



## Full-Length Article

## Comparing different water supplementation in cyclic heat stressed broilers. The effect of selected antioxidant mixtures, betaine, functional amino acids and electrolyzed reduced water

Renée De Baets<sup>a,b,\*</sup> , Sofie Van Nerom<sup>a,b</sup> , Kobe Buyse<sup>a</sup>, Gunther Antonissen<sup>b</sup>, Jeroen Degroote<sup>c</sup> , Evelyne Delezie<sup>a</sup> 

<sup>a</sup> Flanders Research Institute for Agriculture, Fisheries and Food, Animal Science Unit, 9090 Merelbeke-Melle, Belgium

<sup>b</sup> Department of Pathobiology, Pharmacology and Zoological Medicine, Faculty of Veterinary Medicine, Ghent University, 9820 Merelbeke-Melle, Belgium

<sup>c</sup> Laboratory for Animal Nutrition and Animal Product Quality, Department of Animal Sciences and Aquatic Ecology, Faculty of Bioscience Engineering, Ghent University, 9000 Ghent, Belgium

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## ABSTRACT

Combating heat stress (HS) has become an emerging topic in temperate regions. Supplementing drinking water additives may alleviate the adverse effects of HS in broilers. The effects of commercially available additives on performance, body temperature, respiration rate, oxidative status and physiology during induced HS were assessed in two separate trials. In each experiment, 756 Ross 308 male broilers were allocated to four treatments ( $n = 9$ ). Treatments were supplied continuously during the finisher phase until slaughter. In Exp. 1, animals received polyphenols (1 mL/L) with vitamin C (25 %; added at 0.25 mL/L) (PP + VIT C), essential oils (25 %) with vitamin E (1 %; added at 0.1 mL/L) (EO + VIT E), betaine hydrochloride (0.7 g/L) (BET) or unsupplemented water (CON). In Exp. 2, treatments included electrolyzed water at 10 % (ERW10) or 100 % (ERW100), a mixture of crystalline amino acids (AA): L-arginine (4.5 g/L), L-monosodium glutamate (3 g/L) and L-threonine (2.5 g/L), and CON. All pens were subjected to cyclic HS ( $32 \pm 2$  °C; 60 %–70 % RH; 09:30–15:30) until slaughter. On average, birds showed a body temperature of 42.6 °C and a breathing frequency of 186 breaths/minute, while reducing feed intake during daytime HS compared to thermoneutral nighttime (circa 24 °C), which was not affected by any treatment. Likewise, performance, meat quality, oxidative metabolism and physiological responses during HS were not significantly affected by the treatments, except for AA, which decreased feed intake by 9.6 %, but improved FCR by 0.10 compared to CON. AA also reduced the water holding capacity of breast muscle and resulted in paler meat colour. Deviations in water quality (e.g. microbiological and organoleptic parameters) suggested that AA may have promoted microbial growth under HS. In conclusion, despite clear HS responses, characterized by high body temperature and breathing frequency, and a significant difference in feed intake following a cyclic pattern as the model, none of the drinking water additives effectively improved performance or key physiological features during cyclic HS in the finisher phase.

## Introduction

Combating heat stress (HS) has become an emerging topic in poultry farming due to increasingly frequent and intense environmental temperatures, even in temperate regions (Lara and Rostagno, 2013; Mikovits et al., 2019). Broilers are particularly sensitive to heat due to their high growth rate, resulting in a high metabolic heat production (Wasti, et al., 2020; Nawaz, et al., 2021). Additionally, they are often reared in intensive farming systems at a high density, limiting heat loss (Wasti,

et al., 2020). When environmental temperature exceeds the upper critical temperature of animal's thermoregulatory capacity, it will suffer from HS (Saeed, et al., 2019). HS impairs broiler health, welfare and performance through several mechanisms. One major mechanism is the occurrence of oxidative stress, an imbalance between the production of reactive oxygen species (ROS) and the antioxidant system, initiating a cascade of detrimental effects caused by oxidative damage to tissues. The abundance of ROS can affect the intestinal structure leading to barrier disruption and inflammation (Akbarian, et al., 2016).

\* Corresponding author at: Flanders Research Institute for Agriculture, Fisheries and Food, Animal Science Unit, 9090 Merelbeke-Melle, Belgium.  
E-mail address: [renee.debaets@ilvo.vlaanderen.be](mailto:renee.debaets@ilvo.vlaanderen.be) (R. De Baets).

Additionally, increased ROS deteriorates meat quality by lipid peroxidation and protein degradation (Nawaz and Zhang, 2021). Adding exogenous antioxidants may help balancing antioxidant homeostasis and retain tissue integrity.

Supplementing additives via drinking water is a common practical strategy, because these strategies can be easily implemented and adjusted upon a HS episode. A wide range of bioactive compounds of antioxidant nature are available for drinking water applications. In general, adding these compounds during HS may improve growth, feed conversion, carcass yield and meat quality by enhancing oxidative status and intestinal health (Attia, et al., 2011; Şimşek, et al., 2015; Shakeri, et al., 2019; Parveen, et al., 2020).

Several drinking water additives contain polyphenols, which are non-volatile plant secondary metabolites possessing phenol groups (Serra, et al., 2021). They have been shown to support beneficial microflora, improve gut morphology and enhance antioxidant capacity (Hajati, et al., 2015; Lipiński, et al., 2017; Serra, et al., 2021). The volatile aromatic plant derivatives are categorized as essential oils (EO) (Gopi, et al., 2014). EO have demonstrated antioxidant activity, immune modulation, and improvement of intestinal integrity, feed intake and digestion (Gopi, et al., 2014; Yilmaz and Gul, 2024). Generally, natural products like polyphenols and EO, may work synergistically with each other, and with other antioxidants which could increase the antioxidant potential (Sonam and Guleria, 2017). Both water-soluble vitamin C and fat-soluble vitamin E have shown antioxidant effects, protecting cytoplasm and cell membranes against oxidative damage and due to their role as a cofactor in multiple metabolic reactions (Abidin and Khatoon, 2013; Dalólio, et al., 2015). Additionally, vitamin C was observed to retain intestinal structure as demonstrated by increased villi height and width and crypt depth during HS (Hajati, et al., 2015). Betaine has a dual role as an osmolyte and methyl donor. As an osmolyte, it may support cellular homeostasis and could protect cells against osmotic stress. As a methyl donor, betaine showed involvement in several transmethylation reaction e.g. carnitine and creatine synthesis (Ratriyanto and Mosenthin, 2018; Abd El-Ghany and Babazadeh, 2022; Al-Qaisi, et al., 2023).

To mitigate additional detrimental effects on tissue damage during HS, several functional amino acids (AA) show potential as drinking water additive. L-arginine showed to improve blood flow by vasodilatation, allowing the animal to cool itself and helps restore intestinal morphology by increasing villi height and AA transporters as shown in pigs (Liu, et al., 2019; Morales, et al., 2021). L-monosodiumglutamate (L-MSG) may enhance flavor and stimulate broiler performance (Olarotimi and Adu, 2022). Additionally, L-glutamate is involved in the uric acid cycle and an important energy source for enterocytes (Blachier, et al., 2009). L-threonine is the third limiting amino acid, and essential for immunity and intestinal integrity (Dozier Iii, et al., 2000).

Next to additives, electrolyzed reduced water (ERW) is rather novel water treatment characterized by a high pH, low oxidation-reduction potential and low O<sub>2</sub> amount, with active hydrogen acting as ROS scavenger (Akbarian, et al., 2016). ERW has antioxidant properties and shows potential to improve performance and oxidative status during HS (Azad, et al., 2013, 2021; Zhang, et al., 2022).

Multiple studies have tested these drinking water strategies separately under varying conditions. However, comparing them under identical HS conditions would allow to estimate their relative potency in alleviating HS. Moreover, a chronic cyclic HS protocol is indicated to study these effects in Central and Western European regions where heat waves are characterized by a circadian rhythm with a high diurnal temperature range (Katavoutas, et al., 2023), with notably cooler nights that enable birds to partially recover from heat stress and compensate for reduced daytime feed intake.

Therefore, the objective of the study was to evaluate several different drinking water additives under comparable conditions, individually or in combination, as potential mitigation strategies against chronic, cyclic HS in broilers. Their effects on performance, body temperature, respiration rate, oxidative status during HS were assessed in two trials.

## Materials and methods

Two successive studies were performed. All experimental procedures in these studies complied with the European guidelines for the care and use of animals in research (Directive 2010/63/EU) and were approved by the Ethical Committee of the Faculty of Veterinary Sciences under authorization number 2022/058.

### Experimental Design

A total of 1,512, 756 in experiment 1 (Exp. 1) and 756 in experiment 2 (Exp. 2), one-day-old male broiler chicks (Ross 308) were obtained from a commercial hatchery (Vervaeke-Belavi, Tielt, Belgium). In each experiment, birds were randomly allocated to four treatments over 36 pens. Each pen, with pen size measuring 1.36 m<sup>2</sup>, housed 21 broilers. Broilers were housed on a solid floor with wood shavings (1.5 kg/m<sup>2</sup>) and were kept in 18L6D schedule from d7 till d40, 18 light hours were provided from 4:00 am to 10:00 pm. The first week, a 23L1D cycle was used. The initial ambient temperature was set at approximately 34 °C, and gradually decreased by 4 °C per week until 22 °C. All birds received the same 3-phase diet with a starter (d0 to d11), grower (d11 to d25), and finisher (d25 to d40) diet as shown in Table 1. The starter diet was a crumbled pellet feed, while the grower and finisher diets were pelleted. Feed and drinking water were available ad libitum, each pen had 2 drinking nipples. On day 25, before the start of the experimental period, birds were regrouped from 21 to 20 birds per pen based on mean pen weight.

Before each trial, all drinking water lines were disinfected with a 35 % hydrogen peroxide solution to a final concentration of 1 %. Drinking water treatments were continuously supplied from d27 or d26 (Exp. 1 and Exp. 2, respectively) until slaughter. Fresh drinking water treatments were supplied daily via water stock reservoirs connected to the drinking lines. In Exp. 1, animals received a combination of polyphenols (1 mL/L; Impextraco, Heist-op-den-Berg, Belgium) and vitamin C (25 %, added at 0.25 mL/L) (PP + VIT C), essential oils (25 %) and vitamin E (3a700; 1 %) (mixture added at 0.1 mL/L) (EO + VIT E), betaine hydrochloride (0.7 g/L) (BET) or unsupplemented control water (CON). Animals in the EO + VIT E treatment received a commercial blend of EO containing cinnamaldehyde, oregano, anise, tea tree, caraway, mint and eucalyptus oil. In Exp. 2, electrolyzed water was dosed at 10 % (ERW10) or at 100 % (ERW100), a third treatment was a mixture of crystalline amino acids (AA): L-arginine (4.5 g/L), L-monosodium glutamate (3 g/L) and L-threonine (2.5 g/L). Lastly, unsupplemented water was supplied (CON). pH and temperature of the drinking water was analyzed daily in the stock solution and drinking water line by taking a sample of approximately 100 mL and immediately measuring it with a pH meter (HI99161, Hanna Instruments, Temse, Belgium). In Exp. 2, pH and oxidation-reduction potential (ORP) were measured with a dual scale pH/ORP meter (HI991003 with HI12973 electrode, Hanna Instruments, Temse, Belgium). At the end of Exp. 2, a water sample of 2 L was taken to assess microbiological water quality by a commercial laboratory (INAGRO, Roeselare, Belgium).

One day after the start of supplying drinking water additives, from d28 (Exp. 1) or d27 (Exp. 2), all pens were subjected to cyclic HS until slaughter (d40). During HS, the temperature increased to 32 ± 2 °C with a relative humidity (RH) of 60 - 70 % for 6 h (09:30 - 15:30) (Fig. 1). During the rest of the day, the temperature was maintained around 26 ± 1 °C with a RH of 55 - 65 %. Ambient temperature and RH were continuously monitored.

**Electrolyzed-reduced Water Generation.** Electrolyzed-reduced water (ERW) was generated using an electrolyzer (Aquaiox EA-30, Soest, The Netherlands), which consists of a chamber with positively and negatively charged electrodes separated by a semipermeable membrane. The set-up consists of an activated carbon (charcoal) filter, a brine (NaCl) reservoir, the electrolysis device itself, and a storage tank for the produced water.

**Table 1**  
Feed composition for the starter, grower and finisher diet.

	Starter	Grower	Finisher
<b>Ingredient (%)</b>			
Wheat	30.77	49.40	60.61
Corn	25.00	15.00	8.00
Soybean	8.00	5.00	5.00
Soybean meal	23.61	20.40	11.67
Sunflower meal	3.00	3.00	6.00
Oat hulls	2.00	0.00	0.00
Soybean oil	2.00	2.00	2.00
Animal fat	1.01	1.25	2.96
Mineral and vitamin premix <sup>1</sup>	0.50	0.50	0.50
Limestone	1.66	1.46	1.29
Mono-calcium phosphate	0.87	0.42	0.27
DL-methionine	0.35	0.31	0.26
L-lysine HCl	0.35	0.37	0.46
L-threonine	0.15	0.15	0.17
Choline	0.10	0.10	0.10
L-valine	0.12	0.12	0.14
L-arginine	0.06	0.09	0.11
L-isoleucine	0.00	0.02	0.04
Sodium-bicarbonate	0.14	0.11	0.16
Sodium chloride	0.30	0.30	0.27
Cocciostat <sup>2</sup>	0.02	0.02	0.0
NSP enzyme (100 XGU/kg) <sup>3</sup>	0.01	0.01	0.01
6-Phytase (1000 FTU/kg)	0.005	0.005	0.005
<b>Calculated nutrient composition (%)</b>			
Crude protein	21.0	19.5	18.3
Crude fat	6.59	6.01	7.50
Crude ash	5.95	5.08	4.53
Metabolizable energy (kcal/kg)	3002	3100	3200
Dig. lysine (g/kg)	11.8	10.9	10.3
Dig. arginine/lysine	105	105	105
Dig. methionine + cysteine/lysine	75	75	75
Dig. threonine/lysine	67	67	67
Dig. valine/lysine	80	80	80
Calcium	0.90	0.74	0.64
Available phosphor	0.44	0.36	0.33
Na+K-Cl (mEq/100 g)	23.0	20.5	17.5
<b>Analysed nutrient composition (%)</b>			
Crude protein			18.8
Crude fat (hydrolysis)			7.80
Crude ash			4.0
Calcium			0.70
Phosphor			0.43

<sup>1</sup> The premix provides per kg of diet: Vitamin A (retinyl acetate), 10,000 IU; Vitamin D3 (cholecalciferol), 2,500 IU; Vitamin E (dl- $\alpha$ -tocopherol), 50 mg; Vitamin K3 (menadiolone), 2.3 mg; Vitamin B1 (thiamine), 2.0 mg; Vitamin B2 (riboflavin), 7.5 mg; Vitamin B6 (pyridoxine-HCl), 3.5 mg; Vitamin B12 (cyanocobalamin), 20  $\mu$ g; Niacin, 35 mg; D-pantothenic acid, 12 mg; Choline chloride, 460 mg; Folic acid, 1.0 mg; Biotin, 0.2 mg; Iron (Fe, as FeSO<sub>4</sub>·H<sub>2</sub>O), 80 mg; Copper (Cu, as CuSO<sub>4</sub>·5H<sub>2</sub>O), 12 mg; Manganese (Mn, as MnO), 85 mg; Zinc (Zn, as ZnSO<sub>4</sub>·H<sub>2</sub>O), 60 mg; Iodine (I, as KI), 1.2 mg; Selenium (Se, as Na<sub>2</sub>SeO<sub>3</sub>), 0.25 mg.

<sup>2</sup> Diclazuril.

<sup>3</sup> Ronozyme Multigrain, DSM, Heerlen, the Netherlands.

Water was first filtered through activated carbon. A salt solution and water were then introduced into the electrolyzing device, where electrolysis occurred under a voltage of 110-230 V and 10-14 A. This process generates two types of water: electrolyzed oxidized water (EOW) at the anode, which is acidic and contains hypochlorous acid, and ERW at the cathode, which is alkaline, and has a low ORP, and contains a diluted concentration of NaOH. ERW was produced at a rate of 1-10 L/h and freshly produced each day to ensure consistent water conditions.

## Measurements

**Performance.** Live body weight (BW), average daily gain (ADG; g/d), average daily feed intake (ADFI; g/d), and feed conversion ratio (FCR) were recorded at d0, 11, 25, 32 and 39 at the pen level. Mortality was registered daily. Additionally, feed was weighed daily before and

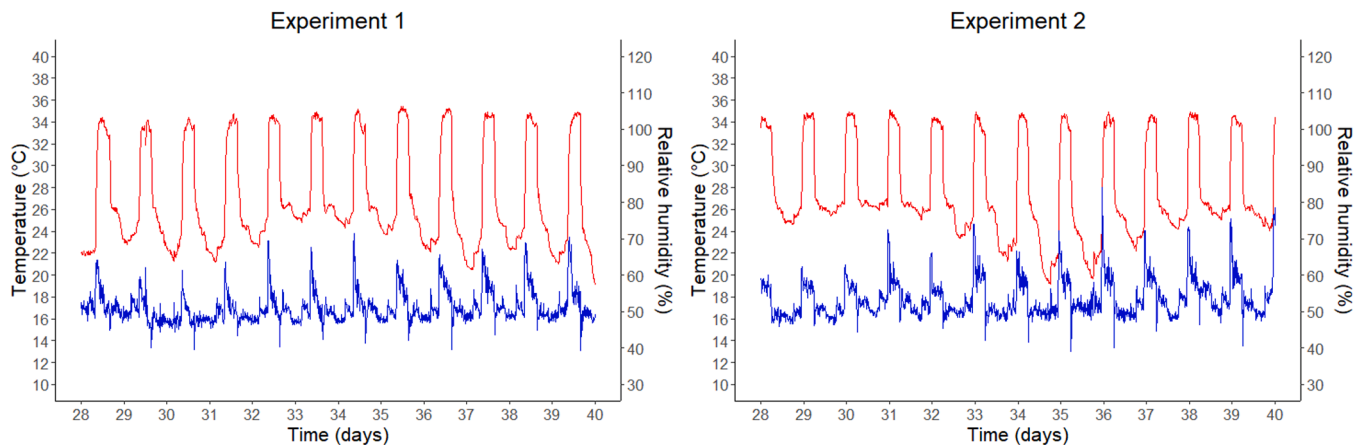
after HS on d31, 32, 35 and 38 (Exp. 1) or d27, 31, 35 and 38 (Exp. 2) to determine feed intake during and after HS, corrected for the number of light hours (09:30 – 15:30: 6 L; 15:30 – 09:30: 12 L). The water stock reservoirs were weighed daily before and after HS from d29-38 to calculate daily water intake.

**Body Temperature and Breathing Frequency.** At the first day of HS, d31, 35 and 38, body temperature (Tb) was measured in four randomly marked chickens per pen, 4 h after HS was initiated, using a digital thermometer (Digi-Vet SC 12, Kruuse, Langeskov, Denmark). At the same time, video recordings of each pen were made to determine breathing frequency of two randomly selected chickens per pen. Afterwards, a human annotator analyzed the videos to determine the breathing frequency by counting the number of breaths in a 60 second interval.

**Sample Collection and Processing.** Sample collection was performed 4 h after HS was initiated in a limited time frame to keep temperature differences to a minimum, hence two subsequent days samples were taken. At both d39 and d40, one chicken per pen (7 pens), with an individual weight close to the average pen weight was sampled, resulting in 14 animals per treatment. BW and Tb were measured before euthanasia, which was induced by intravenous injection with sodium pentobarbital 20 % (Kela, Hoogstraten, Belgium). Blood samples were collected by exsanguination and immediately aliquoted to analyze glucose using a commercial glucose meter (OneTouch Verio Reflect, LifeScan Belgium, Westerlo, Belgium) and, in Exp. 1, lactate (mmol/L) with a lactate analyzer (The EDGE Lactate Analyzer, RDSM, Hasselt, Belgium). The rest of the blood was collected in serum tubes (4 mL; no coagulant) and K<sub>2</sub>EDTA tubes (8 mL). Serum samples were used to measure aspartate transaminase (AST), lactate dehydrogenase (LDH), creatine kinase (CK) and uric acid in a commercial laboratory (DGZ, Torhout, Belgium). The samples in EDTA were centrifuged (1,500 g, 10 min, 4 °C) and plasma aliquots (2 mL) were made in Eppendorf tubes and frozen at –80 °C until analysis. The liver was removed and color (Hunterlab Miniscan 45/0, AMETEK Hunterlab, Reston, VA) was measured with CIE L\*a\*b\* where L\*, a\* and b\* values represent lightness, red/green and blue/yellow respectively as axes on a 3-dimensional color scheme. Additionally, in Exp. 2, a blood-gas analysis was performed using a point-of-care analyser (i-STAT Alinity, Zoetis, Zaventem, Belgium). Cartridges were kept at 4 °C until used. Blood was collected in a 500  $\mu$ L heparin tube and were processed within 15 min of collection. The analysis was done using CG4+ cartridges (Zoetis, Zaventem, Belgium), which measures the following parameters: pH, carbon dioxide partial pressure (pCO<sub>2</sub>), oxygen partial pressure (pO<sub>2</sub>), bicarbonate (HCO<sub>3</sub><sup>-</sup>), total carbon dioxide (TCO<sub>2</sub>), base excess (BE), oxygen saturation (sO<sub>2</sub>) and lactate. Additionally, in Exp. 2, pH of the crop, proventriculus and gizzard were measured (HI99161, Hanna Instruments, Temse, Belgium).

**Oxidative Status.** Malondialdehyde (MDA) was determined according to the thiobarbituric acid reactive substances (TBARS) method as described in Grotto, et al. (2007), with slight modifications (Goethals, et al., 2019). The absorbance of the carbonyl-TBA complexes was measured colorimetrically at 532 nm (Genesys 10S UV-VIS, Thermo Scientific, Madison, USA), quantified using a standard curve with tetramethoxypropane (0 – 30 nmol/mL), and expressed as nmol MDA equivalents/mL sample (U/mL). The glutathione peroxidase (GPx) activity was measured in plasma as described by Goethals, et al. (2019). One unit (U) of GPx was defined as the amount of enzyme required to oxidize 1  $\mu$ mol of NADPH per minute and per litre plasma at 25 °C.

**Intestinal morphology.** Tissue samples from the mid-jejunum were collected in 3.7 % formaldehyde and processed as described by De Grande, et al. (2020). Villus height (VH) and crypt depth (CrD) were measured using a light microscope (Kern OBN 135, Kern Optics, Balingen, Germany). Villus height was measured from the crypt-villus junction to the villus tip, whereas CrD was measured from the crypt base to the villus-crypt junction. The VH and CrD were measured at ten randomly selected sites and mean values were calculated.



**Fig. 1.** Room climate during cyclic HS in Experiment 1 (d28 - 40) and Experiment 2 (d27-40). Treatments include polyphenols and vitamin C (PP + VIT C), essential oils and vitamin E (EO + VIT E), betaine (BET), electrolyzed reduced water at 10 % (ERW10), electrolyzed reduced water at 100 % (ERW100), functional amino acids (AA) and control (CON). Heat stress was induced daily from 9:30 - 15:30. Red represents temperature (°C) and blue represents relative humidity (%).

**Meat Quality.** At the end of the experiment, 21 birds per treatment (3 chickens per pen) were selected from 7 pens per treatment, which were the same pens as used for sampling. In each pen unit, chickens were selected based on average animal weight according to the pen average and were individually marked, weighed, and fasted overnight until transport to a commercial slaughterhouse. The eviscerated carcasses were retrieved and stored at 4 °C until the following day. Carcass yield was calculated as the ratio of eviscerated carcass weight to live weight. Breast fillets were scored for abnormalities (wooden breast, white striping and spaghetti meat) by a trained professional. Meat quality analyses were conducted on the pectoralis major muscles and comprised, in chronological order: temperature (°C), pH, color, thawing loss (%), cooking loss (%) and shear force (N), following the methodology described by [Buyse, et al. \(2021\)](#).

#### Statistical analysis

Statistical analysis was performed with R version 4.1.2 for Windows ([R Core Team, 2022](#)). For performance and mean pen physiological parameter analysis, linear models were made with ‘treatment’ as independent factor. For daily feed intake, a linear mixed model was used with ‘treatment’, ‘timing’ and ‘day’ as fixed factors and ‘pen’ as random factor. Timing has two levels: HS and thermoneutral (TN) on the same day. For meat quality analysis, a linear mixed model was used with ‘treatment’ as fixed factor and animals nested per pen. For respiration rate and body temperature, a linear mixed model was used with ‘treatment’ and ‘measurement day’ as fixed factor and animals nested per pen. For mortality a generalised linear model was used with a binomial distribution and ‘treatment’ as independent factor. Data was analysed by analysis of variance (ANOVA). Linear model assumptions (normality and homoscedasticity) were verified by a visual check of the residuals plots and Levene’s test. Tukey’s range test (Honest Significant Difference, HSD) was used to obtain adjusted p-values to account for multiple comparisons, with level of significance  $\alpha = 0.05$ . Tables and figures report the lsmean  $\pm$  standard error of the mean (SEM). In the Results section, numerical values are presented as mean  $\pm$  standard deviation.

## Results

#### Climate conditions

In Exp. 1, during the HS period (09:30 – 15:30), the average temperature was  $34.02 \pm 0.69$  °C with a RH of  $54.36 \pm 3.92$  %. During the TN period (15:30 – 09:30), the temperature averaged  $23.66 \pm 2.26$  °C, with a RH of  $49.92 \pm 3.62$  % ([Fig. 1](#)). In Exp. 2, during the HS period

(09:30 – 15:30), the average temperature was  $34.08 \pm 0.56$  °C with a RH of  $59.16 \pm 2.87$  %. During the TN period (15:30 – 09:30 h), the temperature averaged  $25.14 \pm 1.71$  °C, with a RH of  $51.67 \pm 4.01$  %.

#### Water characteristics

In Exp. 1, pH of the drinking water treatments was  $7.19 \pm 0.364$ ,  $7.74 \pm 0.316$ ,  $2.81 \pm 0.136$  and  $7.90 \pm 0.256$  for PP + Vit C, EO + Vit E, BET and CON, respectively. In Exp. 2, pH of the drinking water treatments was  $8.53 \pm 0.177$ ,  $10.3 \pm 0.159$ ,  $9.02 \pm 0.207$  and  $7.96 \pm 0.146$  for ERW10, ERW100, AA and CON, respectively ([Supplementary figure 1](#)). Temperature of the water at the source was  $14.1 \pm 3.59$  °C in Exp. 1 and  $10.3 \pm 0.91$  °C in Exp. 2. At 3:30 pm, at the end of HS, the temperature was  $29.3 \pm 0.84$  °C in Exp. 1 and  $29.6 \pm 0.81$  °C in Exp. 2 at the end of the drinking line ([Supplementary figure 2](#)). In Exp. 2, ORP of the drinking water treatments was measured at multiple sample locations. For ERW10, the mean ORP was  $68.9 \pm 64.1$  ( $64.4 \pm 82.9$  (stock solution) and  $72.8 \pm 47.1$  (end of line)). For ERW100, the mean ORP was  $-150.0 \pm 61.1$  ( $-193.0 \pm 41.5$  (stock solution) and  $-97.4 \pm 47.0$  (end of line)). For AA, the mean ORP was  $-6.36 \pm 51.0$  ( $10.7 \pm 48.4$  (stock solution) and  $-14.7 \pm 34.5$  (end of line)). For CON, the mean ORP was  $94.5 \pm 33.8$  ( $101.0 \pm 35.4$  (stock solution) and  $91.4 \pm 32.0$  (end of line)) as shown in [supplementary figure 3](#). The physiochemical, microbiological and organoleptic indices were analyzed in Exp. 2. pH of the treatments was  $8.53 \pm 0.177$ ,  $10.30 \pm 0.159$ ,  $9.02 \pm 0.207$  and  $7.96 \pm 0.146$  for ERW10, ERW100, AA and CON, respectively. Additionally, total plate count 22 °C (CFU/mL) was numerically higher in AA ( $>3.0 \times 10^5$ ) and in water from the source ( $1.9 \times 10^6$ ) compared to the lab references ( $1.0 \times 10^5$ ). Moreover, water from the AA treatment was visibly yellow, with an unpleasant odor and cloudy with particles, while the other treatments were colorless, odorless and bright as shown in [Supplementary figure 4](#).

#### Animal performance

There were no significant differences in the performance parameters (BW, ADG, ADFI and FCR) in Exp. 1 ([Table 2](#)). In Exp. 2, the ADFI was significantly lower ( $p < 0.001$ ) in broilers from the AA group, compared to the other treatments. ADG from d32 - 39 was also significantly lower in AA compared to ERW100 ( $p = 0.008$ ). Nevertheless, the FCR of AA was significantly lower during d25 - 32 and d25 - 39 ( $p = 0.031$  and  $p = 0.002$ , resp.). The use of electrolyzed reduced water (ERW10 and ERW100) did not affect performance. Water intake for both experiments could not be analyzed statistically because it could only be measured at the treatment group level and not the pen level, however, numerically,

**Table 2**

Results in animal performance for Experiment 1 and Experiment 2. Treatments include polyphenols and vitamin C (PP + VIT C), essential oils and vitamin E (EO + VIT E), betaine (BET), electrolyzed reduced water at 10 % (ERW10), electrolyzed reduced water at 100 % (ERW100), functional amino acids (AA) and control (CON). The table shows lsmeans and pooled standard error of the means (SEM). Different significance letters show statistically significant differences between groups within an experiment.

	Experiment 1						Experiment 2					
	PP+VIT C	EO + VIT E	BET	CON	SEM	p-value	ERW10	ERW100	AA	CON	SEM	p-value
Body weight (g)												
d0	40.0	39.9	40.0	39.9	0.069	0.915	42.35	42.35	42.23	42.40	0.046	0.637
d11	373	370	372	370	1.052	0.562	373	365	370	369	1.645	0.449
d25	1552	1553	1542	1559	5.087	0.736	1436	1427	1434	1431	4.836	0.935
d32	2251	2263	2242	2267	9.217	0.774	2149	2138	2139	2135	7.256	0.924
d39	2893	2891	2906	2921	15.013	0.900	2858	2867	2794	2843	11.266	0.150
Average daily feed intake (g/d)												
d0-11	30.1	31.2	29.6	31.5	0.400	0.499	29.2	28.4	28.8	29.0	0.158	0.409
d11-25	111	112	111	111	0.530	0.870	108	107	108	108	0.303	0.944
d25-32	161	161	160	162	1.078	0.935	154 <sup>a</sup>	153 <sup>a</sup>	144 <sup>b</sup>	153 <sup>a</sup>	0.911	< 0.001
d32-39	171	171	172	174	1.427	0.793	197 <sup>a</sup>	195 <sup>a</sup>	170 <sup>b</sup>	191 <sup>a</sup>	1.444	< 0.001
d25-39	166	166	166	168	1.204	0.935	175 <sup>a</sup>	174 <sup>a</sup>	157 <sup>b</sup>	172 <sup>a</sup>	0.888	< 0.001
Average daily gain (g/d)												
d0-11	30.3	30.0	30.2	30.0	0.095	0.577	30.0	29.3	29.8	29.7	0.148	0.437
d11-25	84.2	84.5	83.6	84.9	0.575	0.334	77.2	77.2	77.3	77.1	0.310	0.999
d25-32	93.6	90.3	91.4	90.1	0.799	1.378	92.7	93.6	93.9	90.4	1.333	0.792
d32-39	91.7	89.8	94.8	93.4	1.243	0.556	101.3 <sup>ab</sup>	104.1 <sup>b</sup>	92.9 <sup>a</sup>	101.2 <sup>ab</sup>	1.113	0.008
d25-39	95.7	95.4	96.9	97.4	1.046	0.892	101.4	102.6	97.2	100.8	0.819	0.141
Feed conversion ratio (g feed/g gain)												
d0-11	0.99	1.04	0.98	0.98	0.013	0.341	0.97	0.97	0.97	0.98	0.002	0.491
d11-25	1.32	1.33	1.33	1.31	0.003	0.236	1.42	1.42	1.42	1.42	0.007	0.992
d25-32	1.72	1.79	1.76	1.81	0.019	0.407	1.67 <sup>ab</sup>	1.65 <sup>ab</sup>	1.54 <sup>a</sup>	1.71 <sup>b</sup>	0.020	0.031
d32-39	1.86	1.91	1.82	1.87	0.017	0.388	1.94	1.88	1.84	1.89	0.019	0.259
d25-39	1.73	1.74	1.71	1.73	0.010	0.800	1.73 <sup>a</sup>	1.70 <sup>a</sup>	1.61 <sup>b</sup>	1.71 <sup>a</sup>	0.010	0.002
Average daily water intake (mL/h) <sup>1,2</sup>												
d29-38 (HS; 09:30-15:30)	21.8	22.02	21.9	20.9	0.45		20.9	22.2	18.1	21.5	0.47	
d29-38 (N; 15:30 - 09:30)	23.6	21.4	22.9	22.0	0.47		20.7	18.8	18.7	19.8	0.51	
d29-38 (entire day)	22.0	21.2	21.9	21.6	0.35		21.2	20.2	19.2	20.8	0.32	
Water-to-feed ratio (h/d) <sup>1,2,3</sup>												
d29-38 (HS; 09:30-15:30)	3.30	3.66	3.39	3.52	0.121		3.89	3.83	3.37	3.49	0.079	
d29-38 (N; 15:30 - 09:30)	2.26	2.01	2.14	2.13	0.032		1.84	1.78	1.88	1.86	0.056	
d29-38 (entire day)	2.54	2.38	2.40	2.41	0.033		2.36	2.28	2.30	2.44	0.023	

<sup>1</sup> Measurements were performed at the treatment level and could thus not be statistically analysed. Calculated means and standard errors are presented. HS: during heat stress; N: during the rest of the day.

<sup>2</sup> Corrected for the number of light hours (h). Lighting schedule uses 18 L from 4:00 am to 10:00 pm.

<sup>3</sup> Calculation based on days 31, 32, 35 and 38 (Experiment 1) and days 27, 31, 35, 38 (Experiment 2).

AA appears to have a 7.5 % lower water intake value. Moreover, water intake during HS is numerically 3.8 % lower (Exp. 1) and 5.8 % higher (Exp. 2) compared to the rest of the day, when corrected for the number of light hours (Table 2). Feed intake (g/animal/light hour) during HS (09:30 - 15:30) was statistically lower compared to the rest of the day (15:30-09:30) in both experiments ( $p < 0.001$ ). In both experiments, there were differences between days (Exp. 1:  $p = 0.009$ , Exp. 2:  $p < 0.001$ ). In Exp. 1, there were no treatment differences ( $p = 0.917$ ), in Exp. 2, feed intake was significantly lower in broilers receiving AA ( $p < 0.001$ ) (Fig. 2).

In Exp. 1, EO + VIT E had the highest mortality rate during d32 - 39, at least compared to the PP + VIT C group. Although the mixed model showed a significant effect ( $p = 0.005$ ), post-hoc pairwise comparisons did not confirm this. During d25 - 32 and d25 - 39 no effects on mortality were observed. In Exp. 2, the treatments did not significantly affect mortality during d25 - 32, d32 - 39 and d25 - 39 (Table 3).

#### Physiological parameters

Adding drinking water supplements did not affect the breathing frequency (Exp. 1:  $p = 0.843$ , Exp. 2:  $p = 0.543$ ) and body temperature

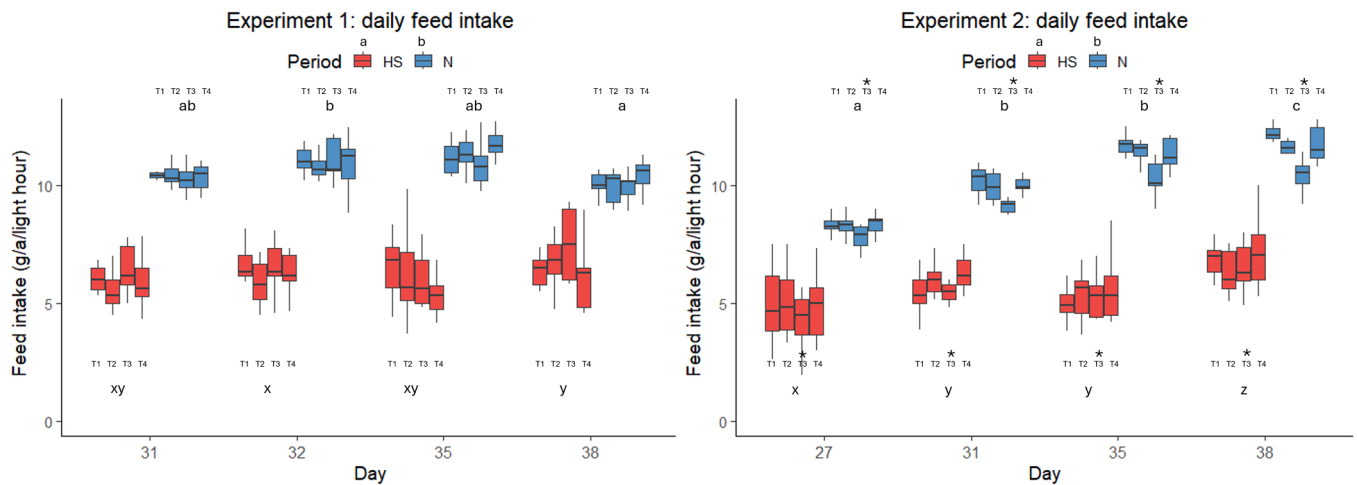
(Tb) (Exp. 1:  $p = 0.713$ , Exp. 2:  $p = 0.455$ ) during heat stress in both experiments. Although, there were some effects of the different observation days. In Exp. 1, breathing frequency was significantly lower on the first day of HS compared to the other days ( $p < 0.001$ ) (Fig. 3). Similarly, Tb was significantly lower on the first day and last day of HS compared to d31, 35, whereas Tb on the last day was still higher compared to the first day ( $p < 0.001$ ) (Fig. 4). In Exp. 2, breathing frequency ( $p < 0.001$ ) and Tb ( $p < 0.001$ ) were also lower on the first day of HS compared to the other observation days.

In Exp. 1, only a significant effect was found between EO + VIT E and BET for LDH ( $p = 0.019$ ). The treatment had no significant impact on other physiological parameters. For the other physiological parameters, no significant differences were found (Table 4).

In Exp. 2, significant differences in liver color were found between AA and ERW10 on the liver color (For L\*,  $p = 0.040$  and for b\*,  $p = 0.021$ ). Furthermore, the pH of the crop was higher in the AA treatment compared to the other treatments ( $p = 0.003$ ).

#### Meat quality

In Exp. 1, no significant differences were observed on the yield of the

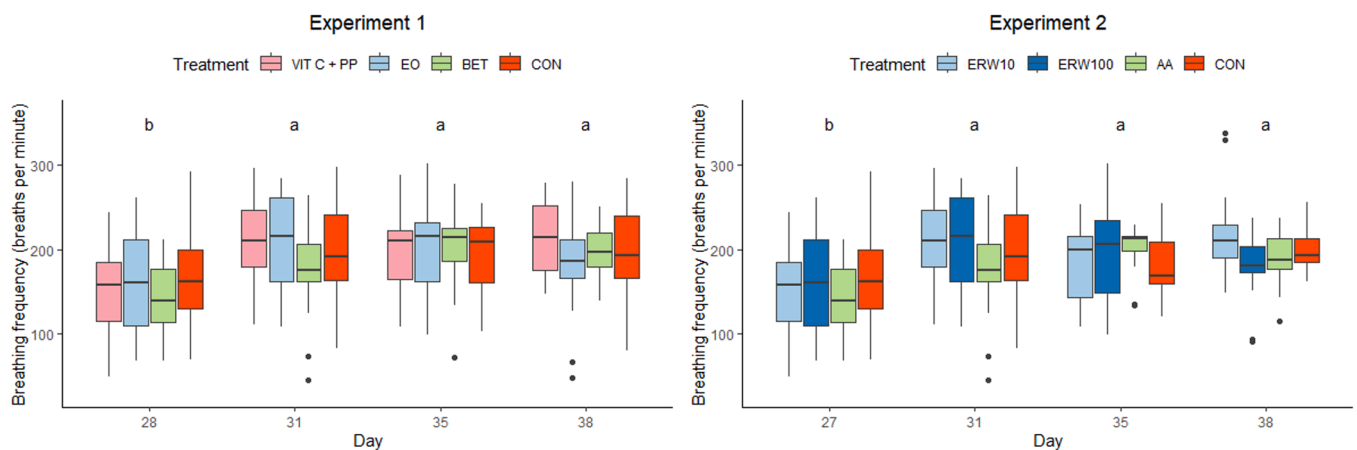


**Fig. 2.** Feed intake corrected for the number of light hours (g/animal/light hour) during heat stress (HS; 09:30 - 15:30) and the rest of the day (N; 15:30 - 09:00). Treatments include polyphenols and vitamin C (T1), essential oils and vitamin E (T2), betaine (T3) and control (T4) in Experiment 1. In Experiment 2, treatments include electrolyzed reduced water at 10 % (T1), electrolyzed reduced water at 100 % (T2), functional amino acids (T3) and control (T4). Lighting schedule uses 18 L from 4:00 am to 10:00 pm. Significance letters indicate differences between days and timings. Asterisks indicate differences between treatments within an experiment.

**Table 3**

Mortality during finisher with treatments applied from d27 – 39 (Experiment 1) and d26 – 39 (Experiment 2). Treatments include polyphenols and vitamin C (PP + VIT C), essential oils and vitamin E (EO + VIT E), betaine (BET), electrolyzed reduced water at 10 % (ERW10), electrolyzed reduced water at 100 % (ERW100), functional amino acids (AA) and control (CON). The table shows mean values ± standard errors. Different significance letters show statistically significant differences between groups within an experiment.

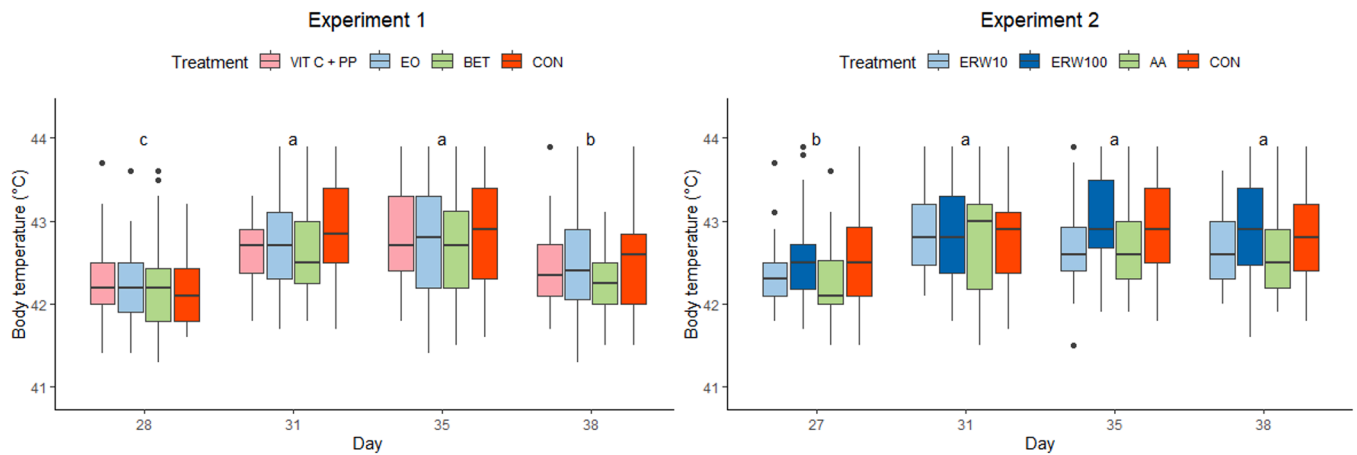
	Experiment 1				p-value	Experiment 2				p-value
	PP+VIT C	EO + VIT E	BET	CON		ERW10	ERW100	AZ	CON	
Mortality (%)										
d25-32	3.89 ± 4.17	1.67 ± 3.54	3.33 ± 3.54	1.67 ± 2.50	0.437	3.89 ± 4.17	1.11 ± 2.20	2.22 ± 3.63	2.22 ± 2.64	0.357
d32-39	0.00 ± 0.00	4.69 ± 5.76	1.73 ± 2.59	1.70 ± 2.54	0.005	2.84 ± 3.67	2.78 ± 3.63	1.11 ± 2.20	2.28 ± 2.70	0.570
d25-39	3.89 ± 4.17	6.11 ± 8.58	5.00 ± 4.33	3.33 ± 3.54	0.636	6.67 ± 4.33	3.89 ± 4.33	3.33 ± 3.54	4.44 ± 3.91	0.517



**Fig. 3.** Breathing frequency (breaths per min) for the different treatments during heat stress (HS) conditions, determined on d28, 31, 35 and 38 (Experiment1) and d27, 31, 35 and 38 (Experiment 2). Treatments include polyphenols and vitamin C (PP + VIT C), essential oils and vitamin E (EO + VIT E), betaine (BET), electrolyzed reduced water at 10 % (ERW10), electrolyzed reduced water at 100 % (ERW100), functional amino acids (AA) and control (CON). Different letters show statistically significant differences between observation days.

different carcass yields, breast meat color, nor meat quality parameters (Table 5). In Exp. 2, also no significant differences were observed on the yield of the different carcass yields, but the AA group had a higher value for lightness (L\*) compared to the other treatments ( $p < 0.001$ ). Furthermore, the AA group had a lower pH compared to ERW10 and CON ( $p = 0.012$ ). Shear force was higher for the AA group compared to

the other treatments ( $p < 0.001$ ). Thawing loss, cooking loss (fresh), cooking loss (thawed), drip loss (24 h) and drip loss (96 h) were also significantly higher in AA compared to the other treatments ( $p < 0.001$ ).



**Fig. 4.** Body temperature (°C) for the different treatments during heat stress (HS) conditions, determined on d28, 31, 35 and 38 (Experiment 1) and d27, 31, 35 and 38 (Experiment 2). Treatments include polyphenols and vitamin C (PP + VIT C), essential oils and vitamin E (EO + VIT E), betaine (BET), electrolyzed reduced water at 10 % (ERW10), electrolyzed reduced water at 100 % (ERW100), functional amino acids (AA) and control (CON). Different letters show statistically significant differences between observation days.

**Table 4**

Physiological parameters of the broilers in Experiment 1 and 2. Treatments include polyphenols and vitamin C (PP + VIT C), essential oils and vitamin E (EO + VIT E), betaine (BET), electrolyzed reduced water at 10 % (ERW10), electrolyzed reduced water at 100 % (ERW100), functional amino acids (AA) and control (CON). The table shows lsmeans and pooled standard error of the means (SEM). Different significance letters show statistically significant differences between groups within an experiment.

	Experiment 1						Experiment 2					
	PP+VIT C	EO + VIT E	BET	CON	SEM	p-value	ERW10	ERW100	AA	CON	SEM	p-value
Body weight (g)	2920	2970	2970	3070	0.021	0.114	2847	2858	2841	2825	13.684	0.877
Tb (°C) <sup>1</sup>	42.4	42.6	42.2	42.3	0.083	0.417	42.9	42.8	42.6	42.6	0.073	0.519
Liver color <sup>2</sup>												
L* liver	27.8	27.3	26.4	29.1	0.742	0.653	27.7 <sup>a</sup>	26.8 <sup>ab</sup>	23.7 <sup>b</sup>	27.1 <sup>ab</sup>	0.488	0.040
a* liver	22.2	22.2	21.9	21.8	0.394	0.978	21.5	21.5	19.9	20.7	0.455	0.552
b* liver	20.7	20.9	20.1	21.3	0.391	0.780	18.7 <sup>a</sup>	17.8 <sup>ab</sup>	15.7 <sup>b</sup>	18.3 <sup>ab</sup>	0.309	0.021
Digestive system pH												
pH crop							4.44 <sup>b</sup>	4.54 <sup>b</sup>	4.99 <sup>a</sup>	4.48 <sup>b</sup>	0.045	0.003
pH proventriculus							3.45	3.87	3.61	3.48	0.066	0.166
pH gizzard							3.67	4.19	4.15	3.72	0.098	0.224
Blood parameters <sup>3</sup>												
AST (U/L)	448	456	476	442	16.5	0.904	558	468	547	393	26.9	0.203
CK (U/L)	19 200	19 021	25 420	26 757	1 376	0.122	25 684	20 549	35 054	20 096	2 179	0.110
LDH (U/L)	4836 <sup>ab</sup>	4105 <sup>a</sup>	5169 <sup>b</sup>	4392 <sup>ab</sup>	109.1	0.019	3827	3892	4041	4087	183.0	0.956
GPx (U/L)	628	646	629	728	19.5	0.289	358	505	410	398	21.2	0.173
Lactate (mmol/L)	8.29	9.20	6.76	9.42	0.975	0.778	3.33	4.53	3.66	3.74	0.367	0.725
Glucose (mg/dL)	306	328	320	318	7.7	0.821	348	378	366	337	12.6	0.679
Uric acid (mg/dL)	5.19	5.50	4.49	4.09	0.301	0.391	4.96	6.53	5.33	5.09	0.416	0.546
MDA (nmol/mL)	14.1	14.9	14.1	13.4	0.450	0.793	15.0	17.8	16.0	15.4	0.704	0.515
pH blood							7.29	7.25	7.28	7.32	0.034	0.897
pCO <sub>2</sub> (kPa)							54.7	60.1	58.3	56.0	3.928	0.965
pO <sub>2</sub> (kPa)							55.6	58.1	58.4	59.8	2.463	0.949
tCO <sub>2</sub> (mmol/L)							27.5	26.7	27.0	27.7	0.634	0.949
HCO <sub>3</sub> (mmol/L)							24.5	24.9	25.2	26.0	0.561	0.831
BE (mmol/L)							-2.42	-2.29	-1.50	-0.21	1.001	0.865
sO <sub>2</sub> (%)							78.3	76.6	75.9	80.1	2.473	0.931
Mid-jejunal gut morphology <sup>4</sup>												
Villus height	1220	1219	1214	1193	47.2	0.971	1062	1106	1038	1129	69.3	0.766
Crypt depth	101.7	95.9	100.0	95.7	7.39	0.898	127.0	112.0	107.0	113.0	10.8	0.576
VC ratio	12.8	13.8	13.1	13.7	0.787	0.724	10.1	10.2	10.0	10.6	0.624	0.929

<sup>1</sup> Tb = body temperature.

<sup>2</sup> L\*: lightness (0 = black, 100 = white); a\* green-red axis (green = negative values, red = positive values); b\* blue-yellow axis (blue = negative values, yellow = positive values).

<sup>3</sup> AST: aspartate aminotransferase; CK: creatine kinase; LHD: lactate dehydrogenase; GPx: glutathione peroxidase; MDA: malondialdehyde; BE: base excess.

<sup>4</sup> VC ratio: villus/crypt ratio.

**Discussion**

Drinking water strategies in this study were tested during chronic, cyclic HS in broilers. Physiological responses confirmed that the applied

protocol effectively induced HS in the finisher phase, as reflected by elevated body temperature  $42.6 \pm 0.6$  °C and respiration rate  $186 \pm 53.8$  breaths per minute compared to TN values of  $41.5 \pm 0.4$  °C and  $72 \pm 22.4$  breaths per minute as observed in earlier research (De Baets

**Table 5**

Meat quality parameters of the broilers in Experiment 1 and 2. Treatments include polyphenols and vitamin C (PP + VIT C), essential oils and vitamin E (EO + VIT E), betaine (BET), electrolyzed reduced water at 10 % (ERW10), electrolyzed reduced water at 100 % (ERW100), functional amino acids (AA) and control (CON). The table shows lsmeans and pooled standard error of the means (SEM). Different significance letters show statistically significant differences between groups within an experiment.

	Experiment 1						Experiment 2					
	PP+VIT C	EO + VIT E	BET	CON	SEM	p-value	ERW10	ERW100	AA	CON	SEM	p-value
<b>Dressing yields (%)</b>												
Carcass yield	66.5	66.7	66.5	65.9	0.488	0.809	67.1	66.2	67.1	66.7	0.353	0.517
Breast yield	32.4	30.7	31.3	30.7	0.562	0.316	31.1	30.6	30.6	30.5	0.331	0.703
Drumstick yield	15.8	14.9	15.2	14.9	0.212	0.079	14.5	14.3	14.4	14.3	0.142	0.699
Thigh yield	26.2	25.8	26.3	25.3	0.389	0.526	25.7 <sup>ab</sup>	25.6 <sup>b</sup>	26.6 <sup>a</sup>	26.3 <sup>ab</sup>	0.196	0.021
Wing yield	10.4	10.6	10.4	10.7	0.194	0.811	10.1	10.3	10.2	10.3	0.141	0.882
<b>Breast meat color</b>												
L*	58.0	58.7	58.5	59.0	0.413	0.555	59.9 <sup>b</sup>	60.0 <sup>b</sup>	63.7 <sup>a</sup>	59.1 <sup>b</sup>	0.411	<0.001
a*	4.73	4.56	4.71	4.73	0.121	0.797	4.48	4.28	4.53	4.44	0.166	0.862
b*	15.1	15.1	15.1	15.4	0.178	0.556	15.5	15.3	16.6	15.3	0.287	0.070
<b>Meat quality</b>												
pH	6.04	5.99	6.03	6.01	0.020	0.521	5.95 <sup>a</sup>	5.95 <sup>ab</sup>	5.85 <sup>b</sup>	5.97 <sup>a</sup>	0.019	0.012
Shear force (N)	6.50	5.98	6.59	6.50	0.218	0.410	6.23 <sup>b</sup>	6.13 <sup>b</sup>	7.81 <sup>a</sup>	5.88 <sup>b</sup>	0.211	<0.001
Thawing loss (%)	4.53	4.40	4.25	4.49	0.432	0.988	3.60 <sup>b</sup>	2.77 <sup>b</sup>	6.14 <sup>a</sup>	3.12 <sup>b</sup>	0.280	<0.001
Cooking loss (fresh) (%)	21.9	21.4	20.7	22.7	0.737	0.503	21.1 <sup>b</sup>	20.8 <sup>b</sup>	28.0 <sup>a</sup>	20.9 <sup>b</sup>	0.634	<0.001
Cooking loss (thawed) (%)	18.1	17.8	17.2	19.1	0.657	0.519	18.5 <sup>b</sup>	18.5 <sup>b</sup>	23.6 <sup>a</sup>	18.4 <sup>b</sup>	0.528	<0.001
Drip Loss 24 h (%)	0.65	0.72	1.37	0.68	0.432	0.411	0.41 <sup>b</sup>	0.45 <sup>b</sup>	0.95 <sup>a</sup>	0.48 <sup>b</sup>	0.053	<0.001
Drip Loss 96 h (%)	1.66	1.83	2.27	1.62	0.247	0.571	1.70 <sup>b</sup>	1.77 <sup>b</sup>	3.29 <sup>a</sup>	1.79 <sup>b</sup>	0.131	<0.001

et al., unpublished). These increases were similar to an elevated body temperature of  $42.9 \pm 0.1$  °C observed by Lin, et al. (2006) after 3 h of HS, which implies that chickens indeed exceeded physiological homeothermic balance. However, cyclic HS protocols are considered milder, and physiological reactions during cooler episodes may be attenuated (de Souza, et al., 2016). Indeed, in the current study, water intake and water:feed ratio was numerically higher during HS (09:30 - 15:30; 6 light hours (L)) compared to TN (15:30 - 09:30; 12L) episodes when corrected for the number of light hours, although water intake warrants cautious interpretation due to the inability to do statistical analysis and is only of indicative nature. In addition, feed intake was statistically higher during HS episodes, again, when corrected for light hours, as feed intake of birds under a 18L:6D lighting is negligible during scotoperiod at around 2 % of their total intake (Rodrigues and Choct, 2019). Despite the recuperation during cooler hours, birds still showed impaired performance and mortality, which agrees with earlier studies (Zhang, et al., 2012; de Souza, et al., 2016; Cheng, et al., 2019). This supports the hypothesis that this model accurately initiates HS as observed in temperate regions and that cumulative heat load was substantial. Moreover, as a mitigation strategy during HS, drinking water additives may be preferred above feed additives, as chickens consumed less feed while increasing water intake during HS.

All drinking water additives dissolved well in water without visible residue. As expected, PP + VIT C was observed to have a slightly acidic pH due to ascorbic acid. Both polyphenols and vitamin C are known for their antioxidant, immunomodulatory and anti-inflammatory properties while also supporting gut morphology (Hajati, et al., 2015; Akbarian, et al., 2016; Lipiński, et al., 2017; Abdel-Moneim, et al., 2020; Shakeri, et al., 2020). Additionally, vitamin C functions as a cofactor in many enzymatic reactions (Shakeri, et al., 2020). Vitamin C is involved in thermoregulation, although no effects on body temperature were seen in the current study (Shakeri, et al., 2020). Papadopoulou, et al. (2017) observed improved antioxidant status after polyphenol supplementation in water at similar concentrations as the current study. In this study, PP + VIT C did not improve antioxidant status, intestinal integrity, performance or meat quality during HS as hypothesized. Despite this, broilers receiving PP + VIT C showed the lowest mortality during HS, which agrees with earlier findings (Al-Fataftah and Abdelqader, 2013). In literature, beneficial effects on performance, body temperature and respiration rate were mostly observed at higher concentrations of polyphenols in drinking water and under continuous high temperatures

(Al-Fataftah and Abdelqader, 2013; Oke, et al., 2017). Under cyclic HS, similar to the present study, performance responses are often limited (Sugito, et al., 2020). Likewise, studies reporting positive effects of vitamin C in drinking water were often conducted during constant HS (Vathana, et al., 2002; Adenkola and Angani, 2017). Additionally, polyphenol efficacy could be hampered by low bioavailability and absorption in the gastrointestinal tract (Abdel-Moneim, et al., 2020).

Both EO and vitamin E have demonstrated antioxidant activity and immune modulation effects, and EO may additionally support gut integrity and microflora during HS (Gopi, et al., 2014; Shakeri, et al., 2020; Yilmaz and Gul, 2024). Combined supplementation has been suggested to enhance antioxidant potential and reduce stress-induced glucocorticoid secretion (Shakeri, et al., 2020). Although this effect was not seen in the current study, which agrees with studies using dietary oregano oil under severe cyclic HS (38 °C) (Ghazi, et al., 2015). Studies observing positive effects on performance and meat quality typically used higher EO concentrations in drinking water, e.g. a mixture at 0.25 mL/L during severe HS (36 °C) (Tekce, et al., 2020) or oregano oil at 400 mg/L (Hernández-Coronado, et al., 2019), which is higher than our final concentration of 0.025 mL/L in which we saw no positive effects on performance or meat quality. Similarly, studies adding both EO and vitamin E to the diet reported no improvement in performance of laying hens (Büyükkılıç Beyzi, et al., 2020). Mortality was expected to decrease through antioxidant effects, however, no antioxidant effects were observed, while mortality was highest in this treatment. Higher mortality has been associated with decreased water intake due to a possible strong taste of certain EO (Khosravinia, 2016), but no decrease in water intake was observed in this study, although this was not statistically verified. Evidence on vitamin E- and EO-supplemented drinking water remains limited, hampering optimal dosing. Studies using 50-100 mg/L vitamin E during continuous HS (28-30 °C) reported improved body temperature, performance and mortality (Mustafa and Othman, 2024), while dietary inclusion of 250 mg/kg vitamin E is generally considered effective in reducing mortality and body temperature during HS (Shakeri, et al., 2020; Calik, et al., 2022).

The pH of drinking water supplemented with betaine (BET) was markedly acidic due to its hydrochloride form (King, 1936), though this did not affect water intake. Betaine is known to enhance antioxidant capacity and intestinal integrity, thereby potentially improving performance during HS (Shakeri and Le, 2022). In this study, however, no effects on antioxidant status, intestinal integrity or performance were

observed. Similarly, earlier research found no effects of betaine supplied at 0.55 g/L in laying hens (De Baets, et al., 2024). The osmoregulatory function of betaine may support sustain body temperature by limiting dehydration and enhancing water retention (Ratriyanto and Mosenthin, 2018). Indeed, earlier research noticed that dietary betaine significantly reduced body temperature during higher temperatures (35 – 38 °C) (Attia, et al., 2009; Ruff, et al., 2021), which may explain the lack of effects in the current study under milder temperatures. Betaine could improve carcass quality via its role in methionine metabolism and carnitine synthesis (Ratriyanto and Mosenthin, 2018). Indeed, Attia, et al. (2009) found improved meat quality, performance, respiration rate, blood pH, and humoral immune response in chickens during HS (38 °C) with dietary betaine (1 g/kg), while we could not confirm these effects with 0.7 g/L in water. Comparable benefits in meat ducks at 0.4 – 1.2 g/L were attributed to improved metabolic homeostasis (Lee, et al., 2019). At similar drinking water concentrations (0.5 g/L), Sayed and Downing (2011) observed only transient effects on BW gain while higher doses (1 g/L) affected performance negatively, suggesting that bird requirements may be exceeded, especially during HS when water intake increased.

ERW is generated through electrolysis, producing both oxidized water, used as a disinfectant, and ERW, which contains high levels of active hydrogen with antioxidant properties, acting as a ROS scavenger (Azad, et al., 2013). ERW was confirmed to be highly alkaline with a low oxidation-reduction potential (ORP) as expected (Akbarian, et al., 2016), yet this did not negatively affect water intake or broiler performance. Although, variation in ORP between sampling points suggested some decline along the drinking line. Electrolyzed, hydrogen-rich water has been shown to enhance glucose metabolism in humans (Kajiyama, et al., 2008). Indeed, earlier studies in broilers have confirmed ERW to modulate glucose metabolism, and improve the oxidative status and growth performance during constant HS (34 °C) (Azad, et al., 2013, 2021). Similar improvements in FCR, villus length, villus/crypt ratio, and antioxidant capacity were reported in laying hens, again, under high temperature (34 °C for 8 h) (Zhang, et al., 2022). In contrast, the milder cyclic HS used in the current study may explain the absence of effects on performance, glucose metabolism or oxidative status. In Exp. 2, blood-gas parameters were analysed as well, as changes were expected in response to ERW's high pH, but no significant effects could be demonstrated.

Functional amino acids, L-arginine, L-monosodium glutamate (L-MSG) and L-threonine, were included in drinking water to compensate for potential deficiencies caused by a reduction in feed intake during HS. More specifically, these AA were selected based on their proposed roles. Arginine was hypothesized to increase performance and meat quality due to its role in guanidinoacetic acid synthesis as a precursor of creatine (Portocarero and Braun, 2021). L-MSG could enhance palatability, stimulating broiler performance (Olarotimi and Adu, 2022). In contrast to expectations, broilers receiving AA had a numerically lower water intake and a significantly reduced feed intake, resulting in reduced growth during HS, although slaughter weights were unaffected. Supplemental AA may have interfered with the physiological signalling pathways regulating feed intake, as previous studies observed that hypothalamic L-glutamate injections reduced feed intake in broilers (Wang, et al., 2012). The reduction in feed intake was proportionally greater than the effect on growth, leading to an improved FCR. Similarly, Williams, et al. (2017) observed reduced feed intake and improved FCR in turkeys fed supplemental L-arginine (0.05 %) in TN conditions. Although, other studies observed improved performance effects with MSG at 5 g/L in drinking water (Osman and Mohammed, 2021). No effects on gut integrity were observed in this study, which aligns with the absence of performance effects. The AA treatment resulted in an elevated drinking water pH, which increased crop pH, while drinking water pH of the other treatments was higher than the AA treatment. Notably, the AA-supplemented broilers had a lower pH, paler colour, higher shear force and water holding capacity of breast muscle, which

may imply that AA increased heat-stress related reductions in muscle glycogen and muscle pH, which affects the WHC and breast muscle colour due to excessive protein denaturation and less functional proteins (Barbut, 1993; Ma, et al., 2021). Williams, et al. (2017) also observed higher thawing and cooking losses in breast muscles of turkeys supplemented with arginine under TN conditions. Additionally, arginine-induced vasodilatation was hypothesized to improve heat dissipation and lower Tb (Liu, et al., 2019). However, these effects were not observed in this study. Literature on drinking water supplementation of amino acids are limited, which makes optimal dosing challenging. Literature on dietary additions recommend the addition of 6600 mg/kg L-arginine, 1000 mg/kg L-threonine and 500 mg/kg L-MSG (Olarotimi and Adu, 2022; Wasman, 2022; Oliveira, et al., 2024) Although, increased water intake during HS may increase the intake beyond requirements, and high doses of L-MSG could be associated with adverse effects (Khadiga, et al., 2009).

Generally, none of the additives improved antioxidant status, tissue damage markers, performance, or thermoregulation under the current mild cyclic HS model, whereas most positive results described in literature originate from constant or more severe HS models (e.g.  $\geq 34$ –38 °C), which may nuance those findings and suggest that such positive effects may be limited to extreme HS.

Studies on optimal dosing, stability and bioavailability in drinking water remain limited. The optimal dosing in drinking water may vary from those in diets, as broilers increase their water intake during HS. Moreover, due to the existence of various mixtures of plant derivatives, it is often difficult to determine the optimum dose of inclusion (Serra, et al., 2021). After all, the biological activity of polyphenols and EO depends on their bioavailability which is defined by the type of compound, chemical and physical properties and the type and presence of functional groups (D Archivio, et al., 2007; Lipiński, et al., 2017; Serra, et al., 2021). Despite daily renewal, the ORP of ERW was observed to increase and thus deteriorate through the drinking line, which may indicate that the stability in drinking water is not yet optimal. Lastly, microbiological issues may have played a role. Water temperature increased rapidly during HS despite daily renewal of the stock solution and a low drinking water flow rate due to the limited number of birds compared to practice may impair continuous uptake of additives, combined with heat, it may stimulate biofilm formation. In Exp. 1, microbiology was not tested. However, upon visual inspection, water samples of the treatment with PP + VIT C showed debris in the drinking water line, clogging nozzles. In the stock solution, no such effects were observed. Moreover, AA in powder form may prove unsuitable for use in drinking water as reflected by a substandard microbiological and organoleptic profile. Many biofilm-forming microorganisms utilize nitrogen-based compounds as a nutrient source, thus AA supplementation may have promoted biofilm formation in the water lines (Liu, et al., 2016). Although all water lines were disinfected beforehand.

## Conclusion

Drinking water additives are often used in the poultry industry during HS, as they can be applied rapidly and broilers increase their water intake while reducing feed intake. Despite their suitability, none of the tested drinking water additives, including polyphenols and vitamin C, essential oils and vitamin E, betaine, electrolyzed reduced water and amino acids (L-arginine, L-monosodium glutamate and L-threonine) improved broiler performance, meat quality, oxidative status, or physiological responses during HS in the finisher phase, which might be associated with the chronic cyclic HS model applied in these experiments, potentially allowing broilers to recuperate during TN nighttime episode. Co-supplementation of L-arginine, L-monosodium glutamate and L-threonine via the drinking water impaired feed intake by 9.6 %, growth by 8.2 % but improved FCR by 0.10. Meat quality parameters such as water holding capacity and shear force were affected negatively. Likely, these effects can be associated with the suboptimal

water quality that arose after supplementing the AA in the drinking water, and thus such an application warrants caution.

### CRedit authorship contribution statement

**Renée De Baets:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Sofie Van Nerom:** Writing – review & editing, Formal analysis, Data curation. **Kobe Buyse:** Writing – review & editing, Supervision, Conceptualization. **Gunther Antonissen:** Writing – review & editing, Supervision, Conceptualization. **Jeroen Degroote:** Writing – review & editing, Supervision, Conceptualization. **Evelyne Delezie:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.psj.2025.106322](https://doi.org/10.1016/j.psj.2025.106322).

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