 mol/L HCl adjusted to pH 6.0 with 1 mol/L NaOH. This pH 6.0 solution serves as a physiologically relevant *in vitro* approximation of the upper avian intestinal environment (duodenum/jejunum), which is more acidic than mammalian intestinal fluids [21]. However, we acknowledge that this simplified *in vitro* model lacks pancreatic enzymes and bile salts, representing a limitation when extrapolating these *in vitro* release profiles to live hens.

This SIF is not a complete conventional intestinal fluid formulation; it was used as a simplified *in vitro* model approximating the mildly acidic-to-near-neutral intestinal environment of poultry. The simplification and the absence of digestive enzymes and other luminal components limit direct extrapolation to the gastrointestinal tract of live hens.

2.2. Experimental Design

A completely randomized 2×3 factorial arrangement was used, comprising two thermal environments (Comfort and Heat Stress) and three dietary peptide doses (0.0 [D0], 2.5 [D1], and 5.0 [D2] mg/kg of feed). A total of 18 Lohmann White laying hens at 40 weeks of age were used, with three hens per temperature $\times$ dose combination. Prior to the study, the hens were balanced and allocated across the experimental groups to ensure there were no significant differences in baseline characteristics, including initial body weight (mean: 1.58 ± 0.05 kg), baseline laying performance (mean laying rate: $94.4 \pm 2.1\%$), and age

(40 weeks). Within each climatic chamber, hens were randomly assigned to individual cages and dietary treatments using a random number generator to ensure unbiased allocation.

The sample size of three hens per treatment combination (18 hens in total) was determined in accordance with the ethical requirements of animal experimentation and the 3Rs framework (Replacement, Reduction, and Refinement), specifically under the Reduction principle to minimize animal use. To mitigate between animal variability while utilizing a reduced sample size, deliberate design choices were implemented, including the use of a genetically uniform commercial line from a single age cohort (40 weeks) housed in individual cages. Additionally, a major technical and independent constraint was the high production cost, labor-intensive manual solid-phase synthesis, and limited yield of the microencapsulated peptide, which restricted the total mass of active material available for in vivo administration. Given these ethical and technical constraints, this study was intentionally designed as an exploratory, proof-of-concept trial. Consequently, this small sample size increases the risk of Type II error and limits the ability to detect small or moderate effects; based on sensitivity power analysis, the design is suited to reliably detect only large treatment effects ($f \geq 0.8$).

The hens were monitored for 35 consecutive days, with the first seven days used for acclimatization before the thermal challenge. Nine hens were housed in each of two climatic chambers of identical dimensions (4.10 m × 3.50 m × 2.85 m), with one chamber assigned to the Comfort condition and the other to Heat Stress. Each hen was housed individually in a wire-floor cage (0.5 m × 0.5 m × 0.5 m).

During acclimatization, both groups were maintained at 26 °C during the light period and 24 °C during the dark period. After acclimatization, the Comfort chamber remained under these conditions, whereas the Heat Stress chamber was programmed to 35 °C during the light period and 26 °C during the dark period. The measured daytime temperature and relative humidity were 34.5 ± 0.72 °C and $56 \pm 2\%$ under heat stress, compared with 25.6 ± 0.94 °C and approximately 68 ± 5% under the 24–26 °C comfort conditions. Using the commonly applied THI equation $THI = 0.8T + [RH \times (T - 14.4)/100] + 46.4$ [22].

Peptide administration began during acclimatization, before the onset of heat stress, and therefore represents a prophylactic/preconditioning intervention rather than a purely therapeutic treatment. The microencapsulated peptide was administered once weekly as a discrete pulse dose homogenized into a 10 g feed aliquot (based on an average weekly intake of 770 g/hen), rather than as a continuous dietary concentration. Individual feeders were used to prevent competition and to monitor intake. The experimental data and samples were collected between June and July 2024.

Study timeline: Days −7 to 0: acclimatization under thermoneutral conditions; during acclimatization: once-weekly prophylactic/preconditioning administration of the assigned peptide dose; from the end of acclimatization through the 35-day experimental period: Comfort or Heat Stress environmental exposure; blood sampling: experimental days 1, 7, 14, 21, and 28; body-weight measurements: experimental days 1, 7, 14, 21, and 28; egg production and egg-quality observations: daily throughout the experimental period.

2.3. Management of Hens and Supplementation of Microencapsulated Ctx(Ile²¹)-Ha Peptide

The hens were fed twice daily, receiving 110 g of feed per day (55 g at 08:30 and 55 g at 16:00), with water available *ad libitum*. The feed ingredients and their nutritional levels are presented in Table 1. The weekly dose of the microencapsulated peptide (1.92 mg for D1 and 3.85 mg for D2) was calculated based on the average weekly feed intake (770 g per hen) and carefully homogenized into a small 10 g portion of the daily feed offered as a once-weekly pulse dose. This portion was offered in individual feeders to prevent competition and ensure exact consumption. Complete consumption of the peptide-containing feed portion

was visually verified for each hen before the remainder of the daily feed was provided. Thus, the reported mg/kg values describe the calculated weekly dose relative to the average weekly feed intake and should not be interpreted as a continuous dietary concentration.

Table 1. Ingredients and nutritional composition of the experimental diets provided to laying hens.

Ingredients	Quantities (%)
Corn	61.38
Soybean meal (46%)	24.00
Wheat bran	2.50
Pirius Emul Dry (plant-based lipid supplement) ¹	0.30
Fine ground limestone (CaCO ₃ source)	6.00
Coarse limestone gravel (CaCO ₃ source)	4.50
Sodium chloride (common salt)	0.44
Toxin sequestrant (Calibrim Afla) ²	0.13
DL-methionine (99%)	0.05
L-Lysine (98%)	0.05
Pigment for egg yolk (PX1017 Pig laying) ³	0.25
Organic mineral and vitamin premix ⁴	0.10
Enzyme blend (phytase and carbohydrases) ⁵	0.10
Dicalcium phosphate (Free Phos)	0.20
Total	100.00
Nutritional levels	Quantities (calculated)
Metabolizable Energy (kcal/kg)	2790
Crude protein (%)	16.96
Total lysine (%)	0.91
Total methionine (%)	0.39
Total tryptophan (%)	0.21
Total threonine (%)	0.66
Calcium (%)	4.02
Available phosphorus (%)	0.44
Total phosphorus (%)	0.68
Ether extract (%)	2.90
Sodium (%)	0.18
Calcium/Potassium ratio	9.11

¹ Prius Emul Dry: Commercial emulsified lipid supplement derived from plant-based ingredients, designed to enhance energy availability and nutrient absorption in poultry diets (Auster Nutri, Brazil; <https://www.austernutri.com.br/prius/>, accessed on 15 August 2025). ² Calibrim Afla: Commercial aflatoxin sequestrant, typically based on bentonite or similar clay minerals. ³ PX1017 Pig laying: Commercial feed additive used for enhancing yolk pigmentation. ⁴ Organic mineral and vitamin premix: Provides essential vitamins (A, D₃, E, K₃, B₁, B₂, B₆, B₁₂, pantothenic acid, niacin, biotin, folic acid) and minerals (Zn, Mn, Fe, Cu, Se, I). ⁵ Enzyme blend: Includes phytase and carbohydrase enzymes to improve nutrient digestibility.

Hens were weighed at housing and on experimental days 1, 7, 14, 21, and 28. Leftover feed was monitored daily. Feed intake was summarized per hen over the experimental period, and the overall feed conversion ratio was calculated from total feed intake and total egg weight over the experimental period.

Body-weight measurements obtained repeatedly from the same hen were retained as longitudinal observations and were evaluated descriptively through individual and group trend trajectories rather than treated as independent observations in the factorial ANOVA, preventing artificial inflation of the sample size.

Based on the daily feed consumption, overall feed conversion ratio was calculated from total feed intake and total egg weight over the experimental period and temperature treatment (comfort or heat stress) using Equation (2).

$$\text{FCR} = (\text{Total feed intake(g)}) / (\text{Total egg weight(g)}) \quad (2)$$

2.4. Analysis of Egg Production and Egg Quality

Eggs were collected and counted daily, and egg quality was assessed daily using specific gravity, egg weight, shell strength, shell thickness, albumen height, and Haugh unit. All daily egg-quality measurements from each individual hen were averaged across the entire experimental period, producing a single independent value per hen for the factorial analysis ($n = 3$ hens per temperature $\times$ dose combination).

2.5. Analysis of Blood and Physiological Parameters

Blood and physiological parameters were monitored on experimental days 1, 7, 14, 21, and 28. The same hens were sampled repeatedly, yielding 90 blood samples in total. Blood collection procedures [23,24], sampling times, and handling durations were standardized as much as possible across treatments. Sampling was performed under the same general feeding and thermal-exposure schedule for the experimental groups. Repeated venipuncture and handling were recognized as potential sources of acute physiological stress and are considered in the interpretation of the blood results.

Hemoglobin concentration was determined by optical spectrophotometry at 540 nm using the cyanmethemoglobin (Drabkin) colorimetric method (Labtest[®] kit, Ref. 43, Lagoa Santa, Brazil). Total plasma protein was measured using a veterinary clinical refractometer (Ion RHC-200[®], Araucária, Brazil) with an accuracy of 0.2 g/dL.

The measured blood variables included glucose, hemoglobin, hematocrit, plasma proteins, erythrocytes, leukocytes, Na^+ , K^+ , Cl^- , normalized calcium, ionic calcium, total calcium, and pH. These measurements constitute a limited physiological panel and should not be interpreted as a comprehensive assessment of hepatic, renal, oxidative, inflammatory, or histopathological safety, which represents a limitation of this preliminary tolerability study.

2.6. Statistical Analysis

Data were analyzed according to a 2×3 factorial arrangement comprising two thermal environments (Comfort: 26 °C; Heat Stress: 35 °C) and three dietary peptide doses (D0: 0.0, D1: 2.5, and D2: 5.0 mg/kg of feed), utilizing 18 laying hens in total ($n = 3$ hens per temperature $\times$ dose combination). The individual hen served as the experimental unit for dietary peptide treatments. This sample size was defined in accordance with the ethical requirements of animal experimentation and the 3Rs framework (specifically the Reduction principle) to minimize animal use, alongside technical constraints regarding peptide mass availability from manual solid-phase synthesis and ionotropic gelation. Consequently, the trial was intentionally structured as an exploratory, proof-of-concept assessment of physiological tolerability.

Because environmental conditions were maintained using a single climatic chamber per thermal regime, the thermal effect is inherently confounded with chamber location; therefore, temperature-related p -values and temperature $\times$ dose interactions are reported as exploratory associations rather than inferential proof of isolated thermal effects. For performance variables summarized over the experimental period (total feed intake, total egg weight, and overall feed conversion ratio), egg quality parameters, and blood parameters, analysis of variance (ANOVA) was conducted using the General Linear Model (GLM) procedure in Minitab 19. The statistical model evaluated the fixed main effects of thermal

condition, peptide dose, and their two-way interaction (Temperature $\times$ Dose). When main effects or interactions were statistically significant ($p \leq 0.05$), mean comparisons were performed using Fisher's protected Least Significant Difference (LSD) test.

3. Results

3.1. Obtaining of the Ctx(Ile²¹)-Ha Peptide

At the end of the synthesis process, the initial peptidyl-resin mass was 1368 mg and the final mass was 3500 mg. The peptide synthesis yielded 2700 mg of crude peptide, corresponding to an overall synthesis efficiency of 77.14%.

The chromatographic profile (Figure 3) showed a peptide purity greater than 95%, and only material meeting this criterion was used in subsequent experiments. The mass-spectrum signals reported in Figure 4 (m/z 764.0, 573.3, and 459.0 for $z = +3$, $+4$, and $+5$, respectively) were consistent with the theoretical molecular mass of 2289.72 g mol⁻¹.

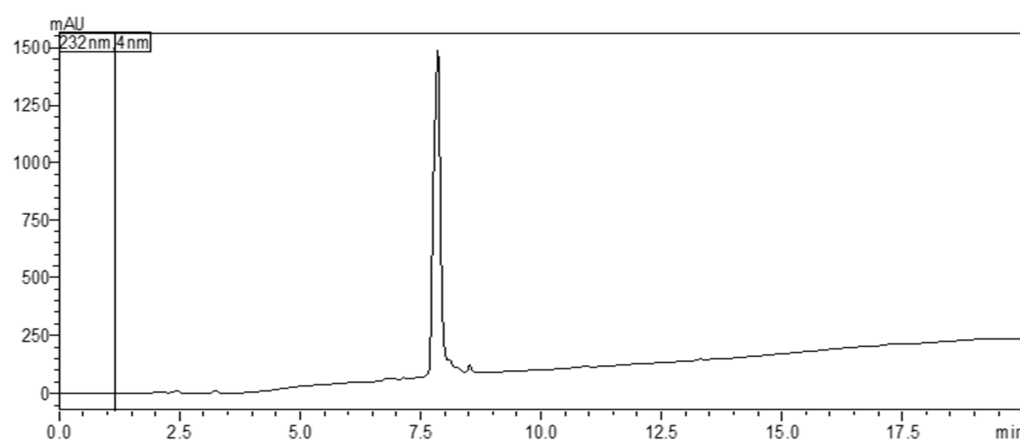


Figure 3. Chromatographic profile (RP-HPLC) of the purified antimicrobial peptide Ctx(Ile²¹)-Ha. [High-resolution profile confirming >95% analytical purity with a sharp retention peak under 5–95% solvent B gradient over 30 min].

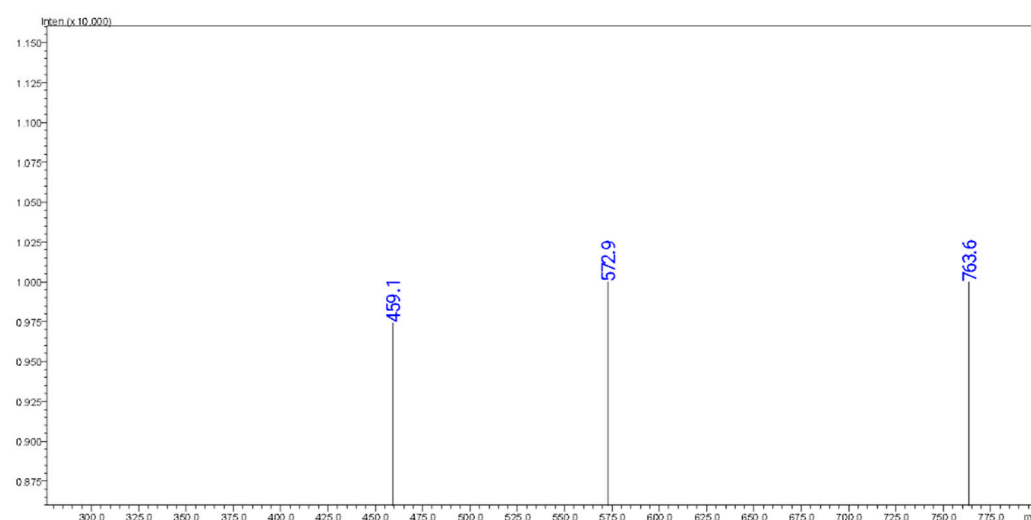


Figure 4. Mass spectrum (ESI-MS) of purified antimicrobial peptide Ctx(Ile²¹)-Ha. Theoretical molecular mass: 2289.72 g mol⁻¹; observed m/z signals: 764.0 ($[M + 3H]^{3+}$), 573.3 ($[M + 4H]^{4+}$), and 459.0 ($[M + 5H]^{5+}$), confirming successful peptide synthesis and identity.

3.1.1. Microencapsulation and Coating of the Antimicrobial Peptide Ctx(Ile²¹)-Ha

The formulation prepared from 100 mg of peptide incorporated 79.84 mg, corresponding to an encapsulation efficiency of 79.84%. The formulation prepared from 200 mg

incorporated 116.94 mg, corresponding to an encapsulation efficiency of 58.47%. These values therefore represent two peptide-loading preparations using the same ionotropic-gelation and HPMCP-coating approach, not different coating materials.

3.1.2. Release Tests of Microcapsules in Stomach and Intestinal Solutions

The release data do not represent cumulative release. They represent the fractional (instantaneous) percentage released at each sampling interval; therefore, values may increase or decrease between successive time points. At 5 min, the highest fractional release observed in SGF was 9.74% for the 200 mg preparation (Table 2).

In SIF, high fractional release values were observed at several individual time points, including 93.28% for the 100 mg preparation at 5 min and 97.48% for the 200 mg preparation at 5 min. These measurements indicate the pattern of release under the simplified laboratory conditions but do not establish in vivo intestinal release, bioavailability, or biological activity.

The highest fractional release in SIF occurred at 5 min for the 200 mg preparation; because the values are instantaneous rather than cumulative, this peak should not be interpreted as total peptide release over time (Table 3).

Table 2. Fractional (instantaneous) percentage Ctx(Ile²¹)-Ha release per sampling interval in simulated gastric fluid (SGF) from HPMCP-coated microcapsules (non-cumulative values).

	1 Min	2 Min	5 Min	10 Min	15 Min	20 Min
100 mg	3.02	4.48	3.53	5.46	0.00	0.00
200 mg	0.82	6.62	9.74	2.46	7.19	4.76
HPMCP	0.00	8.10	8.60	1.20	2.30	2.80

Table 3. Fractional (instantaneous) percentage Ctx(Ile²¹)-Ha release per sampling interval in simplified simulated intestinal fluid (SIF) from HPMCP-coated microcapsules (non-cumulative values).

	1 Min	2 Min	5 Min	10 Min	15 Min	20 Min	30 Min	45 Min	60 Min
100 mg	20.67	14.11	93.28	12.10	13.86	12.10	12.86	10.59	23.95
200 mg	7.57	17.03	97.48	95.58	10.88	10.13	11.17	96.53	12.50
HPMCP	2.60	5.20	7.00	7.90	8.80	10.20	10.40	12.40	7.60

3.2. Analysis of Feed Intake, Feed Conversion and Body Condition

No significant temperature $\times$ peptide-dose interactions were detected for total feed intake, total egg weight, feed conversion, or rectal temperature ($p > 0.05$). The peptide dose did not show a statistically detectable main effect on these variables. However, the heat-stress chamber was associated with lower total feed intake (17.8% less) and total egg weight (18.3% less), together with a small increase in mean rectal temperature (41.2 °C versus 41.0 °C; $p < 0.01$). These findings indicate a biologically relevant thermal response under the tested conditions, but the temperature effect cannot be separated statistically from the chamber effect because only one chamber was used per thermal condition. The non-significant peptide effects should therefore be interpreted cautiously given the limited sample size (Table 4).

Table 4. Total feed intake, total egg weight, feed conversion, and rectal temperature of hens under Comfort (26 °C) and Heat Stress (35 °C) conditions and D0, D1, and D2 peptide treatments. Values are summarized per hen over the experimental period.

Variables	Experimental Doses of Peptides (P)			Temperature Conditions (T)		<i>p</i> -Value (ANOVA)			SEM ¹
	D0	D1	D2	Comfort	Heat Stress	P	T	P × T	
Total feed intake (g)	3.243	3.084	3.091	3.4468 ^A	2.833 ^B	0.26	<0.01	0.99	84.3
Total egg weight (g)	1.914	1.746	1.790	1.999 ^A	1.635 ^B	0.32	<0.01	0.66	61.6
Feed conversion	1.69	1.80	1.73	1.73	1.76	0.51	0.69	0.35	0.03
Rectal temperature (°C)	41.1	41.1	41.1	41.0 ^B	41.2 ^A	0.84	<0.01	0.93	0.04

¹ Standard error of the mean (SEM). Different uppercase letters within a row indicate a significant main effect of the thermal condition at $p \leq 0.05$ according to the F test. Peptide-dose effects and temperature × dose interactions were evaluated in the factorial analysis. Because only one chamber was used per temperature condition, temperature-related comparisons are exploratory and are confounded with chamber.

Hens maintained under Comfort conditions showed relatively stable body-weight trajectories across peptide doses, whereas hens in the Heat Stress chamber showed progressive weight loss over the experimental period (Figure 5). These plots are descriptive and illustrate temporal patterns within the same experimental units; they do not provide independent inferential evidence for a temperature effect or demonstrate that the observed trajectory was caused exclusively by the thermal treatment.

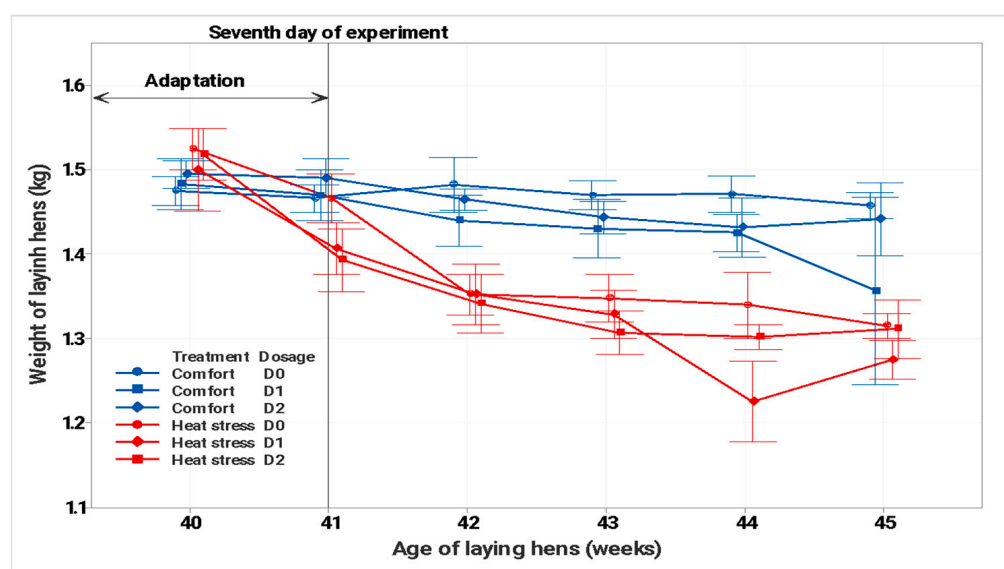


Figure 5. Variation in the mean and standard error of the weight of laying hens during the experimental period, for each dosage tested (D0: 0.0 mg/kg of feed, D1: 2.5 mg/kg of feed, and D2: 5.0 mg/kg of feed) in each temperature treatment (Comfort: 25.6 °C and approximately 68% relative humidity, and Heat stress: 34.5 °C and 56% relative humidity).

3.3. Egg Production and Egg Quality

Within each thermal environment, shell quality varied numerically among peptide doses without a consistent dose-related pattern across variables. We therefore refrain from drawing conclusions about dose-specific effects on eggshell quality from the present design (Figure 6).

Egg quality results are summarized in Table 5. No statistically significant interaction was observed for Haugh unit or albumen height ($p > 0.05$). Significant temperature × peptide-dose interactions were observed for egg weight, eggshell thickness, eggshell strength, and specific gravity ($p < 0.05$). Because these interactions were estimated with

only three hens per temperature $\times$ dose combination and multiple endpoints were tested, they should be regarded as exploratory and interpreted primarily as interaction patterns rather than definitive main effects. Under the Heat Stress condition, the 5.0 mg/kg dose was associated with lower shell thickness and strength than D0 and/or D1, whereas the lower-dose groups did not show the same pattern.

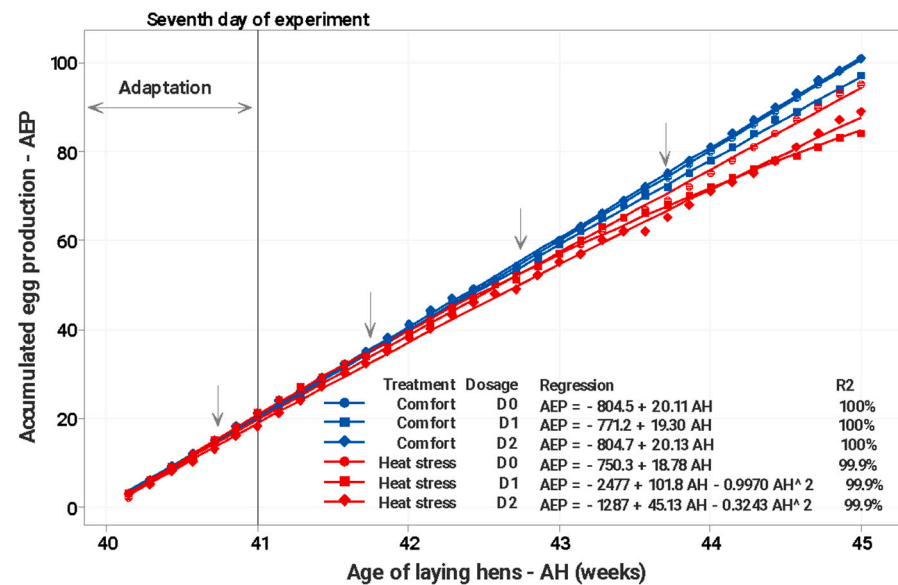


Figure 6. Cumulative production of eggs laid during the experimental period, for each dosage tested in each temperature treatment (Comfort: 25.6 °C and approximately 68% relative humidity, and Heat stress: 34.5 °C and 56% relative humidity). The downward arrows indicate the days on which the peptide doses were applied (D0: 0.0 mg/kg of feed, D1: 2.5 mg/kg of feed, and D2: 5.0 mg/kg of feed).

Table 5. Egg-quality values for the combined thermal conditions (Comfort: 26 °C and approximately 68% relative humidity; Heat Stress: 35 °C and approximately 56% relative humidity) and peptide doses (D0: control; D1: 2.5 mg/kg; D2: 5.0 mg/kg). Daily measurements were averaged within each hen before factorial analysis.

Variables	Comfort			Heat Stress			Comfort	Heat Stress	<i>p</i> -Value			SEM ³
	D0	D1	D2	D0	D1	D2			<i>p</i> ¹	T ²	P $\times$ T	
Weight (g)	59.28 ^{ab}	60.64 ^a	58.65 ^b	54.95 ^c	53.97 ^c	54.25 ^c	59.48 ^A	54.47 ^B	0.14	<0.01	0.01	0.20
Haugh Unit	87.19	86.62	88.24	89.37	88.44	85.60	87.34	88.01	0.44	0.62	0.06	0.44
Albumen height (mm)	7.73	7.62	7.87	7.85	7.83	7.46	7.74	7.73	0.77	0.87	0.19	0.07
Eggshell thickness (mm)	0.43 ^{ab}	0.41 ^{bc}	0.44 ^a	0.39 ^d	0.40 ^{cd}	0.37 ^e	0.43 ^A	0.39 ^B	0.24	<0.01	<0.01	0.01
Eggshell strength (kgf)	4.81 ^a	3.91 ^b	4.85 ^a	3.51 ^{bc}	4.05 ^b	3.31 ^c	4.58 ^A	3.60 ^B	0.25	<0.01	<0.01	0.05
Specific gravity (g/cm ³)	1081 ^a	1077 ^b	1081 ^a	1073 ^c	1077 ^b	1070 ^c	1080 ^A	1073 ^B	0.08	<0.01	<0.01	0.30

¹ Experimental peptide doses. ² Thermal conditions. ³ Standard error of the mean (SEM). Different uppercase letters indicate a significant main effect of thermal condition at $p \leq 0.05$ according to the F test. Different lowercase letters indicate differences among temperature $\times$ peptide-dose combinations at $p \leq 0.05$ according to the F test. Because multiple endpoints were evaluated with $n = 3$ hens per combination, these interaction results should be interpreted as exploratory.

3.4. Blood Parameters

The mean blood parameters are presented in Table 6. No statistically significant peptide-dose effects or temperature $\times$ dose interactions were detected for the measured blood variables ($p > 0.05$). Additionally, heat stress induced a slight but significant increase in leukocyte counts ($p = 0.04$), a typical immunological response to environmental stress.

Table 6. Blood parameters for the thermal conditions (Comfort and Heat Stress) and peptide doses (D0, D1, and D2). Repeated blood measurements from the same hen were pooled across sampling days to obtain one overall mean per hen for the factorial analysis.

Variable	Experimental Doses of Peptides (P)			Temperature Conditions (T)		P	p-Value T	P × T	SEM ¹
	D0	D1	D2	Comfort	Heat Stress				
Glucose (mg/dL)	194.79	199.83	193.69	196.20	195.75	0.23	0.82	0.76	1.49
Hemoglobin (g/dL)	8.68	8.49	8.96	8.80	8.61	0.26	0.49	0.21	0.11
Hematocrit (%)	28.85	27.44	28.75	28.50	28.30	0.11	0.81	0.76	0.30
Plasma proteins (g/dL)	7.92	8.35	7.71	8.19	7.79	0.09	0.09	0.99	0.12
Erythrocytes (10 ⁶ /mm ³)	2.17	2.08	2.18	2.07	2.22	0.43	0.22	0.21	0.03
Leukocytes (10 ³ /mm ³)	48.44	44.63	47.65	46.81 ^B	47.31 ^A	0.54	0.04	0.96	1.45
K ⁺ (mmol/L)	4.38	4.28	4.45	4.58	4.16	0.88	0.09	0.95	0.13
Na ⁺ (mmol/L)	150.76	150.65	151.38	152.72 ^A	149.10 ^B	0.92	0.02	0.83	0.74
Cl [−] (mmol/L)	119.71	120.03	120.06	120.84 ^A	119.01 ^B	0.61	<0.01	0.06	0.20
nCa (mg/dL)	6.74	6.85	6.77	6.96 ^A	6.60 ^B	0.82	0.03	0.35	0.08
iCa ²⁺ (mg/dL)	6.08	6.20	6.14	6.29 ^A	5.98 ^B	0.75	0.04	0.35	0.07
TCa (mg/dL)	11.86	12.10	11.99	12.27 ^A	11.67 ^B	0.76	0.04	0.35	0.14
pH	7.25	7.26	7.26	7.25	7.27	0.94	0.43	0.97	0.01

¹ Standard error of the mean (SEM). Different uppercase letters within a row indicate a significant association with the thermal condition at $p \leq 0.05$ according to the F test. Because the same hens were sampled repeatedly and only one chamber was used per temperature condition, these results are exploratory and do not establish independent temperature effects.

The Heat Stress chamber was associated with lower Na⁺, Cl[−], and calcium fractions and a small increase in leukocyte counts compared with the Comfort chamber. These observations are consistent with a physiological response to the thermal environment, but because chamber and temperature were confounded, they should be interpreted as associations with the assigned chamber condition rather than as definitive independent temperature effects. The repeated blood measurements were pooled across sampling days to characterize overall exposure rather than temporal responses; consequently, day-specific physiological dynamics were not evaluated inferentially.

4. Discussion

This exploratory proof-of-concept study evaluated two distinct questions: whether microencapsulated Ctx(Ile²¹)-Ha could mitigate selected productive and physiological consequences associated with heat stress and whether its administration was associated with detectable adverse changes in the measured endpoints. The primary hypothesis of a protective effect on feed intake, egg weight, feed conversion, and rectal temperature was not supported statistically. At the same time, no adverse peptide-dose effects were detected in the measured blood variables. These findings provide preliminary information on tolerability under the specific conditions tested, but they do not constitute a comprehensive systemic safety assessment.

The lower feed intake and egg weight observed in the Heat Stress chamber are consistent with the expected consequences of elevated environmental temperature in laying hens [9–12]. The relatively small difference in rectal temperature (41.0 versus 41.2 °C) should not be interpreted as evidence that the thermal challenge was mild. Laying hens are effective homeotherms and can maintain core temperature through compensatory responses such as panting and peripheral vasodilation, but this homeostasis can occur at the cost of reduced feed intake and productive performance. In the present experiment, the combination of approximately 34.5 °C daytime temperature and 56% relative humidity corresponded to an estimated THI of 85.3, well above the estimated comfort condition. Thus, the observed productive response is a more informative indicator of thermal challenge than the small absolute change in core temperature. Nevertheless, the absence of independent chamber replication prevents complete separation of temperature from chamber effects.

Ambient temperature alone does not fully characterize thermal load because relative humidity also affects the ability of birds to dissipate heat. We therefore included the recorded relative humidity and an estimated THI in the description of the challenge. The Heat Stress condition was sustained during the light period after a seven-day acclimatization period, making the intervention a prolonged environmental challenge rather than a brief acute exposure. The resulting interpretation should remain cautious because the THI equation used here is an auxiliary environmental descriptor and the present design included only one chamber per thermal condition.

The reduction in eggshell thickness observed under the Heat Stress chamber is biologically consistent with altered calcium availability and acid–base balance during the thermal challenge [9,14,25]. Heat stress can reduce feed intake and alter respiratory physiology, which may affect calcium absorption, transport, and shell-gland function. These mechanisms provide a plausible explanation for the observed shell response, but calcium transport and shell-gland physiology were not directly measured in this experiment.

Previous studies reported biological activity of microencapsulated Ctx(Ile²¹)-Ha at 2.5 mg/kg in laying hens challenged with *Salmonella Enteritidis* [17,18]. The present study addressed a different physiological context: prolonged environmental heat stress. The absence of a detectable protective effect on the principal productive variables therefore indicates that the efficacy observed in an acute infectious model cannot be assumed to transfer to chronic thermal stress. The proposed distinction is that direct antimicrobial activity may be more relevant when a defined microbial challenge is present, whereas prolonged heat stress involves broader metabolic, thermoregulatory, electrolyte, and calcium disturbances. These mechanisms were not directly measured here and should be regarded as hypotheses for future work rather than explanations demonstrated by the present data.

The eggshell interactions observed at 5.0 mg/kg under Heat Stress deserve particular attention. The further reductions in shell thickness and strength at the higher dose represent a potential adverse signal under thermal challenge rather than evidence supporting a generalized safety conclusion. One possible explanation is additional physiological strain in calcium and electrolyte regulation when birds are already experiencing reduced feed intake and heat-associated acid–base disturbances; however, this interpretation remains speculative because the relevant metabolic pathways were not directly assessed.

The systemic safety, structural optimization, microbial membrane permeabilization, and physiological tolerance of peptide additives in poultry have been increasingly investigated [26–30]. Overall, while dietary Ctx(Ile²¹)-Ha did not counteract the severe productive losses caused by heat stress, the absence of overt blood biochemical alterations supports its preliminary physiological tolerability at tested levels.

Another important limitation is the absence of mechanistic analyses. Gut microbiota composition, intestinal morphology or permeability, inflammatory cytokines, oxidative-stress biomarkers, and other molecular indicators were not measured. The present biochemical panel also did not include a comprehensive assessment of hepatic or renal function, and histopathology was not performed. Consequently, the study cannot establish comprehensive systemic safety or identify the biological mechanism underlying the observed responses. Future experiments should integrate microbiota profiling, inflammatory and oxidative biomarkers, broader hematological and biochemical panels, and histopathological evaluation.

The main limitations are the lack of independent climatic-chamber replication, the small sample size ($n = 3$ per temperature $\times$ dose combination), the repeated nature of blood, body-weight, and egg observations, the pooling/averaging procedures used to maintain statistical independence, the limited power to detect small or moderate effects, and the risk of Type I error from testing multiple eggshell-quality endpoints. Because

chamber and temperature were confounded, the thermal treatment cannot be generalized independently of the chamber used. A repeated-measures mixed-effects model would be preferable for longitudinal outcomes, but the present exploratory analysis used per-hen summaries for repeated blood and egg-quality measurements and descriptive trend analysis for body weight. These constraints mean that non-significant findings should not be interpreted as equivalence and that significant interaction patterns should be viewed as hypothesis-generating.

Additionally, given the very small sample size ($n = 3$ per temperature $\times$ dose combination), 95% confidence intervals for treatment comparisons were not calculated; instead, standard errors of the mean (SEMs) are reported throughout the Results. We acknowledge that the SEM alone does not convey the same information as a confidence interval regarding the precision or range of plausible treatment effects, and this represents an additional limitation to the interpretation of the reported estimates.

5. Conclusions

The present findings should be interpreted as preliminary and exploratory. Under the tested conditions, no adverse changes attributable to peptide dose were detected in the measured blood variables, but the study does not establish comprehensive systemic safety or absence of toxicity. The principal productive hypothesis—that Ctx(Ile²¹)-Ha would mitigate the effects of heat stress—was not supported by the measured feed intake, egg-weight, feed-conversion, or rectal-temperature responses.

The interaction pattern observed for eggshell quality, particularly the lower shell thickness and strength at 5.0 mg/kg under heat stress, should be considered a signal requiring further investigation rather than evidence of safety. Larger studies with independent chamber replication, adequate statistical power, repeated-measures modeling, longer exposure, broader biochemical and hematological profiling, and histopathological and mechanistic analyses are needed before conclusions regarding efficacy, dose optimization, or safety can be generalized.

Author Contributions: L.E.B.-N. contributed to the conceptualization, methodology, formal analysis, investigation, data curation, and both original draft preparation and review & editing of the manuscript. S.G.G. assisted with methodology and investigation and also contributed to the review and editing process. D.d.L.S. aided in the conceptualization of the work and its subsequent review and editing. D.D.S. contributed to formal analysis and data curation. D.F.P. deceased, contributed to the conceptualization, methodology, formal analysis, investigation, data curation, writing of the original draft, review & editing, visualization, and the acquisition of funding. E.F.V. contributed to conceptualization, methodology, data curation, original draft writing, review & editing, and visualization and, importantly, also secured funding. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The Institutional Animal Care and Use Committee certify that the proposal “Evaluation of the Ctx(Ile²¹)-Ha antimicrobial peptide supplementation in laying hens and its effect on egg quality”, protocol number 01/2024, under the responsibility of Prof. Dr. Eduardo Festozo Vicente, and his team (Prof. Dr. Danilo Florentino Pereira, Prof. Dr. Diogo de Lucca Sartori and Me. Lorenza Eivazian **Brandão-Neves**), which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research or teaching, is in accordance with the Law 11794 of 8 October 2008, of Decree 6899 of 15 July 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was APPROVED by the Institutional Animal Care and Use

Committee (CEUA) of the São Paulo State University (UNESP), School of Sciences and Engineering, Tupã, in the meeting of 26 February 2024.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the author's decision to make the data available if needed.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ACN	Acetonitrile
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
D0	Dosage, control, no antimicrobial peptide
D1	Dosage 1 (2.5 mg/kg of feed)
D2	Dosage 2 (5.0 mg/kg of feed)
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DIEA	Diisopropylethylamine hexafluorophosphate
DMF	Dimethylformamide
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FCR	Feed conversion ratio
FMOC	Fluorenylmethyloxycarbonyl protecting group
Hb	Hemoglobin
HATU	2-(1-H-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBT	N-hydroxybenzotriazole
HPMCP	Hydroxypropyl methylcellulose phthalate
iCa	Ionic calcium
nCa	Normalized calcium
NMM	N-methylmorpholine
pH	Potential of hydrogen
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SPPS	Solid-phase peptide synthesis
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
tCa	Total calcium
UHPLC	Ultra-high-performance liquid chromatography

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