



**MORPHOLOGICAL CHANGES IN THE LUNGS AND AIR SACS
OF THE JAPANESE QUAIL (*Coturnix japonica*) EXPOSED TO
HEAT STRESS**

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fulfilment of the requirements for the degree

of

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Declaration

I **Abdurrahman Abdulkadir** declare that this thesis is my own unaided work unless otherwise stated. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



Signed on the 17 August 2023

Dedication

In memory of my Father

To my Mother – Hajiya Salamah

My Wife – Aishah

My Daughters – Ramlah, Salamah-Ihsan, Aishah-Ummi

Publications and Presentations

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Abstract

The Japanese quail presents a potential protein food security alternative in rural sub-Saharan Africa because of its small size and easy husbandry. The nutritional and therapeutic value of its meat and egg makes it an interesting and better choice than chicken in some parts of the world. In addition, researchers used Japanese quail as an animal model of human genetic and developmental disorders because of its short generational interval. However, global warming threatens its welfare by propagating heat stress. The physiological response of Japanese quail under heat stress causes negative performance and sometimes instant mortality. Hence, in this study, lung of the quail under heat stress was microscopically examined as it is the most important organ for survival under heat stress. All other organs depend on the lungs for oxygen, and it is also the most crucial in evaporative cooling.

A total of 38 Japanese quail were used in this study. A pilot study was conducted that used eight quails, to ensure the possibility of survival under the proposed experimental temperatures. Afterwards, 30 quails were randomly allocated — based on initial body mass — to five groups of six quails each. The control groups (CT and CT2) were maintained at a thermoneutral temperature of 25°C throughout the experiment while acute heat stress group (AH) were maintained at 38°C for 24 hours only. The chronic heat stress groups (CH1 and CH2) were maintained at 35°C for seven days and 28 days respectively. Body mass, cloacal temperature, and respiratory rate of quails were measured daily to monitor health and detect any serious ill health from heat exposure. Food and water were provided *ad libitum*. At the end of the experiment, all quails were terminated using an overdose of anaesthetic and lungs

were harvested and processed for microscopy. Lung weight, volume and size were measured before sampling. Tissue samples were processed, and sections were cut with a microtome and stained with Mayers H&E, new pentachrome stain and Gomori's one-step trichrome stain. Other tissue samples were triple immunolabelled with anti- α -SMA and Collagen 1 antibody and DAPI nuclear stain. Tissue samples were also processed for scanning and transmission electron microscopy.

No significant difference in body mass, cloacal temperature, respiratory rate and lung parameters was found in heat-stressed quails compared with control. However, microscopic examination revealed blood congestion and excessive leakage of blood into airway of lungs in heat-stressed groups compared with the control. In addition, there was structural damage to parenchyma and blood vessels, which incites an inflammatory response causing deposition of collagen fibres in some areas of the lungs in heat-stressed groups. Interestingly, these effects occur in a time-dependent pattern. The most impact is seen in AH and CH1 groups while CH2 shows signs of recovery.

In conclusion, Japanese quail lung was negatively impacted by heat stress, which can lead to instant mortality or long-term reduction of performance. Despite the evidence from this study suggesting that Japanese quail can adapt to the effects of heat stress if it survives the initial impact, a conscious effort must be made to alleviate or remove heat stress for quality outcomes.

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Table of Contents

Declaration.....	I
Dedication	II
Publications and Presentations	III
Abstract.....	IV
Acknowledgements.....	VI
Table of Contents.....	VII
List of Figures	XII
List of Tables	XVI
List of Abbreviations	XVII
1 CHAPTER ONE — INTRODUCTION	1
1.1 Background.....	2
1.2 The Japanese quail	4
1.3 Japanese quail as a model for experimental research.....	11
1.4 Economic importance of Japanese quail.....	17

1.5	Negative impact and mitigation of heat stress on Japanese quail	22
1.6	Anatomy of the avian respiratory system.....	40
1.7	Highlights and rationale of the study.....	50
1.8	Aim of the study	51
1.9	Objectives of the study	52
2	CHAPTER TWO — SCOPING REVIEW	53
2.1	Introduction.....	54
2.2	Methodology.....	54
2.2.1	Inclusion criteria.....	54
2.2.2	Search strategy	55
2.2.3	Sources of evidence selection.....	58
2.2.4	Data extraction	59
2.2.5	Data analysis and presentation	62
2.3	Results	62
2.3.1	Study inclusion	62

2.3.2	Characteristics of included studies	66
2.3.3	Review findings	70
2.4	Discussion	73
2.5	Limitations of the scoping review	77
2.6	Conclusions.....	77
3	CHAPTER THREE — MATERIALS & METHODS.....	79
3.1	Ethics approval	80
3.2	Study animals	80
3.3	Experimental setup	82
3.4	Terminal procedures	85
3.5	Latex casting.....	85
3.6	Lung volume estimation using Scherle’s method	87
3.7	Sampling.....	90
3.8	Light microscopic procedures	93
3.8.1	Mayer’s H&E staining.....	93

3.8.2	The new pentachrome staining	93
3.8.3	Gomori's one-step trichrome staining.....	93
3.9	Immunofluorescence labelling procedures	94
3.9.1	Optimisation of antibodies	94
3.9.2	Immunofluorescence triple labelling.....	97
3.10	Scanning electron microscopy procedures	97
3.11	Microscopy	98
3.12	Morphometry.....	98
3.13	Statistical analyses	99
4	CHAPTER FOUR — RESULTS.....	100
4.1	Body vitals measurements	101
4.2	Lung parameters	105
4.3	Light microscopy analyses.....	106
4.4	Fluorescence microscopy analysis	115
4.5	Electron microscopy analyses	121

5	CHAPTER FIVE — DISCUSSION & CONCLUSION	126
5.1	Discussion	127
5.2	Limitations of the study	137
5.3	Conclusion	138
6	REFERENCE LIST	140
7	APPENDICES	167
7.1	Appendix 1 — Similarity index report	167
7.2	Appendix 2 — Ethics Approval Certificate	168
7.3	Appendix 3 — Pilot Study Report.....	171
7.4	Appendix 4 — Registered scoping review protocol	175

List of Figures

Figure 1: Drawing of the Japanese quail male and female showing the approximate size and plumage.	9
Figure 2: A diagram of the cellular mechanism of ROS processes.	26
Figure 3: A latex cast of the Japanese quail lung showing the trachea, lungs, and some of the air sacs.....	41
Figure 4: A photograph of an intact Japanese quail lung.	44
Figure 5: The representative micrographs of the Japanese quail lungs.....	46
Figure 6: Diagram of unidirectional airflow in the avian respiratory system.....	50
Figure 7: A flow chart diagram of the study selection criteria.	65
Figure 8: The number of included studies categorised based on country and year.	69
Figure 9: A diagram of the impact of heat stress on Japanese quail.....	72
Figure 10: A diagram of the process of ROS generation and the mechanism of some dietary interventions.....	75
Figure 11: A flow of the animal grouping and experiment procedures.	81
Figure 12: A schematic floor plan of the housing used for the experiment.....	83

Figure 13: The photographs of the cage used for the experiment.....	84
Figure 14: A picture of the latex cast apparatus.....	86
Figure 15: The photographs of the method used to estimate the lung size using a vernier calliper.....	87
Figure 16: A diagram showing Scherle's method of lung volume measurement.	89
Figure 17: The photographs of modified Scherle's method used in lung volume estimation.	90
Figure 18: The sampling processes for light and scanning electron microscopy analyses.....	92
Figure 19: The line graphs of vital parameters of Japanese quails in CH1 and control group over seven days.....	103
Figure 20: The line graphs of vital parameters of Japanese quails in CH2 and control groups over 28 days.....	104
Figure 21: The representative micrographs of the H&E-stained Japanese quail lungs of the control and heat-stress groups.....	106
Figure 22: The representative micrographs of new pentachrome-stained Japanese quail lungs of control and heat-stressed groups.....	108
Figure 23: The representative micrographs of Gomoris' trichrome stained Japanese quail lung of control and heat-stressed groups (AH, CH1 and CH2).	110

Figure 24: The representative micrographs of Gomoris' trichrome stained Japanese quail lung of control and heat-stressed groups.....	111
Figure 25: A representative micrograph of Gomoris' trichrome stained Japanese quail lung of control and heat-stressed groups.....	112
Figure 26: A representative micrograph of the immunolabelled Japanese quail lungs of the control and heat-stressed groups.....	116
Figure 27: A representative micrograph of the immunolabelled (merged) Japanese quail lungs of the control and heat-stressed groups.	117
Figure 28: A representative micrograph of the immunolabelled (merged) Japanese quail lungs of the control and heat-stressed groups.	118
Figure 29: A representative micrograph of the immunolabelled (Col1) Japanese quail lungs of the control and heat-stressed groups.	119
Figure 30: The representative micrographs of the immunolabelled (merged) Japanese quail lungs of the control and heat-stressed groups.....	120
Figure 31: The representative scanning electron micrographs of the Japanese quail lungs of the control and heat-stressed groups.	123
Figure 32: The representative scanning electron micrographs of the Japanese quail lungs of the control and heat-stressed groups.	124

Figure 33: The representative scanning electron micrographs of the Japanese quail lungs of the control and heat-stressed groups.	125
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List of Tables

Table 1: Standard biological data for Japanese quail.	7
Table 2: Japanese quail lines used to study human hereditary diseases.	13
Table 3: Comparison of the chemical composition of Japanese quail and chicken eggs.	20
Table 4: Comparison of Japanese quail and chicken eggs mineral content.	21
Table 5: Comparison of vitamin content of Japanese quail and chicken eggs.	21
Table 6: Comparison of Japanese quail and chicken eggs' fatty acids.	22
Table 7: Comparison of Japanese quail and chicken eggs' amino acids content.	22
Table 8: Some of the selected studies on the measures of mitigating the effect of heat stress in the Japanese quail.....	31
Table 9: A sample of the data extraction used for the scoping review.	61
Table 10: The characteristics of included studies for the scoping review.....	66
Table 11: The table of vital parameters of Japanese quails in AH and control group.....	102
Table 12: The table of the gross measurements on the Japanese quail lungs.....	105
Table 13: The morphometric values of cell and nuclei in Japanese quail lungs.....	114

List of Abbreviations

ACTH: Adrenocorticotrophic hormone

ADP: Adenosine diphosphate

ALT: Alanine transferase

AST: Aspartate transferase

ATP: Adenosine triphosphate

AvUCP: Avian uncoupling protein

CAT: Catalase

DNA: Deoxyribonucleic acid

COX-2: Cyclooxygenase 2

GSH-Px: Glutathione peroxidase

H/L: Heterophil/Lymphocyte ratio

HO-1: Haem-oxygenase 1

HSP: Heat shock proteins

I κ B α : Nuclear factor of kappa light polypeptide gene enhancer in B cells alpha

IPCC: Intergovernmental panel on climate change

KEAP1: Kelch-like ECH (eryol CoA hydratase) associated protein 1

MDA: Malondialdehyde

NAD: Nicotinamide adenine dinucleotide

NADH: Reduced nicotinamide adenine dinucleotide

NF- κ B: Nuclear factor kappa B

Nrf2: Nuclear factor (erythroid-derived) related factor 2

NVRI: National veterinary research institute

OSF: Open science framework

PHA-P: Phytohaemagglutinin-P

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SOD: Superoxide dismutase

SRBC: Sheep red blood cell

TCA: Tricarboxylic acid

TNF- α : Tumor necrosis factor-alpha

UCP: Uncoupling protein.

1 CHAPTER ONE — INTRODUCTION

1.1 Background

One of the many dangers of global warming is the extreme heat weather conditions (Law et al., 2018). Other hazards associated with global warming are loss of biodiversity, rising sea levels, ocean acidification and deoxygenation (Law et al., 2018). The concern about climate change is that it exacerbates poverty and food insecurity (Law et al., 2018). This concern includes its adversity on poultry production and product quality because poultry farming provides a source of income and nutrition (Nyoni et al., 2019).

Extreme heat continues to rise globally with global warming (McCarthy, 2017), threatening animal welfare, including poultry. It leads to heat stress in humans, birds, and other animals. Heat stress is the physiological response to high environmental temperatures (Nazar et al., 2019). There are two types of heat stress, namely acute heat stress and chronic heat stress. Acute heat stress results from sudden and short periods of high ambient temperatures from a few hours to less than seven days. In contrast, chronic heat stress refers to a prolonged period of high ambient temperature for more than seven days (Marchini et al., 2016).

Avian species are homeothermic, capable of dissipating heat to surroundings to maintain normal core body temperature (Saeed et al., 2019). The body temperature in most birds ranges between 38.5 – 41°C, but it is up to 38.9 – 41.4°C in quail (Prinzinger et al., 1991). Birds incapable of maintaining core body temperature because of a rise in ambient temperature above their thermoneutral comfort are said to be heat-stressed. The thermoneutral environmental temperatures for most birds are 16 – 25°C (Abdel-Moneim et al., 2021) and up to 30°C for Japanese quail (Alagawany et al., 2017). Any rise in environmental temperature

above thermoneutral range, being for a short or long period, will propagate heat stress in poultry (Saeed et al., 2019). The bird's response to regulate heat stress includes a reduction of feed intake and minimising heat production from metabolism (Akbarian et al., 2016). Meanwhile, growth performance, productivity and immune response will decline due to insufficient dietary energy, protein, and minerals (Alagawany et al., 2017). These lead to low meat and egg quality and economic loss (Alagawany et al., 2017; Barrett et al., 2019). Thus, heat stress is responsible for a huge economic loss in general poultry production in some countries (Bohler et al., 2021). Furthermore, heat stress response also initiates excessive production of reactive oxygen/nitrogen species more than the body's antioxidant capacity (Del Vesco and Gasparino, 2013), leading to mitochondrial dysfunction and gross tissue damage affecting normal organs structure and functions (Akbarian et al., 2016).

Aengwanich and Simaraks (2004) reported damage to heart, lungs, kidneys and liver in broilers exposed to 33°C chronic temperatures. The pathophysiology could be mitochondrial dysfunction or direct impact of hot air delivered directly into respiratory system (Marder, 1983). In addition, Xie et al. (2020) observed diffuse congestion and parabronchial haemorrhage in lungs of budgerigars exposed to heat stress. However, respiratory system morphology of heat-stressed Japanese quail was not investigated. The respiratory system plays a vital role in thermoregulation through panting and evaporative cooling during heat-stress conditions (Weathers and Schoenbaechler, 1976). Increasing the respiratory rate in heat-stressed birds leads to respiratory alkalosis by offsetting blood pH, thereby reducing blood acidity (Saeed et al., 2019). Furthermore, heat stress causes physiological responses and morphological adaptations in lungs and other vital organs of birds to return the system

to normal homeostasis. Considering the Intergovernmental Panel on Climate Change (IPCC) sixth assessment report, which suggested that temperatures will continue to rise with increased occurrence of heat waves in foreseeable future (IPCC, 2021), is one of the reasons that motivated this study.

Morphology of Japanese quail's lung will be examined in this study, because there exists published literature on several organs of quail affected by heat stress (Al-Ali et al., 2017; Pu et al., 2020), however no comprehensive study on lungs, despite its significance in thermoregulation. In addition, quail is an essential economic commodity and a source of quality nutrition in Nigeria, South Africa, and other sub-Saharan countries, especially among rural households. Morphological and morphometric data from this study will help refine husbandry practices, especially among rural farmers with limited resources to control climate. In the following section, Japanese quail history and behaviour will be briefly discussed.

1.2 The Japanese quail

The small, terrestrial, compact bird *Coturnix japonica*, the Japanese quail, is indigenous to Japan, China, Korea, and Mongolia, from where it was introduced to other parts of the world (Mills et al., 1997; Mizutani, 2003).

The wild Japanese quail's natural habitat is grassland, cropland and riverside (Cheng et al., 2010) with a complicated seasonal migration (Baer et al., 2015) probably because quails do not fly for long distance. It was domesticated in Japan or brought in from China in domesticated form around the eleventh century for their song (Cheng et al., 2010). Later, in twentieth century, it was mainly bred in Japan for egg production (Huss et al., 2008).

However, during the second world war, most of the lines of Japanese quail were lost, and revival of the colony was mainly from a few surviving non-singing quail (Mills et al., 1997). Thus, most Japanese quail colonies worldwide came from post-war colonies (Mills et al., 1997). A colony of Japanese quail was established in dental research institute at the University of the Witwatersrand around 1978 from an unidentified line of *Coturnix coturnix japonica* (Ichilcik and Austin, 1978).

Japanese quail belongs to the order Galliformes, family Phasianidae, genus *Coturnix*. The Japanese quail are classified as old-world quail, distinct from new-world quail such as Bobwhite or California quail (Huss et al., 2008; Baer et al., 2015). Although closely related to common quail (*Coturnix coturnix*), interbreeding between the two species is almost impossible except in captivity (Cheng et al., 2010; Baer et al., 2015). Hence in 1983, Japanese quail was given a full species status *Coturnix japonica* (Cheng et al., 2010). It is noteworthy that *Coturnix japonica* was initially considered a subspecies of common quail and was given the scientific name *Coturnix coturnix japonica* (Cheng et al., 2010). The confusion regarding the scientific name of the Japanese quail still exists in the literature to date (Mills et al., 1997). Some of these examples in recent publications include Santos et al. (2017) and Mehaisen et al. (2019), who referred to Japanese quails as *Coturnix coturnix japonica* with the word *Coturnix* repeated. Other examples are the *Coturnix coturnix* with no *japonica* ever mentioned (Nazar et al., 2018) or the *Coturnix japonica* which is the correct nomenclature (Vitorino Carvalho et al., 2020). Sometimes researchers avoid the scientific name altogether and use “Japanese quail” phrase only (Nazar et al., 2019). In this thesis, Japanese quail phrase will be interchanged with scientific name *Coturnix japonica* (*C. japonica*) as mentioned in the title.

Some biological data available from literature regarding Japanese quail include approximate length of 17cm. The females usually weigh around 120g, while males weigh around 100g and females are considerably larger (Baer et al., 2015). However, certain broiler quail genetically bred for meat can weigh up to 300g at six weeks of age (Cheng et al., 2010). **Table 1** summarises some of the standard biological parameters of Japanese quail. The quail has a relatively rapid growth reaching sexual maturity within five to six weeks post-hatching (Baer et al., 2015). This feature makes it attractive for business turnover and modelling in laboratory experiments.

Table 1: Standard biological data for Japanese quail.

Body weight (g)	
1 day old	6 – 8
Adult male	100 – 130
Adult female	120 – 160
Organ weight (% body weight)	
Liver	1.95
Heart	0.91
Kidney	0.73
Testes	2.88
Body temperature (°C)	40.7 – 43.2
Performance and longevity	
Egg weight (g)	9 – 10
Egg number/100 bird days	80 – 90
Age at sexual maturity (days)	38 – 42
Life span (months)	24 – 26
Reference: (Baer et al., 2015)	

The plumage of wild type, as preserved in domestic Japanese quail (Mills et al., 1997), is predominantly cinnamon-brown in both sexes but distinguishable on breast and cheek feathers (**figure 1**). The females have pale-coloured breasts and cream-coloured cheek feathers, whereas the males have dark rust-red breasts and cheek feathers (Cheng et al., 2010). The distinction in plumage colour between the male and female Japanese quail usually appears in the third week of life (Mizutani, 2003). Furthermore, there is a testosterone-dependent plumage change during winter among male Japanese quail, during which it develops a white collar (Mills et al., 1997).

The Japanese quail feed and drink throughout the day, but frequency of eating and drinking is most at beginning and end of the day, depending on photoperiod (Mills et al., 1997). The sense of taste and smell helps quail to select certain food preferences and insects during foraging. It preferred sweet and sour solutions over salty and bitter ones (Mills et al., 1997). In addition, quail can differentiate and avoid aposematic insects — insects using body colour to indicate that it is potentially dangerous — based on smell alone (Mills et al., 1997). While foraging, the myopic lower vision field of the quail enables it to focus on the ground and monitor the sky and horizon for predators concurrently. However, colour vision is more predominant than form and shape vision (Mills et al., 1997).



Figure 1: Drawing of the Japanese quail male and female showing the approximate size and plumage.

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[http://offline.whatbird.com/obj/1189/portrait/Japanese Quail.aspx](http://offline.whatbird.com/obj/1189/portrait/Japanese%20Quail.aspx)

Furthermore, ambient temperature changes affects feed intake pattern if quail were offered a choice. In an experiment by MacLeod and Dabutha (1997), Japanese quail were seen to reduce intake of carbohydrate diet and preferred diet with high protein when exposed to higher ambient temperature. This mechanism avoids heat production related to energy expenditure during thermoregulation while maintaining an appropriate growth rate (MacLeod and Dabutha, 1997).

Body temperature in Japanese quail is affected by photoperiod, as Woodard and Mather (1964) suggested. Light periods coincide with higher body temperature indicating more physical activity compared to lower body temperature during dark period, which is primarily a resting time (Woodard and Mather, 1964). However, while maintaining similar photoperiods and ambient temperatures, female quail tend to have higher body temperatures than males. Still, body weight was not found to influence this difference in body temperature (Gilbreath and Ko, 1970). The level of body temperature is also affected by thermal manipulation during incubation and early life. The earlier experiment by McNabb and McNabb (1977) and a recent experiment by Vitorino Carvalho et al. (2020) indicated that thermal manipulation during embryogenesis and early life interferes with quail chicks' ability to develop normal body temperature. This is most likely due to energy expenditure in evaporative cooling mechanisms that happened during manipulation process. The experiments further revealed that quail chicks reared in higher air temperature conditions had slowed growth compared to thermoneutral comfort because energy is expended in cooling the body (McNabb and McNabb, 1977). Hence, energy required for growth was traded to cool body due to elevated body and environmental temperatures.

1.3 Japanese quail as a model for experimental research

The Japanese quail was first described as an experimental animal model by Carol Padgett and William Ivy of Auburn University in 1959 (Huss et al., 2008). It was selected for this role because of its small size, rapid growth, and easy husbandry. The study of human developmental stages gained a lot of insight from studying avian embryos. This is because eggs are easily accessible to be studied directly by removing a small portion of the eggshell and incorporating computer-assisted video microscopy (Huss et al., 2008). With video-microscopy quail, embryo development can be recorded or viewed live on a computer screen where live images might be captured (Huss et al., 2008). Quail and chicken were the avian models mostly used, which even led to making of quail-chick chimaera (Huss et al., 2008). The chimaera is a suitable model for understanding the fate of cell differentiation during embryogenesis (Huss et al., 2008; Baer et al., 2015). The 16-day developmental stages of Japanese quail are close to chicken's development but only five days apart (Baer et al., 2015). Both Japanese quail and chicken are warm-blooded, which resembles human physiology and reason for their use in understanding human development (Huss et al., 2008; Baer et al., 2015). However, Japanese quail is comparably more efficient than chicken and mouse in producing knockout and transgenic lines with fewer experiments and costs (Huss et al., 2008; Baer et al., 2015). This is because Japanese quail have shorter embryogenesis, mature earlier, produce eggs of the next generation of transgenic lines quicker, and quail embryo can withstand the introduction of a transgene into blastoderm due to its resilient nature (Huss et al., 2008). For example, a fluorescent-expressing transgenic line of Japanese quail was created

to study tissue-specific embryogenesis, which permits the understanding of myogenesis, skeletogenesis, angiogenesis, vasculogenesis, teratology, et cetera (Baer et al., 2015).

In addition, Japanese quail is a model for studying human diseases such as congenital disabilities, hereditary diseases, immune and endocrine diseases, et cetera (Baer et al., 2015). Several closed lines were developed to mimic human genetic disorders, as summarised in **table 2**.

Table 2: Japanese quail lines used to study human hereditary diseases.

	Quail strain	Gene	Feature
1	Silver (S) strain	Autosomal incomplete dominant gene	Homozygous quail show white plumage and retinal defect. There is hypopigmentation in the posterior retina with thinning of the subjacent sclera leading to scleral ectasia. Homozygous quail also shows impaired osteoclastic bone resorption. The heterozygous quail show greyish plumage.
2	Dark feather nervous disorder (DNFD) strain	Autosomal recessive gene	Mutants show dark brown plumage. It is characterised by cerebellar functional disorders involving fine tremors in the extremities and trunk, gait disturbance and tumbling when excited or active.
3	RWN strain	Autosomal recessive gene	The mutants have glycogen storage disease type II (acid maltase deficiency, AMD). The AMD quail have wing dysfunction.
4	LWC strain	Autosomal dominant gene	The mutants show generalised myotonia, muscle stiffness and muscle weakness. Affected birds cannot lift the wing vertically or right when placed on their dorsum.
5	Quv strain	Autosomal recessive gene	The mutants show head and body quivering because of neurofilament deficiency in some nerves.
6	ET strain	Autosomal recessive gene	The mutants show ear tufts and irregularly shaped ear openings. The

			mutant ear opening is oval with a fissure on its ventral margin.
7	TT strain	Autosomal recessive gene	The mutant shows a throat tuft consisting of an epidermal peduncle and covering feathers. Sometimes it shows abnormal ear openings showing a wide cleft at the ventral margin and an extra bony projection on the peduncle.
8	Hereditary multiple malformations (HMM) strain	Autosomal recessive gene	The mutant shows several malformations, including delayed ossification and protruding viscera through the umbilicus. This strain is especially useful in morphogenesis research.
9	Short lower beak (SLB) strain	Autosomal recessive gene	The mutant shows shortened lower beak, opened mouth, and small body size. Skeletal abnormalities are also seen in the hyoid apparatus, cervical vertebrae, ribs, and appendicular skeleton. This strain is important in the morphogenesis research of mandible and appendicular skeletons.
10	ZN strain	Autosomal dominant gene and sex-linked recessive gene.	The mutant shows crossed legs, opened eyes, and an abnormal beak. All ZN strains die in the late embryonic stages. The strain might be helpful in the study of developmental leg skeletons.

Reference: (Mizutani, 2003)

Furthermore, Japanese quail is a perfect model for studying old age diseases owing to its predictable ageing and maturity process comparable to mammal physiology (Huss et al., 2008; Baer et al., 2015). First, the significance of using quail in senescence study is that the hypothalamic system of Japanese quail exhibits neuroplasticity (Baer et al., 2015). This allows for behaviour modification, for example, in male quails, testosterone replacement therapy can reverse entire sexual behaviour after it is lost (Huss et al., 2008; Baer et al., 2015). Secondly, bone physiology — especially in female quail — offers an opportunity to study hormonal effect on bone fragility and role of vitamin D (Baer et al., 2015). This is because, in female Japanese quail, the hollow bone serves as a reservoir for minerals, especially calcium — used in egg production — thus predisposing ageing female quail to decline bone in density and osteoporosis (Baer et al., 2015).

Regarding pathogens research, Japanese quail is quite susceptible to most poultry infections and serve in the study of avian infections (Cheng et al., 2010). For example, Japanese quail was experimentally used as a model for the study of *Chlamydia psittaci*, which affects several organ systems of birds, including liver and lungs (Takashima et al., 1996). Avian chlamydiosis is a latent zoonotic infection which can be a source of human infection (Takashima et al., 1996). In birds, immunosuppression can lead to a lethal form of chlamydiosis infection with a significant mortality rate (Takashima et al., 1996). As will be discussed later, heat stress immune-modulation suppresses immune system and expose the birds to infections (Mehaisen et al., 2019), which means heat stress could activate a latent chlamydia infection in Japanese quail, which can be transmitted to humans.

Similarly, Japanese quail was modelled for the study of vibronic hepatitis induced by *Campylobacter jejuni* (Misawa et al., 1996). Hepatic lesions similar to those reported in chicken liver were observed, despite the absence of clinical signs (Misawa et al., 1996). In addition, to develop an efficient diagnostic test for *Mycobacterium avium*, Japanese quail was experimentally inoculated to induce mycobacteriosis. This helps the research team to develop a protocol for studying avian mycobacteriosis (Tell et al., 2003). Granulomas with necrotic centres were seen in liver, intestine, kidney and testis (Tell et al., 2003). Furthermore, Tell et al. (2010) tested the efficacy of antifungal drug voriconazole in Japanese quail by inducing aspergillosis, a common fungal infection caused by *Aspergillus fumigatus*, which naturally infects birds. Recently, during the COVID-19 pandemic, the pathogenic effects of *Angiostrongylus cantonensis* were published by Fecková et al. (2021). Angiostrongyliasis is a parasitic infection which mainly affects the nervous system and is found in several hosts, including humans and dogs (Fecková et al., 2021). However, Japanese quail model showed mild symptoms with no gross or histopathological lesions suggesting low susceptibility to angiostrongyliasis and can serve as pest control for the disease (Fecková et al., 2021). In addition, some avian species were diagnosed with fatal cases of angiostrongyliasis (Reece et al., 2013). Thus, Japanese quail was used as a model to understand the disease's pathogenicity among domesticated birds (Fecková et al., 2021). This study was followed by another critical analysis of *Eimeria tenella*, a parasite which causes coccidiosis among avian species (Khan et al., 2022). Japanese quail were used as a model in this study to identify a dietary alternative in treating avian coccidiosis (Khan et al., 2022). These studies are examples of experiments that employed Japanese quail as a model for studying poultry diseases. It is noteworthy that

Newcastle disease, avian influenza (Adebiyi et al., 2022) and some *Eimeria* spp. (Berto et al., 2013) were recovered from naturally infected Japanese quail.

1.4 Economic importance of Japanese quail

Japanese quail meat and eggs are generally accepted as an alternative to chicken meat owing to perceived healthier and therapeutic value (Olorunfemi et al., 2016). This perception and shorter breeding period make it a favourite and lucrative poultry business worldwide. The domesticated Japanese quail was first introduced into Europe, and USA around 1930-1950 for meat production (Ichilcik and Austin, 1978; Mnisi et al., 2021), with about 100 million reared quail in Europe (Spain, France, Italy and Portugal) and about 19 million in USA as estimated in the 1990s (Lukanov, 2019).

In South Africa, a publication by Ichilcik and Austin (1978) reported that a colony of Japanese quail was established at University of the Witwatersrand for toxicological screening studies. This colony was sourced from a local farm of exotic animals (although the time of establishment is unknown) with an undefined chain of sources (Ichilcik and Austin, 1978). Now, six large-scale and over a hundred small-scale Japanese quail farms are registered in South Africa, and several more have been identified but not registered (Mnisi et al., 2021). Some of the best-performing quail farms in South Africa produce an average of 16,000 birds per production cycle and about 435 kilograms of eggs per year (Mnisi et al., 2021).

Furthermore, local farmers introduced Japanese quail into Zimbabwe in 2011 as a game bird for meat and egg, which was marketed as therapeutic (Jeke et al., 2018). However, Zimbabwean government banned the sale of Japanese quail products in 2016 due to a lack of

scientific evidence to back the marketed therapeutic values of Japanese quail meat and eggs (Jeke et al., 2018), which led to a significant decline in availability of Japanese quail products in Zimbabwe.

In west African Nigeria, Japanese quail was brought by National Veterinary Research Institute (NVRI) in 1992 and was promoted by both National Television Authority and print media (Adeoti and Baruwa, 2019). Since then, several quail farms have been established in Nigeria, yet no official figures can be found stating the estimated number of quail farms to date. However, Oladimeji et al. (2019) collected data from 193 quail farmers to evaluate quail production's technical and scale efficiencies in two states of Nigeria. In addition, the volume of quail products in Nigeria could not be ascertained, but a high-profit margin was reported by a few researchers that conducted economic and profit analysis of some quail farms in Nigeria (Muhammad-Lawal et al., 2017; Sekumade and Owoeye, 2017; Adeoti and Baruwa, 2019). These publications indicated that there are significant quail farming activities in Nigeria.

Japanese quail farming was started in Bangladesh around the same time as Nigeria in 1992, but it remained static for more than a decade and then experienced a boom in 2009 (Redoy et al., 2017). Since then, quail farming has declined in Bangladesh due to bird sensitivity and cannibalism among the birds (Redoy et al., 2017). In a survey by Nasar et al. (2016), only 52 quail farmers were interviewed from 14 districts of Bangladesh, and majority of the farmers had less than 5000 birds on their farms (Nasar et al., 2016; Redoy et al., 2017).

In India, quail was imported from Davis, California, in 1974 by Central Avian Research Institute for diversification (Prabakaran et al., 2020). Now, quail is third largest poultry in India (Prabakaran et al., 2020), with a high-profit margin (Kandangal and Bashir, 2019). In a survey of 120 quail farmers from three districts in northern Kerala, India, there are eight large-scale quail farms with up to 30,000 birds per farm and 112 small to medium-scale farms, all reporting high profits (Kandangal and Bashir, 2019).

Having mentioned the status of quail farming in some countries, it is important to briefly discuss the nutritional value of quail meat and egg from mysteries and facts that made Japanese quail nutritious and lucrative. It is noteworthy that, in Nigeria, Bangladesh, India, and other developing countries, quail farming is vital to bridging protein supply gap among increasing rural populace (Mohammed and Ejiofor, 2015).

The Japanese quail eggs and meat are rich sources of high-quality protein and essential fats necessary to prevent undernutrition and malnourishment (Jeke et al., 2018; Mnisi et al., 2021). Furthermore, quail eggs and meat claimed therapeutic purposes in improving diseases such as hypertension, vision problems and stomach ulcer (Olorunfemi et al., 2016; Mnisi et al., 2021). Japanese quail eggs are used in Chinese culture to treat asthma, tuberculosis, and diabetes (Bayomy et al., 2017). Japanese quail eggs also help improve immune status, memory, fertility, liver disease and gallbladder stone (Olorunfemi et al., 2016; Bayomy et al., 2017).

The reported benefits of Japanese quail eggs and meat resulted from its nutritional composition, which contains low cholesterol, saturated fatty acids, and low calories

(Olorunfemi et al., 2016; Bayomy et al., 2017). A comparison of quail and chicken egg nutritional values is presented in **tables 3-7**.

Table 3: Comparison of the chemical composition of Japanese quail and chicken eggs.

Chemical composition (%)	Quail eggs	Chicken eggs
Moisture	72.9±1.26	74.86±2.33
Protein	12.99±0.91	11.91±1.18
Fats	11.4±1.02	10.63±2.19
Ash	1.07±0.17	0.93±0.15
Carbohydrates	1.64±2.16	1.67±1.27
Energy (Kcal/100g)	161.12±5.41	149.99±6.06
Cholesterol (mg/g)	12.26±0.78	12.48±0.42
Reference: (Ali and Abd El-Aziz, 2019)		

Table 4: Comparison of Japanese quail and chicken eggs mineral content.

Mineral content (mg/100g)	Quail eggs	Chicken eggs
Calcium	31.45±1.51	35.46±1.19
Iron	3.01±2.17	2.85±2.04
Copper	2.28±2.08	1.95±1.86
Zinc	3.16±2.56	3.54±2.50
Magnesium	19.8±0.53	17.32±0.48
Sodium	25.7±1.36	31.83±2.44
Potassium	20.86±1.46	23.81±1.63
Phosphorus	302.5±4.61	236.3±2.68
Reference: (Ali and Abd El-Aziz, 2019)		

Table 5: Comparison of vitamin content of Japanese quail and chicken eggs.

Vitamin content (mg/100g)	Quail eggs	Chicken eggs
Vitamin A	0.36±1.63	0.40±1.86
Vitamin B1	0.12±1.27	0.15±1.19
Vitamin B2	0.47±1.18	0.30±0.78
Vitamin B3	0.09±0.17	0.2±0.32
Vitamin B6	0.65±1.51	0.60±1.62
Vitamin D	0.45±0.64	0.45±0.53
Vitamin E	5.9±1.26	5.3±2.17
Reference: (Ali and Abd El-Aziz, 2019)		

Table 6: Comparison of Japanese quail and chicken eggs' fatty acids.

Fatty acids content (%)	Quail eggs	Chicken eggs
Σ SFA	34.07	36.97
Σ MUFA	47.47	44.9
Σ PUFA	18.46	18.13
Reference: (Ali and Abd El-Aziz, 2019)		

Table 7: Comparison of Japanese quail and chicken eggs' amino acids content.

Amino acids content (g/100g)	Quail eggs	Chicken egg
Σ EAA	39.22	39.04
Σ NEAA	52.24	51.83
Reference: (Ali and Abd El-Aziz, 2019)		

1.5 Negative impact and mitigation of heat stress on Japanese quail

High temperatures are considered the most significant bioclimatic factor affecting poultry in developing countries, especially in Africa and Asia (Nyoni et al., 2019). Although all birds react similarly to heat stress, research shows that little individual response exists between bird species (Nyoni et al., 2019). For example, chicken broilers have lower thermal tolerance than Japanese quail (Aengwanich and Simaraks, 2004). The Japanese quail core body temperature range between 38.9 – 41.4°C (Prinzinger et al., 1991), which might fluctuate higher during exposure to heat.

Heat-stressed Japanese quail develop mechanisms to maintain normal body temperature during extreme temperature exposures. They reduce feed intake to minimise heat produced during metabolism of macromolecules to generate cellular energy (Batool et al., 2021) and increase respiratory rate and gular flutter (Weathers and Schoenbaechler, 1976) to help in evaporative cooling. Furthermore, cutaneous water evaporation exists in Japanese quail despite the absence of sweat and sebaceous glands (Marder and Ben-Asher, 1983). Although not as efficient as columbids (for example, pigeons), the cutaneous water loss helps Japanese quail cope with high environmental temperatures (Marder and Ben-Asher, 1983). In addition, quail will also increase water intake (Batool et al., 2021) to help lower body temperature. However, increased respiration will lead to respiratory alkalosis (Olgun et al., 2021).

The mechanism for respiratory alkalosis is that there is increased removal of Carbon-dioxide (CO_2) from gas exchange surface, leading to a decrease in CO_2 and bicarbonate (HCO_3) in circulation (Teeter et al., 1985). This led to a change in blood pH and electrolyte imbalance due to loss of calcium carbonate (Olgun et al., 2021), ultimately affecting bone growth and egg quality. In addition, increased cellular energy demand is increased in heat-stressed birds despite reduced dietary intake resulting in generation of excessive reactive oxygen species (Akbarian et al., 2016).

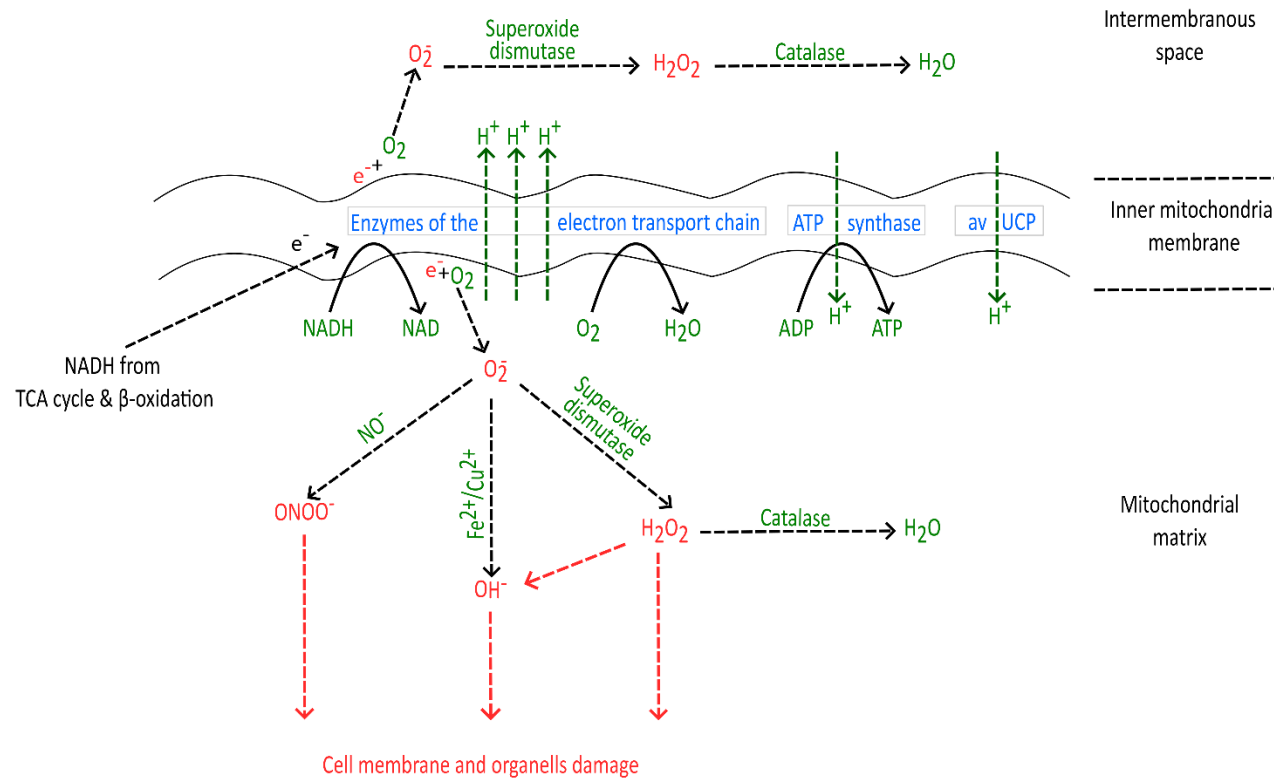
The mechanism of reactive oxygen species production (**figure 2**) begins with glucose and free fatty acids metabolism, leading to production of reduced nicotinamide adenine dinucleotide (NADH) through acetyl CoA metabolism (Mayes and Botham, 2003). The NADH molecules donate their electron to respiratory complexes of oxidative phosphorylation pathway — also called electron transport chain — situated within the inner mitochondrial membrane, in

which oxygen is converted to water (Mayes and Botham, 2003). During the process of oxidative phosphorylation, hydrogen ions (H^+) are transported from the matrix of mitochondria to intermembranous space, which increases membrane potential (Mayes and Botham, 2003; Akbarian et al., 2016). The enzyme ATP synthase uses the H^+ ion molecules within the intermembranous space to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP), thereby transporting H^+ back to mitochondrial matrix and reducing membrane potential (Mayes and Botham, 2003; Akbarian et al., 2016).

However, during the transfer of electrons in electron transport chain, there is leakage of electrons causing production of reactive oxygen species via superoxide (O_2^-) production, because not all oxygen molecules get converted completely to water (Akbarian et al., 2016). The antioxidant enzyme superoxide dismutase (SOD) can convert O_2^- to H_2O_2 and H_2O thereby reducing the potentially damaging effects of oxygen radicals and maintaining homeostasis (Akbarian et al., 2016). Subsequently, catalase (CAT) converts H_2O_2 and H_2O , because H_2O_2 is still potentially dangerous and can traverse cell membrane and acts as a secondary messenger (Akbarian et al., 2016). In addition, H_2O_2 can be further reduced to hydroxyl radical ($HO\bullet$), which is a highly dangerous molecule in causing cell damage (Akbarian et al., 2016).

The cells have a control mechanism using uncoupling protein to transport H^+ back from intermembranous space into mitochondrial matrix without the involvement of ATP synthase (Mayes and Botham, 2003). This uncoupling protein is identified in birds as avian uncoupling protein (avUCP) (Akbarian et al., 2016). The avUCP helps to decrease membrane potential and improve efficiency of electron transport chain so that more energy can be provided and reduce the production of superoxide (O_2^-) (Akbarian et al., 2016).

Therefore, due to increased energy demand by cells in heat stress, there is an overwhelming increase in membrane potential (Akbarian et al., 2016) and downregulation of avUCP (Del Vesco and Gasparino, 2013) activity decreasing the efficiency of electron transport chain and increased electron leakage (Akbarian et al., 2016). Consequently, antioxidant capacity is overwhelmed by excessive reactive oxygen species generation leading to oxidative cell damage. This is because reactive oxygen species can interact freely with cellular proteins, lipids and DNA causing catastrophic changes that can lead to apoptosis and necrosis (Akbarian et al., 2016). Thus, for example, interaction of oxygen radicals with lipid bilayer membrane causes lipid peroxidation and increased permeability of the cell membrane, leading to influx of calcium, sodium and water leading to cell swelling (Swanson et al., 2010). While interaction with nuclear proteins and DNA will cause transcription and cell transportation systems irregularities and can lead to activation of cell death pathways (Swanson et al., 2010).



Key: e^- = electron, OH^- = hydroxyl ion, $ONOO^-$ = peroxynitrite, $O_2^{\cdot -}$ = superoxide, H_2O_2 = hydrogen peroxide, H_2O = water, H^+ = hydrogen ion, O_2 = oxygen, NO^- = nitric oxide
 NADH = reduced nicotinamide adenine dinucleotide, NAD = nicotinamide adenine dinucleotide, ADP = adenine diphosphate, ATP = adenine triphosphate,
 avUCP = avian uncoupling protein, TCA = tricarboxylic acid

Figure 2: A diagram of the cellular mechanism of ROS processes.

The level of lipid peroxidation is assessed by measuring the amount of malondialdehyde (MDA) in serum and tissues (Erişir et al., 2018). Elevated MDA in the serum, liver, heart, kidney (Sahin et al., 2003a), ovary (Sahin et al., 2017), testis (Turk et al., 2015), muscle (Halici et al., 2012), and egg yolk (Akdemir et al., 2015) were observed in heat-stressed Japanese quail suggesting oxidative stress and cell damage. As mentioned earlier, the antioxidant defence enzymes synthesized by the body will suffice to handle generated oxidants under normal homeostasis. However, excessive production during stress will overwhelm the body's antioxidant capacity leading to oxidative stress. Research showed decreased activity of SOD, CAT, and glutathione peroxidase (GSH-Px) in serum and tissues of Japanese quail exposed to acute or chronic heat stress (Halici et al., 2012; Sahin et al., 2013; Sahin et al., 2017). In addition, most antioxidant vitamins such as vitamins A, E, C, B12 and folic acid (Sahin et al., 2003a; Sahin et al., 2008; Tuzcu et al., 2008), including certain minerals such as zinc, chromium, iron and phosphorus (Sahin et al., 2003a) were observed to be depleted in heat-stressed Japanese quail. Sahin and co-workers reported that increased excretion of these minerals is one of the reasons for depletion, although the mechanism for the phenomenon is yet to be elucidated (Sahin et al., 2003a; Sahin et al., 2004; Sahin et al., 2005).

Following the production of reactive oxygen species in mitochondria, some of the oxygen radicals escape into cytosol, especially H_2O_2 , which activate the release of nuclear factor erythroid 2-related factor 2 (Nrf2) from Kelch-like ECH (erythroid CoA hydratase)-associated protein 1 (KEAP1) (Sahin et al., 2017). Note that KEAP1 binds Nrf2 in cytosol, acting as regulator of Nrf2 in normal physiological conditions and releases it upon stress signal (Sahin et al., 2017). Hence, upon release, Nrf2 is translocated into nucleus where it binds to

antioxidant response element (ARE) and stimulates transcription of more antioxidants (Akbarian et al., 2016; Sahin et al., 2017) this serves as a control mechanism to increase antioxidant capacity. In addition, pro-inflammatory factors such as the tumour necrosis factor α (TNF- α) are activated by stress (Mehaisen et al., 2019). Tumour necrosis factor- α is synthesized de-novo following stress signal as the body does not create reserve (Zelova and Hosek, 2013). Tumour necrosis factor- α in turn stimulates nuclear factor kappa B (NF- κ B), which is otherwise inactive within cytosol (Albensi, 2019). Also, NF- κ B can be activated by cell stressors directly instead of TNF- α , from where it translocate into nucleus for protein transcription related to the stress response (Albensi, 2019). In addition, NF- κ B regulates mitochondrial gene expression, respiratory complex and even metabolism (Albensi, 2019). In heat-stressed Japanese quail, elevated serum levels of TNF- α (Mehaisen et al., 2019) and NF- κ B and a decreased level of Nrf2 (Orhan et al., 2012) were observed. The decrease in the antioxidant enzymes, vitamins and minerals coupled with decreased antioxidant enzymes and transcription factor (Nrf2) indicates that heat stress depletes antioxidant capacity and promotes inflammation in most tissues. Heat shock proteins (HSPs) were observed to be significantly elevated in Japanese quail during heat stress (Orhan et al., 2013; Akdemir et al., 2015). Heat shock proteins are a complex system of protein families responsible for protein's metabolic activities, transport, folding, assembly, and degradation (Brancolini and Iuliano, 2020). Heat shock proteins attempts to repair damaged protein during stress-induced proteotoxicity to maintain proteostasis (Brancolini and Iuliano, 2020; Lang et al., 2021). Therefore, serum HSPs elevation is a sign of stress-induced protein damage not unique to heat stress.

Furthermore, quail increases blood glucose through gluconeogenesis, in response to increased serum level of corticosterone (Cockrem, 2007) due to reduced feed intake. Corticosterone is known to increase blood sugar level in addition to its function in behavioural changes to help bird cope with heat stress (Cockrem, 2007). Furthermore, thyroid hormone triiodothyronine (T3) was observed to be decreased in heat-stressed Japanese quail (Mehaisen et al., 2019), to reduce metabolic heat production. The reduced T3 results from decreased levels of reduced glutathione, which is a cofactor required to produce T3 (Mancini et al., 2016). The thyroid hormone regulates thermogenesis and has a significant influence on metabolism of glucose, fatty acids and cholesterol (Mancini et al., 2016). Hence, inappropriate levels of T3 will negatively affect the metabolism of these macromolecules (Mancini et al., 2016). For example, reduced serum T3 levels will lead to reduced cellular uptake and consequently elevated serum levels of cholesterol and triglycerides (Sinha et al., 2014). Thus, heat stress elevates cholesterol levels in serum (Sahin et al., 2004) and egg (Akdemir et al., 2015) of Japanese quail.

Similarly, immune response and bone density of Japanese quail were reported to decline upon heat stress exposure. Low white cells count, and decreased stimulation index of T-cells were observed in quails exposed to extreme heat (Mehaisen et al., 2017; Mehaisen et al., 2019). In addition, bone mineral density, osteocalcin, and alkaline phosphatase measured by Sahin et al. (2007) were reduced by heat stress. The indices of egg quality such as egg weight, shell thickness, egg yolk index and haugh units were observed to decline in heat-stressed Japanese quail (Akdemir et al., 2019). The decline in immune response and egg quality could be a manifestation of biochemical derangements and cellular damage.

However, as mentioned earlier oxidative damage will cause cell injury that could lead to cell repair or cell death. Cell repair can lead to increase deposition of collagen fibres, which if prolonged can lead to fibrosis and contraction of affected organ (Wynn, 2008). But in the event of cell death, necrosis was reported (Turk et al., 2015), which might be promoted by pro-inflammatory factors such as NF- κ B during heat stress (D'Arcy, 2019). The resultant effect of necrosis is spillage of cell content affecting neighbouring cells (D'Arcy, 2019). Consequently, widespread uncontrolled cellular death follows a cascade of inflammatory processes and could manifest negative outcomes on organ weight (Turk et al., 2015) and body weight (Mehaisen et al., 2019). The mechanism of this could be related to reduced cell number as a result of removed dead cells or tissue shrinkage from increased collagen deposition and contraction.

However, it is not only the problems of heat stress that were studied, but researchers also investigated ways of mitigating the problem. For example, *Alchemilla vulgaris* was found to improve egg production in Japanese quails exposed to heat stress of 34°C, eight-hour cycle for 75 days experiment (Akdemir et al., 2019). *Alchemilla vulgaris* is reported to have high flavonoid content which can activate antioxidant enzymes such as superoxide dismutase (Akdemir et al., 2019), hence, it can contribute to free radical scavenging. A synopsis of some studies conducted on different dietary supplements to mitigate heat stress in Japanese quail is presented in **table 8**. The mechanisms of dietary strategies could be protecting lipid membrane from peroxidation or quenching of reactive oxygen species directly or by stimulating antioxidant enzymes (Abdel-Moneim et al., 2021). Other mechanisms include cell signalling for heat shock protein to protect cells and in some cases improve the efficiency of

electron transport chain to reduce proton leakage (Abdel-Moneim et al., 2021). Normally, vitamins are known to be lipid bilayer protectors while minerals will activate and increase efficiency of antioxidant enzymes (Abdel-Moneim et al., 2021). The phytochemicals and probiotics activity depends on their vitamins and antioxidant contents, for example, lycopene in tomato (Sahin et al., 2008) and flavonoids in moringa (Abou-Elkhair et al., 2020). However, few have direct reactive species scavenging ability, for example, *Berberis vulgaris*.

Table 8: Some of the selected studies on the measures of mitigating the effect of heat stress in the Japanese quail.

S/no	Reference(s)	Temperature (°C)/Duration	Mitigation(s)	Mechanism	Reported benefits
1	(Akdemir et al., 2019)	34, 8-hour cycle	<i>Alchemilla vulgaris</i>	High flavonoid content – activates antioxidant enzymes.	<i>Alchemilla vulgaris</i> improved egg production, but not eggshell quality.
2	(Hajati et al., 2020)	34, 8-hour cycle	<i>Arthrospira platensis</i> (microalgae)	Vitamins, Gamma-linoleic acid, phycocyanins, phenolic acids, β -carotene and chlorophyll content.	The microalgae decreased serum MDA and H/L ratio.
3	(Sahin et al., 2004)	34, 8-hour cycle	Ascorbic acid and melatonin	Antioxidant – Melatonin stimulates growth hormone.	Combining ascorbic acid and melatonin helps reduced serum and liver MDA. They also improve weight gain.

4	(Sahin et al., 2013)	34, 8-hour cycle	<i>Berberis vulgaris</i>	Berberine is one of the isoquinoline alkaloids content. Antioxidant, anti-inflammatory.	<i>Berberis vulgaris</i> reduced hepatic MDA and increased antioxidant enzymes. It also improves NF-kB, HSP70, Nrf2 and HO-1.
5	(Sahin et al., 2017)	34, 8-hour cycle	Capsaicinoids	Capsaicin is an alkaloid that Inhibits lipid peroxidation and ROS generation.	Capsaicinoids decreased serum and ovarian MDA and improve antioxidant enzymes. It also improves NF-kB, AKT (protein kinase), Nrf2 and HO-1.
6	(Akdemir et al., 2015)	34, 8-hour cycle	Chromium-histidinate	Chromium (Cr) is involved in carbohydrate, lipid and protein metabolism through activating certain enzymes in poultry and being an active component of a glucose tolerance factor that enhances insulin actions at the receptor level.	Chromium reduces serum cholesterol but not egg cholesterol. It also decreases serum and egg MDA, NF-kB and HSPs 60, 70,90.
7	(Orhan et al., 2012)	34, 8-hour cycle	Chromium histidinate	Same as no.6.	Chromium improves feed intake, weight gain and final body weight. It also inhibited hepatic NFkB

					expression and enhance IkB α .
8	(Sahin et al., 2005)	34, 8-hour cycle	Chromium picolinate and Biotin	Chromium same as no.6. Biotin acts as a cofactor for various carboxylation enzymes including pyruvate carboxylase (gluconeogenesis) and acetyl coenzyme A carboxylase (lipogenesis).	Chromium improves final body weight, weight gain and feed intake. It decreases hepatic and serum MDA, glucose and cholesterol levels. It also enhances serum vitamin C and E levels.
9	(Sahin et al., 2002b)	32.5, continuous	Chromium picolinate	Same as no.6.	Chromium increases body weight, feed intake, egg production and eggshell thickness. It also increases serum insulin and decreases corticosterone and glucose levels.
10	(Gitoe et al., 2018)	35, 8-hour cycle	Organic (Cr-L- Met) and inorganic (CrCl ₃) chromium	Chromium (Cr) is a cofactor of insulin, decreases lipid peroxidation, and decreases corticosterone production, and therefore could be considered a good antioxidant.	Chromium improves egg production, humoral (SRBC, Newcastle) and cellular (PHA) immune response.

11	(Turk et al., 2016)	34, 8-hour cycle	Rosemary oil	Antioxidant and free radical scavenging activities.	Rosemary oil improves the testicular weight, spermatogonium count, spermatocyte count, spermatid count, sperm count and gonadal-somatic index. It also decreases testicular MDA and increases rGSH, GSH-Px and CAT. In addition, it also improved the diameter of seminiferous tubules and the germinal cell layer thickness. It also improves androgen receptors, the proapoptotic (Bax) and antiapoptotic (Bcl-2) markers in the testis.
12	(Sahin et al., 2003a)	34, 8-hour cycle	Vitamin C and Folic acid	Antioxidant - Folic acid is required in the methylation of homocysteine to form methionine and in the biosynthesis of amino acids and deoxynucleotides needed for DNA replication and repair. Vitamin C has a role in the	Combined vitamin C and folic acid improved live weight and feed intake. They decrease hepatic, heart, kidney and serum MDA. They also decrease serum homocysteine and ACTH concentrations. However, they improve serum vitamins C, E, A, B12 and folic acid. They also improve the retention of nitrogen, ash,

				utilization and perhaps absorption of folic acid.	calcium, phosphorus, zinc, copper, iron, and chromium.
13	(Abou-Elkhair et al., 2020)	35, continuous	<i>Moringa oleifera</i> seed powder 0.3%	<i>Moringa oleifera</i> comprises significant concentrations of vitamins A, B, and C, proteins, phosphorus, and calcium. It is rich in flavonoids, carotenoids, tocopherols, selenium, and vitamin E and has potent antioxidant activity.	Moringa improves feed intake, egg production and egg weight. It also improved the expression of oestrogen receptor 2, follicle-stimulating hormone receptor, prolactin receptor, and steroidogenic acute regulatory protein.
14	(Kaplan and Koksai, 2021)	35, continuous	Digestarom® Poultry - a blend of 8% peppermint, 2% eugenol or clove, 3.4% anethole or anise and thyme and sodium chloride	Not specified	Digestarom® improved weight gain and feed intake. It also decreases the H/L ratio and serum MDA. In addition, also improved the cooking loss and water-holding capacity of the meat. However, it did not affect meat colour.
15	(Zeweil et al., 2016)	25-30 (environmental)	<i>Aspergillus awamori</i> (probiotic)	Antioxidant activity	The probiotic improved egg production and weight gain. It decreases

					egg yolk lipids and cholesterol. It also increases serum lipids, cholesterol and total proteins. It improves antioxidant capacity and reduces MDA levels.
16	(Ahmed et al., 2014)	27-37 (environmental)	<i>Withania somnifera</i> roots ethanolic extract	Antioxidant activity	<i>Withania somnifera</i> roots extract improved haemoglobin level and PCV at 2g/kg diet. It also improved TSH levels but not T3 and T4 levels.
17	(Sahin et al., 2009)	34, 8-hour cycle	25-hydroxycholecalciferol (25-OH-D3) and soy isoflavones	Isoflavones act as immune enhancers and lower plasma cholesterol.	The combination improved bone mineral density, tibia ash, calcium, and phosphorus. It also increases serum vitamin D and alkaline phosphatase activity.
18	(Erişir et al., 2018)	34, 16-hour cycle	Grape seed contains high tocopherols antioxidant	Lowers plasma cholesterol, and antioxidant activity.	Grape seed decreased plasma total cholesterol, glucose, triglyceride, AST, and ALT levels. It also decreases hepatic and kidney MDA levels.
19	(Del Vesco et al., 2014a)	38, 24-hour only	Methionine	Antioxidant activity – production of glutathione.	Methionine reduced TBARS levels and increase the activity of GSH, GPx, and uric acid concentration.

20	(Del Vesco et al., 2017)	38, 24-hour only	Selenium	Antioxidant activity – production of glutathione family.	Selenium increases the expression of UCP and the glutathione enzymes family.
21	(Sahin et al., 2002a)	34, continuous	Vitamins C and E	Antioxidant activity	The combination of vitamin C and E increases serum T4 and TSH. However, they decreased serum glucose, urea, triglycerides and cholesterol. They also reduced serum and hepatic concentrations of MDA.
22	(Sahin and Kucuk, 2001)	34, continuous	Vitamin E and selenium	Antioxidant activity	The combination improved feed intake, final body weight and digestibility of nutrients.
23	(Halici et al., 2012)	34, 8-hour cycle	α -lipoic acid, vitamins E and C.	α -lipoic acid is involved in the cycling of antioxidants including vitamins C and E and GSH.	The combination decreased MDA level and increase GSH, CAT and SOD activity.
24	(Ahmed and Saleh, 2017)	40-45, (environmental)	Dates by-product (HAFSH) and yeast.	Dates by-products as energy and protein sources. Yeast (<i>Saccharomyces cerevisiae</i>) as probiotic.	The dates by-product increases egg production by 10%. It also improves proteins level, HDL and T3 and decreased cholesterol and glucose levels.

25	(Tuzcu et al., 2008; Orhan et al., 2013)	34, 8-hour cycle	<i>Epigallocatechin gallate</i> (EGCG) in green tea.	Antioxidant activity – free radicals scavenger.	EGCG improved feed intake and weight gain. It also decreased hepatic and serum MDA, serum cholesterol, triglycerides and glucose. In addition, EGCG also reduced hepatic expressions of COX-2, c-Jun, c-Fos, HSP60, HSP70 and HSP90.
27	(Sahin et al., 2003b)	34, 8-hour cycle	Melatonin	Stimulate growth hormone.	Melatonin did not affect feed intake but improved live weight gain. It also decreased the excretion of nitrogen, ash, calcium, phosphorus, zinc, iron, and chromium.
28	(Mehaisen et al., 2017; Mehaisen et al., 2019)	35, continuous	Propolis – produced by worker honeybees	Not specified	Propolis improved feed intake and live weight gain. It also alleviates intestinal desquamation and increases crypt depths. In addition, it decreases serum corticosterone, MDA and TNF- α but increases T3.
29	(Youssef et al., 2021)	34-36 (environmental)	Fresh lemon	Antioxidant vitamins content	Fresh lemon improved weight gain and total antioxidant capacity.
31	(Onderci et al., 2004)	34, 8-hour cycle	Genistein	Antioxidant activity – free radical scavenging.	Genistein increased feed intake and live weight gain. It also decreased

					serum and hepatic levels of homocysteine and MDA.
32	(Sahin et al., 2008)	34, 8-hour cycle	Tomato powder	Contain the antioxidant lycopene and vitamins A and C.	Tomato powder increases feed intake and live weight gain and serum vitamin C, E and A and lycopene. It reduces hepatic, serum and muscle MDA.
33	(Dosoky et al., 2021)	32-34 (environmental)	Licorice	Licorice is a phytobiotic that stimulates GIT and has antibiotic activity.	Licorice improved egg laying rate and feed consumption, serum total protein and reduce MDA levels.
34	(Nazar et al., 2019)	34, 14-hour cycle	Thymol	Antioxidant activity	Thymol supplementation increased albumin concentrations and inflammatory responses and decreased antibody titers. Improved H/L ratio.

The results reported in table 8 are only studies conducted on Japanese quails, which is the interest of this project, however, comprehensive reviews of nutritional strategies used in general poultry were published by Abdel-Moneim et al. (2021) and Olgun et al. (2021).

1.6 Anatomy of the avian respiratory system

The avian respiratory system is built on efficiency to provide for high oxygen extraction capacity (Maina, 2008). The avian respiratory system comprises nasal cavity, trachea, syrinx, primary and secondary bronchi, tertiary bronchi or the parabronchi and air sacs (Dogan and Takici, 2018). The functional respiratory components are gas exchanger i.e. lungs and the mechanical ventilator i.e. air sacs (**figure 3**). The lungs are situated between the ribs on dorsolateral surface and a membrane on ventral surface, hence it is allowed only minimal movement during respiratory cycles (Maina, 2008). The air sacs — although avascular and not involved in gas exchange — continuously ventilate the lungs in bellow-like movements (Maina, 2008).

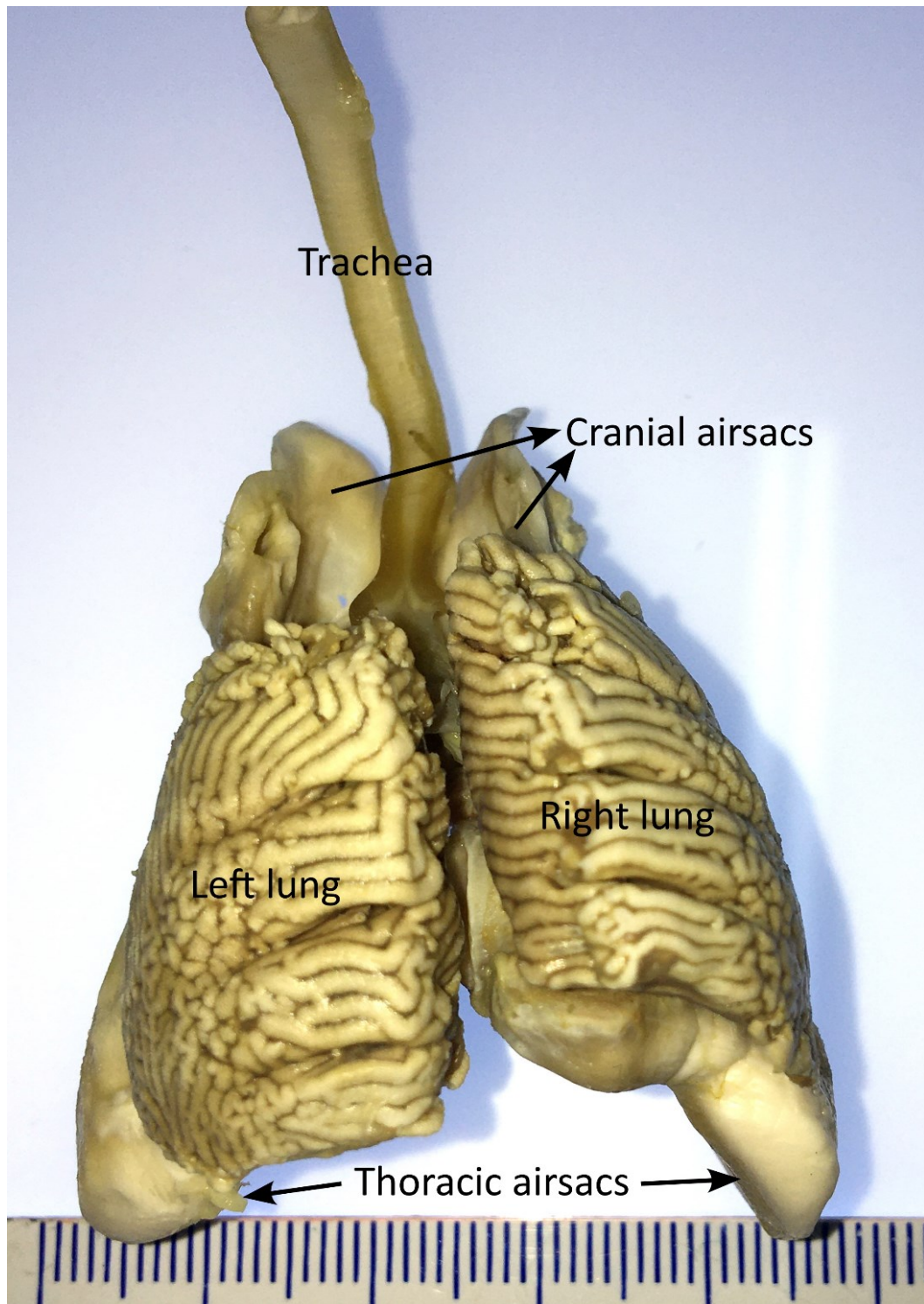


Figure 3: A latex cast of the Japanese quail lung showing the trachea, lungs, and some of the air sacs.

The evolution of avian respiratory system ensures that size and position of the different components conform to the size of the bird, therefore, differ between bird species (Maina, 2008). In Japanese quail, trachea continued from larynx and extends about 83-91 rings which are complete rings except for the first three rings (Cevik-Demirkan et al., 2007), and unlike the C-shaped mammalian trachea. In addition, upper two-thirds of trachea is oval while lower one-third is circular (Cevik-Demirkan et al., 2007).

The syrinx is a modification of last two or three cartilages of trachea and first two or three cartilages of bronchi (Cevik-Demirkan et al., 2007). The syrinx situated at the point of bifurcation of trachea is responsible for production of sounds in birds and not larynx (Cevik-Demirkan et al., 2007). The trachea and syrinx are covered by ciliated pseudo-stratified cuboidal epithelium with goblet cells and intraepithelial glands (Cevik-Demirkan et al., 2007).

The larynx is covered by non-keratinised stratified squamous epithelium at vestibule and a pseudo-stratified cuboidal epithelium at caudal region (Cevik-Demirkan et al., 2007). The laryngeal epithelia contain goblet cells and mucus-producing intraepithelial glands (Cevik-Demirkan et al., 2007).

The extrapulmonary primary bronchi arising from the level of syrinx entered the lungs at hilum and become intrapulmonary primary bronchi (Cevik-Demirkan et al., 2006). The avian lung is wedge-shaped (**figure 4**), not divided into lobes and is contained within the ribs (Maina, 2008). The lung has three surfaces, dorsolateral or costal surface, dorsomedial or vertebral surface and ventromedial or septal surface (Maina, 2008).

The intrapulmonary primary bronchi traverse the lung and ends at abdominal air sac (Cevik-Demirkan et al., 2006), air sacs will be discussed later in this section. The gross examination shows no cartilaginous rings in primary bronchi (Cevik-Demirkan et al., 2006), but microscopic examinations show it is supported by plates of cartilage (Maina, 2008). Generally, there are four categories of secondary bronchi arising from intrapulmonary primary bronchi as it traverses the lung (Maina, 2008). The medioventral secondary bronchi arising from dorsomedial wall of cranial one-third of intrapulmonary primary bronchi, mediodorsal, lateroventral and laterodorsal secondary bronchi arise from caudal two-thirds of intrapulmonary primary bronchi (Maina, 2008). The medioventral secondary bronchi are usually four (Cevik-Demirkan et al., 2006; Maina, 2008), mediodorsal secondary bronchi are usually 7-10, number of lateroventral secondary bronchi varies and lateromedial secondary bronchi are small and numerous (Maina, 2008). The secondary bronchi give rise to tertiary bronchi also called parabronchi (Maina, 2008). The primary bronchi and beginning of secondary bronchi are lined by pseudostratified columnar epithelium ciliated with goblet cells, and the remainder of secondary bronchi is covered by cuboidal to squamous epithelium lacking mucous glands (Maina, 2008).

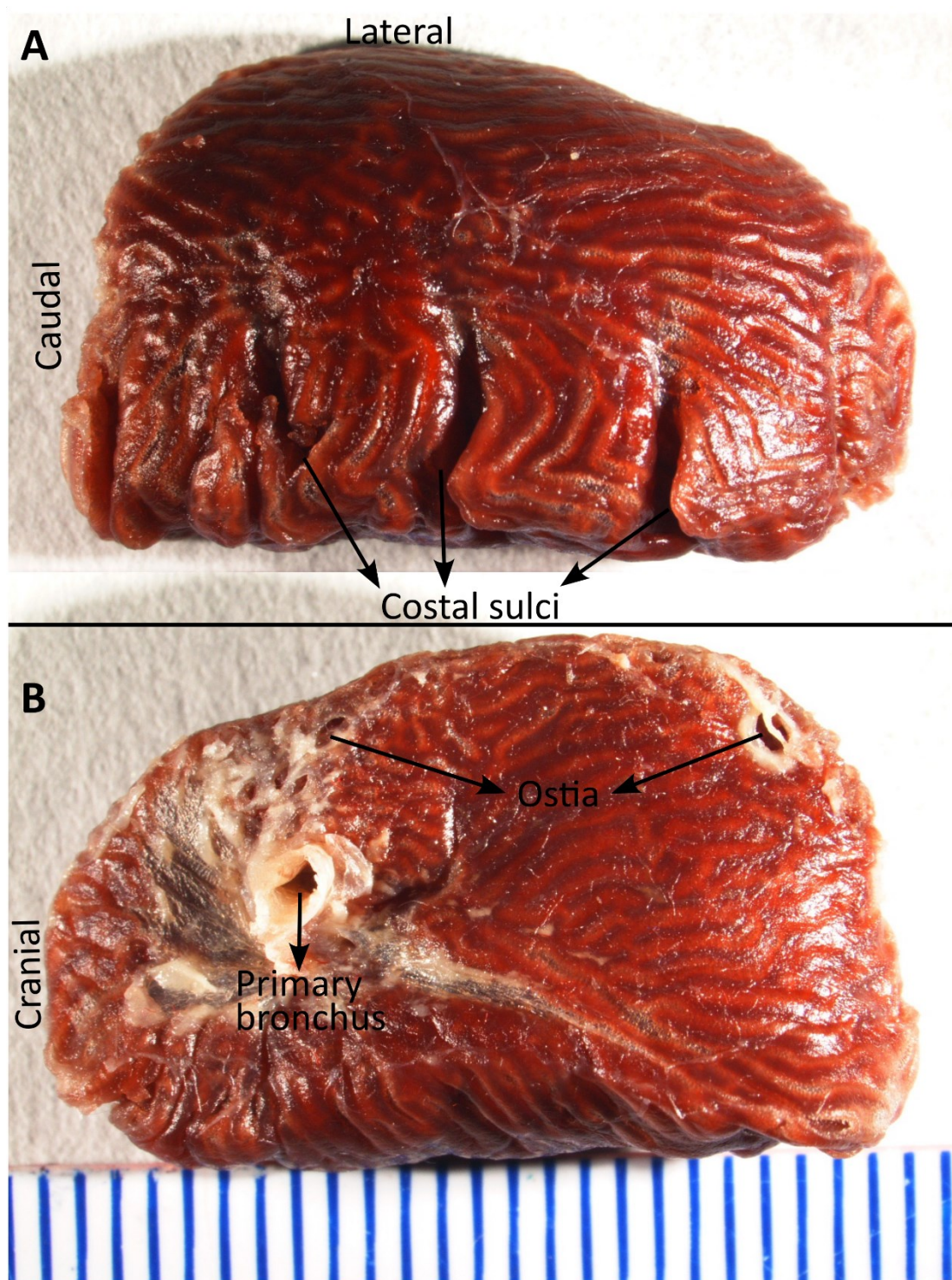


Figure 4: A photograph of an intact Japanese quail lung.

The dorsal (A) and ventral (B) surfaces of the lung. Note the costal sulci as the site where the lungs were held by the ribs. The primary bronchus as it enters and traverses the lung to end in the abdominal air sac. The caudal large ostium is the termination of the primary bronchus.

The parabronchi interconnect within lung parenchyma connecting the secondary bronchi, as such, medioventral and mediodorsal secondary bronchi are joined to form paleo-pulmonary part of the lung (Maina, 2008). The lateroventral and laterodorsal secondary bronchi together with the caudal air sac form neo-pulmonary part (Maina, 2008). The paleo-pulmonary part is found in all birds due to evolutionary superiority while the neo-pulmonary part is less developed or absent in some birds (Maina, 2008). However, microarchitecture and morphometry of paleo-pulmonary and neo-pulmonary parts are the same (Maina, 2008). In Japanese quail, parabronchi are circular in microscopic cross-section (**figure 5A and 5B**), and interparabronchial septa are not well developed; however, prominent interparabronchial septa appear in some birds for example, domestic fowl separating each parabronchial unit (Maina, 2008). In addition, openings into the atria (**figure 5C**), a small chamber delineated by interatrial septa, are visible throughout lumen of the parabronchi (Maina, 2008). Furthermore, infundibulae are seen (**figures 5C and 5D**), which are ducts arising from atria and branching into air capillaries (Maina, 2008). It is important to note that atria, infundibulae and air capillaries are situated within the wall of parabronchi (Maina, 2008).

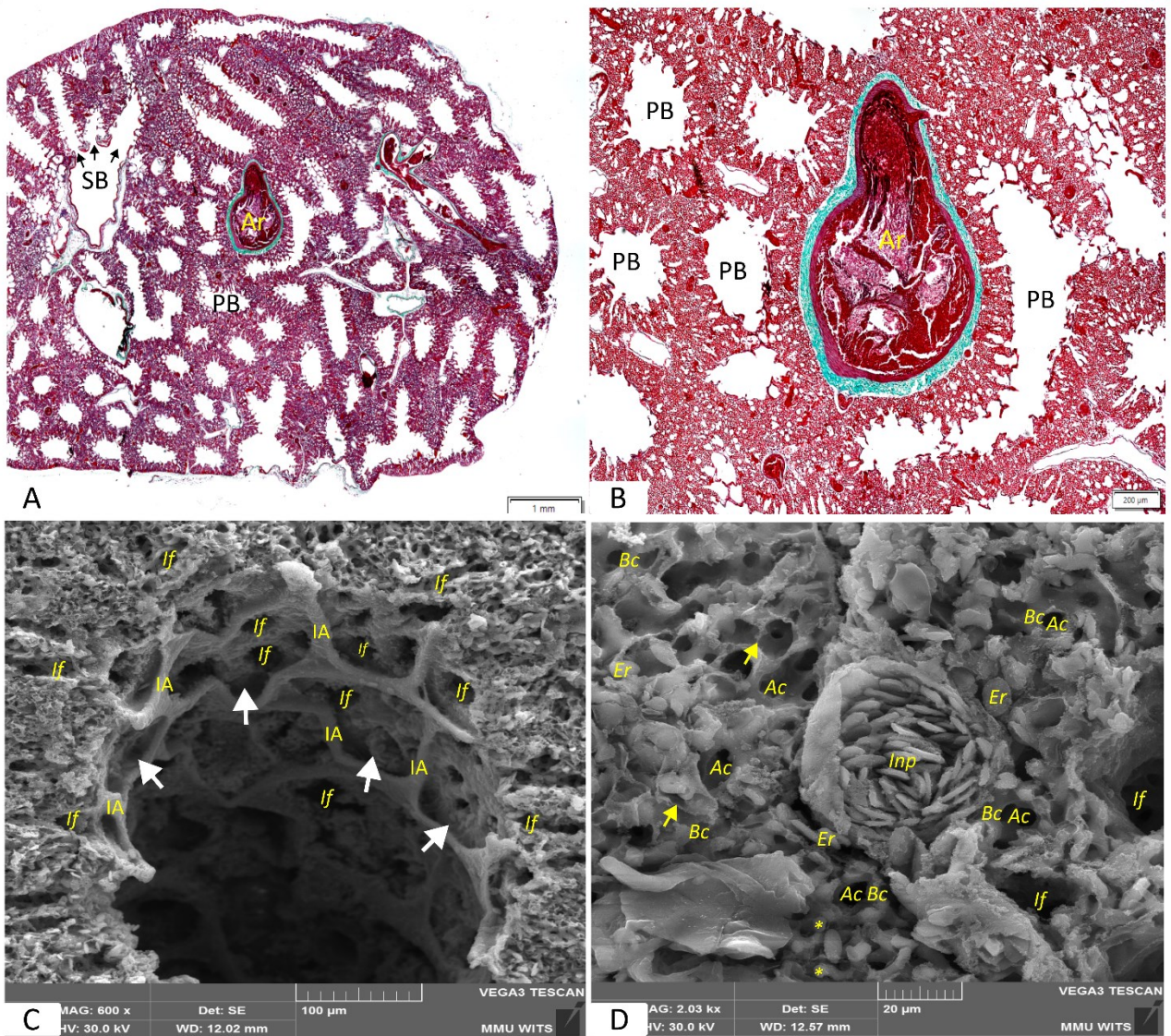


Figure 5: The representative micrographs of the Japanese quail lungs.

*The micrographs A and B are Gomori's trichrome stained while C and D are scanning electron micrographs (SEM). In figure 5A — Gomori's trichrome at 40x magnification — artery (Ar), a secondary bronchus (SB) and parabronchi (PB) are within the field of view but interparabronchial septa are not visible or absent. Note the black arrows showing the SB openings into PB. Figure 5B — Gomori's trichrome at 100x magnification — shows a closer look at the artery and parabronchi, still, interparabronchial septa are absent. Note how the stain becomes brighter at higher magnification. Figure 5C — SEM at 600x magnification — shows a parabronchi unit with atria (white arrows) and infundibulae (If) easily discernible. Note the interatrial septa (IA) between atria. Figure 5D — SEM at 2000x magnification — shows the intraparbanchial artery (Inp), the air capillaries (Ac) and blood capillaries (Bc) intermingled at a perpendicular angle. The blood-gas barrier (yellow arrow) is visible. The erythrocytes (Er) are situated within the blood capillaries, while some are visible out of the blood capillaries. Some thrombocytes (indicated with an asterisk *) are visible.*

The air capillaries mix with blood capillaries to bring air closer to erythrocytes at blood-gas barrier (BGB) for oxygen exchange (Maina, 2008). The BGB is formed by endothelial cell of blood capillaries, intermediate extracellular matrix and epithelial cell of air capillaries (Maina, 2008). The thickness of exchange mantle is between 200 – 500µm, diameter of atria is 60 – 100µm by 120 – 130µm, infundibulae diameter is 25 – 40µm and infundibulae depth into exchange mantle is 100 – 150µm and air capillaries diameter is 3 – 20µm (Maina, 2008). However, these morphometric values are mainly from small birds such as dove and pigeon and thus could relate to Japanese quail. The relationship of blood flow to bulk of airflow in parabronchi is rather perpendicular, called cross-current system (Maina, 2005), to enhance efficiency of gas exchange by increasing duration of interaction between erythrocytes and air capillaries (Maina, 1988).

Furthermore, blood supply to the lung by pulmonary artery is mainly via three branches, cranial, caudomedial and caudolateral branches, then sometimes there is an accessory branch (Maina, 2005). The cranial branch and the accessory branches — when present — supply cranial region while caudal region is supplied by caudomedial and caudolateral branches (Maina, 2005). The main arterial branches give rise to the interparabronchial branches, which in turn give rise to intraprabronchial arterioles ending in blood capillaries. The blood capillaries meet air capillaries at BGB forming cross-current system for efficient gas exchange (Maina, 2005).

The BGB has random attenuations of thick parts which provide strength, connective tissue, and thin parts of least resistance for gas diffusion (Maina, 2008). The epithelial side of BGB is covered by two types of surfactants, lamellated osmiophilic bodies (LOS) and trilaminar

substance (TLS) (Maina, 2008). The surfactant could be lowering surface tension and respiratory impedance to prevent collapse of air capillaries (Maina, 2008). However, some researchers suggested a rigid avian lung will not require surfactants to reduce tension, hence, they suggested surfactants could be involved in prevention of transudation of plasma into respiratory surface (Maina, 2008). In addition, TLS might be involved with the coagulation of blood leaked via BGB or absorption of fluid accumulated in air capillaries (Maina, 2008).

Furthermore, avian respiratory system has free avian respiratory macrophages (FARM) to depend on against invading organisms and particles (Maina and Cowley, 1998). The FARMS are cytotoxic and phagocytic against foreign pathogens and particles entering respiratory system (Mutua et al., 2016). In addition, Maina and Cowley (1998) characterise FARMS on surfaces of atria and infundibulae of the rock dove using scanning electron microscopy. Naturally, the design of avian pulmonary system supplements defence system by developing cilia on trachea and bronchial system up to the beginning of secondary bronchi (Mutua et al., 2016). The mucociliary escalator system as it is termed, traps, and removes invading pathogens and particles, in addition to phagocytic activity of epithelial cells of atria (Mutua et al., 2016).

Before delving into airflow in avian respiratory cycle, a brief description of air sacs will enlighten the whole process. Every bird usually has six air sacs or less, the cervical air sacs are small and paired and situated in craniodorsal aspect of thoracic cavity (Maina, 2005). Next is clavicular air sac, unpaired, usually large and sits in cranioventral part of thorax, thoracic air sacs are pairs (caudal and cranial) of comparable size sometimes one is larger, situated ventral

to lungs and horizontal septum (Maina, 2005). Finally, abdominal air sacs are paired occupying peritoneal cavity dorsal to the viscera (Maina, 2005).

All air sacs are connected to the lungs via ostia and connection is direct when it is to primary or secondary bronchi, while an indirect connection is to parabronchi (Maina, 2005). Most air sacs have one or two direct connections and several indirect ones. The wall of air sacs is mainly a simple squamous epithelium on a thin layer of connective tissue but possesses cuboidal epithelium near ostia, it is avascular with few adrenergic and cholinergic nerve fibres (Maina, 2005).

The air sacs expand during inspiration for air to flow into the primary bronchus to reach caudal thoracic and abdominal air sacs bypassing openings of ventral bronchi (Cieri and Farmer, 2016). The bypass is facilitated by an aerodynamic inspiratory valving largely dependent on convective momentum of the inspired air and progressive narrowing of primary bronchi. Furthermore, air within parabronchi moves to cranial air sacs (Cieri and Farmer, 2016). During expiration, air within caudal thoracic and abdominal air sacs is squeezed to parabronchi via dorsal bronchi facilitated by aerodynamic expiratory valve, while air stored in the cranial air sacs passed to ventral bronchi to reach trachea and is expired to atmosphere (Cieri and Farmer, 2016). In brief, unidirectional airflow in the avian lungs implies that air moves from caudal part of the lung to cranial part via the dorsal bronchi to parabronchi to the ventral bronchi during both inspiratory and expiratory cycles, this is called the Hazelhoff's d-p-v system (Cieri and Farmer, 2016).

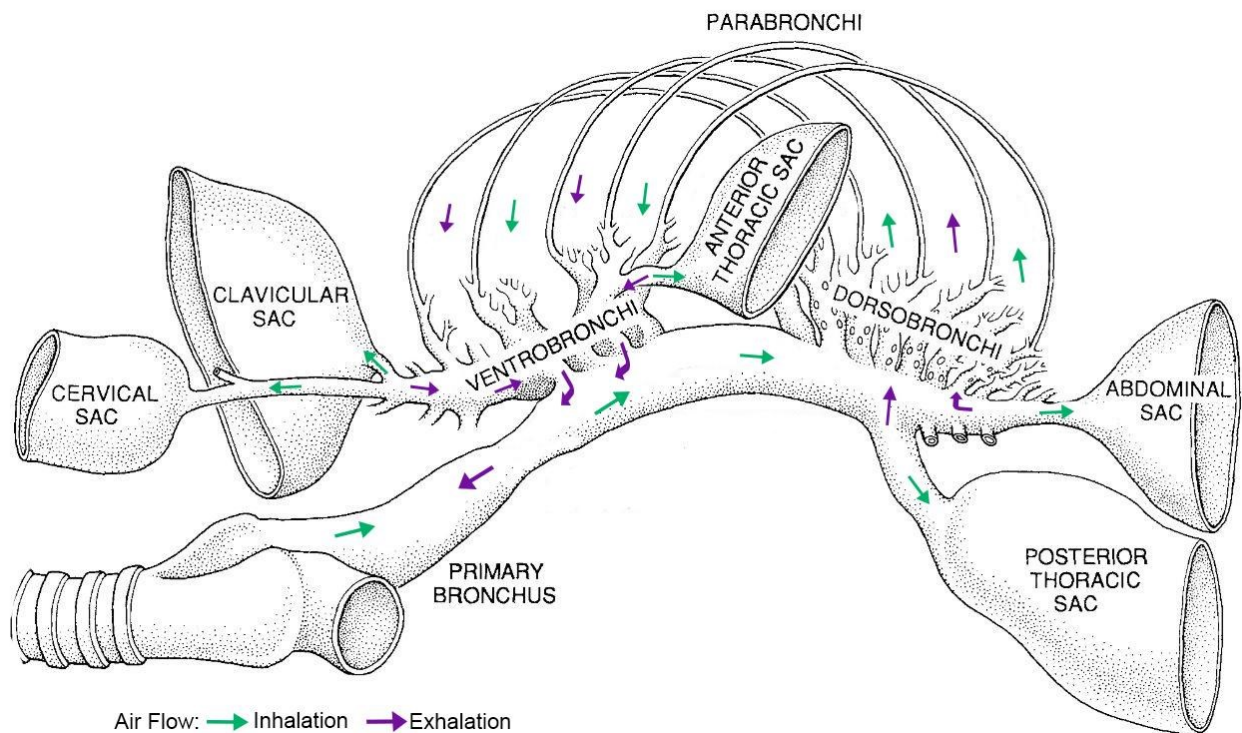


Figure 6: Diagram of unidirectional airflow in the avian respiratory system.

At the commencement of inspiration, air moves from the trachea through the primary bronchus to reach the caudal group of air sacs (green arrows). While air within the parabronchi moves to the cranial group of air sacs (green arrows). On exhalation, the air within the caudal group of air sacs moves to the dorsobronchi and then into the parabronchi (purple arrows). While air within the cranial group of air sacs moves into the ventrobronchi and then through the primary bronchi into the trachea (purple arrows). Note that the parabronchi connect the dorsobronchi to the ventrobronchi and the direction of airflow in them did not change during both inhalation and exhalation. Also note that, the air sacs expand during inhalation and collapse during exhalation, like balloons, and that the posterior thoracic and abdominal air sacs are the caudal group of air sacs, while the cervical, clavicular, and anterior thoracic air sacs are the cranial group of air sacs. Diagram modified from Wang et al. (1992).

1.7 Highlights and rationale of the study

The Japanese quail reaches sexual maturity within six weeks post-hatching and is easily maintained making it a reliable model for biomedical research. The short development period makes it a better model for embryological studies than chicken and mice. The economic

significance of Japanese quail arises from its perceived healthier market products and is a lucrative poultry business because of its rapid growth. However, heat stress resulting from global warming affects its welfare. Researchers examined the impact of heat on some organs of Japanese quail using morphological experiments, but the lungs were morphologically understudied (**next chapter**). Hence, this research investigated the effects of heat stress on the lungs of Japanese quail. The respiratory system receives a direct supply of heat during exposure which could have a negative consequence on function of the system. This study examined the gross morphology, light microscopic and ultrastructural changes in the lungs of Japanese quail as it relates to heat stress. The outcomes of this research might help refine husbandry practices and improve welfare and survival of Japanese quail and other poultry. A scoping review was conducted — next chapter — to identify and analyse literature published on the impact of heat stress on the Japanese quail organs. The scoping review narrowed the possibility of repeating research already conducted and provides enough information in this area of research.

1.8 Aim of the study

This study aims to investigate the morphological changes in the lung and air sacs of the Japanese quail exposed to heat stress.

1.9 Objectives of the study

1. To conduct a scoping review of literature on the effect of heat stress on Japanese quail to identify the research conducted to date.
2. To document gross anatomy of Japanese quail lung using formalin-fixed lung tissue morphologically and morphometrically. This will encompass describing lung structures using gross dissection, measurements of volume changes and size. This will provide a clear understanding of gross pulmonary architecture.
3. To conduct light microscopy using Mayer's H&E, new pentachrome, and Gomori's trichrome stains to document visible changes in arrangement of cells and connective tissues. These procedures will provide an understanding of light microscopic pulmonary morphology and an idea of the system's integrity.
4. To conduct immunofluorescence of the lung's smooth muscles and collagen fibre composition. This procedure will enable the study of strength of the system.
5. To investigate the three dimensional (3D) spatial arrangement of parabronchi and the changes from heat stress and conduct morphometry on identified structures using scanning electron microscopy (SEM).

2 CHAPTER TWO — SCOPING REVIEW

2.1 Introduction

Few published literatures highlighted morphological changes in organs of Japanese quail. Hence, this scoping review aims to identify reported impacts of heat stress on Japanese quail organs, especially the lungs. Morphological change in this study is apparent gross or microscopic change which can be compared to control, i.e., normal tissue, and is attributable to heat stress.

2.2 Methodology

This scoping review was conducted following Joanna Briggs Institute (JBI) methodology for scoping reviews. Preferred reporting of items for systematic reviews and meta-analyses extension for scoping reviews (PRISMA-ScR) is used for reporting. This scoping review is according to a registered protocol (**appendix 4**), which can be found on open science framework (OSF) website (<https://doi.org/10.17605/OSF.IO/VMYQT>).

2.2.1 Inclusion criteria

Studies that reported Japanese quail of any age and sex were eligible; any other type of quail, such as Common quail or California quail, were excluded. Studies that exposed Japanese quail to acute or chronic heat stress and either continuous or cyclical exposure were included. In contrast, studies on Japanese quail exposure to cold stress were excluded. Furthermore, gross morphological and histological studies of Japanese quail organs subjected to heat stress were included. In addition, morphological studies of cell cultures, eggs, and molecular analyses were excluded. Finally, morphological studies of Japanese quail organs not exposed to heat

stress were excluded. This scoping review considers all primary experiments involving Japanese quail; however, systematic reviews, theses, and dissertations that meet the criteria were selected.

2.2.2 Search strategy

The search strategy for this scoping aimed to locate both published and unpublished studies. A three-step approach was used to identify records for this scoping review. The first step was conducted using key terms (Japanese quail, Heat stress, Morphology) to identify major keywords and availability of literature related to the topic. This step was part of pilot study conducted, details of which can be found in the registered protocol. The second step is search of databases and grey literature using search query. The databases searched for records are MEDLINE (via PubMed), SCOPUS, CAB Abstracts (via CAB Direct), and Web of Sciences (All databases). The grey literature search was conducted in ProQuest (eBook central, agricultural sciences database, biological sciences database, environmental science database, health and medical collection, science database, ProQuest dissertation and theses global), Biorxiv & Medrxiv, and Google scholar. All searches were conducted on the 27th and 28th of June 2022. The search strategies are as follows:

1. **Pubmed** searched on 27/06/2022

- a) ((((((((((((((Japanese quail) OR (Coturnix japonica)) OR (Coturnix coturnix japonica)) AND (heat stress)) OR (heat exposure)) OR (heat exposed)) OR (thermal stress)) OR (thermal exposure)) OR (thermal exposed)) OR (high temperature)) AND

(morphology)) OR (morphological)) OR (histology)) OR (histological)) OR (morphometry)) OR (morphometric): 5,850,070 results.

b) (((Japanese quail) OR (Coturnix japonica)) AND (heat stress)) OR (thermal stress)) AND (morphology): 2,676 results.

2. **Scopus** searched on 28/06/2022

(TITLE-ABS-KEY (Japanese AND quail) OR TITLE-ABS-KEY (coturnix AND japonica) AND TITLE-ABS-KEY (heat AND stress) OR TITLE-ABS-KEY (thermal AND stress) OR TITLE-ABS-KEY (heat AND exposure) OR TITLE-ABS-KEY (thermal AND exposure) AND TITLE-ABS-KEY (morphology) OR TITLE-ABS-KEY (morphological) OR TITLE-ABS-KEY (histology) OR TITLE-ABS-KEY (histological) OR TITLE-ABS-KEY (morphometric) OR TITLE-ABS-KEY (morphometry)): 16 document results.

3. **Web of Sciences** searched on 27/06/2022

((ALL= (Japanese quail OR Coturnix japonica OR Coturnix coturnix japonica)) AND ALL= (Heat stress OR Thermal stress OR Heat exposure OR Heat exposed OR Thermal exposure OR Thermal exposed)) AND ALL= (Morphology OR Histology OR morphometry OR morphometric OR Morphological or histological) | 22 results

4. **CAB Direct** searched on 27/06/2022

("Japanese quail") OR ("Coturnix japonica") OR ("Coturnix coturnix japonica") AND ("Heat stress") OR ("Thermal stress") OR ("Heat exposed") OR ("Thermal exposed") OR ("Heat

exposure") OR ("Thermal exposure") AND (Morphology) OR (Morphometry) OR (Histology) OR (Histological) OR (Morphological) OR (Morphometric). 26,866 results (first 1075 provided)

5. **Biorxiv & Medrxiv** searched on 27/06/2022

77 Results for term "Japanese quail Heat stress Morphology"

6. **ProQuest** searched on 27/06/2022

Included databases: eBook central, agricultural sciences database, biological sciences database, environmental science database, health and medical collection, science database and ProQuest dissertation and theses global.

(Japanese quail) AND (heat stress) AND Morphology. 3,710 results. (First 500).

7. **Google Scholar** searched on 27/06/2022

morphology, heat stress "Japanese quail". 6930 results. (First 203).

The search results from CAB abstracts were sorted based on relevance, and the first 1075 records were exported. A similar approach was used for searches of ProQuest (first 500 records) and Google scholar (first 204 records), as suggested by Haddaway et al. (2015). This limitation was applied to avoid redundancy because the titles of CAB abstract results were screened up to first 8,000, which showed no record related to the topic after first 1,000 records. The third step searched for eligible records in references cited by included studies.

2.2.3 Sources of evidence selection

Following the search, all identified citations were exported as a .ris file and collated, then uploaded into EndNote 20.2.1 (The EndNote Team, 2013). After that, collated records from databases and grey literature sources were exported from EndNote as one .txt file. This makes it easier to import into Rayyan web application for systematic reviews (Ouzzani et al., 2016). At this point, all records identified as eligible from pilot study were uploaded into the Rayyan Web application just before removal of duplicates. After uploading the documents into Rayyan web application, duplicates were removed immediately. Subsequently, title and abstracts of records were screened by two reviewers based on the following questions:

1. *Is the study on Japanese quail or involved Japanese quail?*
2. *Is the experimental design on heat exposure or involved heat exposure?*
3. *Does the study outcome include gross morphology, morphometry, or histology of any organ?*

All records that ticked 'yes' in the three questions were selected for full-text screening. However, any study that answered 'no' to one question was excluded. Any conflict between reviewers from level 1 screening was resolved by consensus. Then, level 2 screening of full texts was conducted based on the following questions:

1. *Does the study report explicitly gross or histological evaluation of any organ of the Japanese quail?*
2. *Does the study design have a control group and a heat-stressed group to compare heat stress effect on organs?*

3. *Does the study outcome explicitly mention the effects of heat stress on any organ of the Japanese quail?*

The criteria for full-text screening are that a study must answer 'yes' in first question and another 'yes' in second or third question to be eligible. If a study answered 'no' in question 1, it is excluded, and if it answered 'no' in both question 2 and question 3, it is excluded. Conflicts were resolved by consensus.

All eligible studies were included for data extraction without critical appraisal. The vital information necessary for scoping review question did not warrant strict methodology appraisal.

2.2.4 Data extraction

The reviewers developed a unique data extraction tool (**table 9**) during the protocol development and tested it during the pilot study. The data extracted from the records included the country where the study was conducted and aim of the study. The total number of Japanese quail used, age, sex, weight range and the species name used was extracted as the essential characteristics of bird's sample. The species name used is vital to understanding the standing conflict in using scientific name of Japanese quail. The data extracted regarding heat stress included type and duration of heat exposure, temperature level for both heat stress group and the thermoneutral group, and study duration.

The type of heat stress exposure can be either continuous or cyclic. Continuous heat exposure is when temperature above thermoneutral level is maintained for 24 hours throughout

experimental period for heat stress group. While cyclic exposure is when higher temperature level is increased over certain hours and then reduced to a thermoneutral temperature for remaining hours and repeated the next day. The duration of the study can be acute or chronic. Acute exposure is when the period of heat stress exposure, whether continuous or cyclic, is 24 hours or less, while chronic exposure is more than 24 hours. The Japanese quail body temperature measurement method used by researchers is also extracted to know if body temperature of Japanese quails was monitored.

The second level of data extraction is organ(s) of interest in the study, descriptive and quantitative data reported. The descriptive data is simply observed changes effected by exposure to heat stress on the organ(s) of interest. In contrast, quantitative data is measurements reported in the study, i.e., villus height, crypt depth etc. Numerical values were not extracted because no meta-analysis or meta-synthesis was intended for this scoping review. The study title was added during data extraction, which was not in the protocol, for completeness of data and to help reviewers navigate through data table quickly and precisely.

Table 9: A sample of the data extraction used for the scoping review.

1	Study ID (Author, Year)
2	Country of the study
3	Title of the study
4	Aim of the study
5	Number of Quails
6	Specie name used
7	Age
8	Sex
9	Weight range
10	Type of heat exposure (cyclical/continuous)
11	Temp level (control)
12	Temp level (Heat stress)
13	Duration of heat exposure (Acute/Chronic)
14	Duration of the study
15	Method of temp check
16	Organ studied
17	Descriptive data
18	Quantitative data

2.2.5 Data analysis and presentation

The critical component of this scoping review, which is impact of heat stress on Japanese quail organs, is summarised narratively in a table. Basic features of included studies and data on heat stress are presented in table and figures where appropriate. In addition, a flow chart of screening process of sources is presented. No meta-analysis or meta-synthesis of the extracted data was conducted.

2.3 Results

2.3.1 Study inclusion

The identification, screening, inclusion, and exclusion process of records are summarised in **figure 7**. This scoping review identified 4598 records through databases and grey literature searches. Following removal of 105 duplicates, 4493 records were screened at level 1 title and abstracts. We excluded 4461 records for the following reasons: 4272 experiments did not use or involve Japanese quail, 131 used Japanese quail but did not include heat stress, and 47 studies exposed Japanese quail to heat stress but did not report any morphological outcome. Other reasons for excluding records are studies conducted on Japanese quail eggs and cell lines. However, amongst the 131 records that used Japanese quail without heat stress exposure, 36 reported morphological outcomes. Although excluded, it is significant to outline this observation because this scoping review is interested in morphological consequences.

During second level screening of 32 full texts, we excluded nine records for the following reasons: seven records had an ambiguous methodology, one full text was not in English

language, and one full text was not retrieved even after an online request from the author.

The records excluded after full-text screening are as follows:

1. Al-Mashhadani, T. M. 2021. Effects of sesame seeds on reproduction process in Japanese quails males exposed to heat stress. *Biochem Cell Arch*, 21, 4185-4191. (Al-Mashhadani, 2021) **Reason – full text not retrieved.**
2. Bayrakdar, A., Dalkiliç, B., Yaman, M., Şimşek, Ü. G. & Çiftçi, M. 2017. Effect of dietary orange peels essential oil and thermotolerance on histo-morphometry and serotonin-immunoreactive endocrine cell numbers in the small intestines of heat-stressed Japanese quails. *Kafkas Univ Vet Fak Derg*, 23, 177-184. (Bayrakdar et al., 2017) **Reason – The reported outcome could not be explicitly attributed to heat stress.**
3. Dalkilic, B., Simsek, U. G., Ciftci, M. & Baykalir, Y. 2015. Effect of dietary orange peel essential oil on physiological, biochemical and metabolic responses of Japanese quails as affected by early age thermal conditioning and fasting. *Revue Méd Vét*, 166, 154-162. (Dalkilic et al., 2015) **Reason – The reported outcome could not be explicitly attributed to heat stress.**
4. Egbuniwe, I. C., Uchendu, C. N. & Obidike, I. R. 2020. Effects of betaine and ascorbic acid supplementation on serum gonadotropin, testicular histological analysis and sperm quality in male Japanese quails during the dry season. *Theriogenology*, 158, 391-405. (Egbuniwe et al., 2020) **Reason – The reported outcome could not be explicitly attributed to heat stress.**
5. El-Daly, E. F., Abd El-Azeem, N. A., El-Wardany, I., Abd El-Gawad, A. H. & Hemid, A. E. A. 2014. Effect of different anti-stress feed additives on some blood metabolites and

lymphoid organs histology in laying Japanese quail. *World Appl Sci J*, 29, 1574-1585. (El-Daly et al., 2014) **Reason – Adult Japanese quail were not exposed to heat stress, only the eggs were exposed.**

6. Ozdemir, D., Ozudogru, Z., Imik, H., Can, M. & Sunar, M. 2011. Effects of dietary antioxidant supplementation on the adrenal glands in quails (*Coturnix coturnix japonica*) reared under heat stress. *Rev Med Vet (Toulouse)*, 162, 8-12. (Ozdemir et al., 2011)

Reason – The reported outcome could not be explicitly attributed to heat stress.

7. Porto, M. L. & Fontenele-Neto, J. D. 2020. Effect of thermal manipulation during incubation on the haematological variables, serum biochemistry and morphometry of cloacal bursa of Japanese quails submitted to chronic heat stress. *Arq Bras Med Vet Zootec*, 72, 505-516. (Porto and Fontenele-Neto, 2020) **Reason – Full text was not written**

in English language, only the title and abstract were in English.

8. Porto, M. L., Teofilo, T. S., Cavalcanti, D. M. L. P., Freitas, C. I. A., Oliveira, M. F. & Fontenele-Neto, J. D. 2021. Incubation variables, performance, and morphometry of the duodenal mucosa of Japanese quails (*Coturnix coturnix japonica*) submitted to different incubation temperatures and thermally challenged after hatching. *Arq Bras Med Vet Zootec*, 73, 495-507. (Porto et al., 2021) **Reason – The reported outcome could not be**

explicitly attributed to heat stress.

9. Seyrek, K., Erbas, H., Serter, M., Yenisey, C., Kiral, F. & Bardakcioglu, H. E. 2010. The effects of dietary ascorbic acid supplementation on collagen and amino acid concentrations in Japanese quails exposed to heat stress. *Turk J Vet Anim Sci*, 34, 69-74. (Seyrek et al., 2010)

Reason – Molecular study is the only reported outcome.

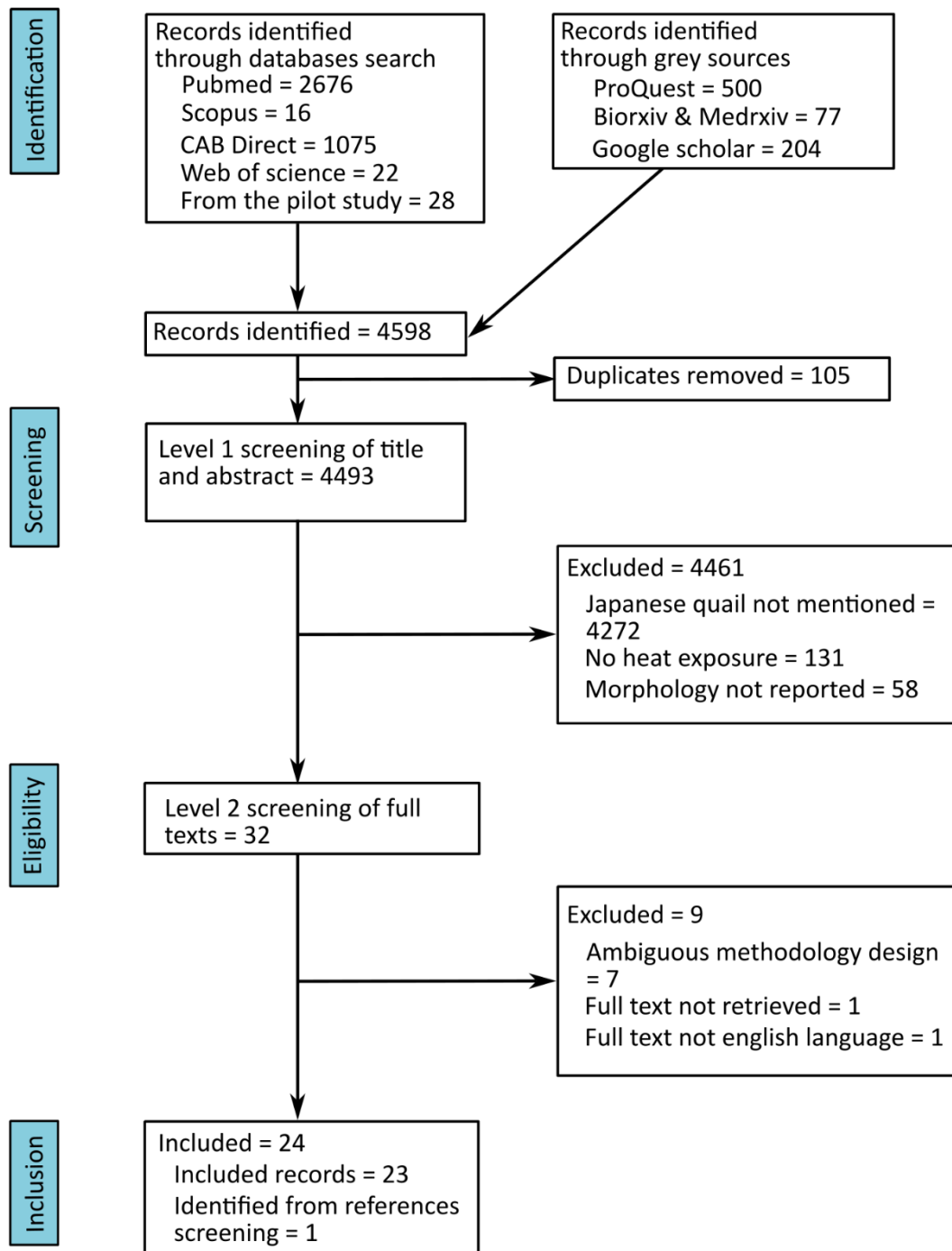


Figure 7: A flow chart diagram of the study selection criteria.

2.3.2 Characteristics of included studies

All studies included in this scoping review are primary animal experiments, and characteristics of included studies are summarised in **table 10**.

Table 10: The characteristics of included studies for the scoping review.

	Reference	Country	heat exposure	Temperature °C	Organ
1	(Al-Ali et al., 2017)	Iraq	Continuous, Chronic	42	Spleen
2	(de Moraes et al., 2021)	Brazil	Continuous, Chronic	32	Intestines, Liver, Uterus, Kidney
3	(Furtado et al., 2022)	Brazil	Cyclic, Chronic	32	Internal organs weight
4	(Hassan et al., 2003)	Egypt	Continuous, Chronic	40	Oviducts
5	(Imik et al., 2010)	Turkey	Cyclic, Chronic	34	Skeletal muscle
6	(Jimenez and De Jesus, 2021)	USA	Continuous, Acute	39	Skeletal muscle
7	(Jimenez et al., 2018)	USA	Continuous, Acute	39	Skeletal muscle
8	(Kaplan and Koksai, 2021)	Turkey	Continuous, Chronic	37	Skeletal muscle

9	(Mehaisen et al., 2017)	Egypt	Continuous, Chronic	35	Intestines
10	(Mohamed et al., 2015)	Egypt	Cyclic, Chronic	34	Liver, Kidney, Brain, Lungs, Heart, Spleen
11	(Ozcelik et al., 2014)	Turkey	Cyclic, Chronic	34	Liver
12	(Pu et al., 2019a)	Japan	Cyclic, Chronic	34	Ovary and Adrenal gland
13	(Pu et al., 2019b)	Japan	Cyclic, Chronic	34	Liver
14	(Pu et al., 2020)	Japan	Cyclic, Chronic	34	Liver and Ovary
15	(Razooqi et al., 2019)	Iraq	Continuous, Chronic	39.4	Testis
16	(Rodrigues et al., 2022)	Brazil	Cyclic, Chronic	32	Internal organs weight
17	(Sandikci et al., 2004)	Turkey	Continuous, Chronic	34	Intestines
18	(Sarica and Ozdemir, 2018)	Turkey	Cyclic, Chronic	34	Morphological asymmetry and tonic immobility
19	(Shahri, 2020)	Iran	Continuous, chronic	25-41.7	Brain

20	(Sritharet et al., 2002)	Japan	Continuous, Chronic	40	Liver and Pituitary gland
21	(Tekce et al., 2020b)	Turkey	Continuous, Chronic	37	Internal organs weight, Meat quality
22	(Tekce et al., 2020a)	Turkey	Continuous, Chronic	37	Internal organs weight, Meat quality
23	(Turk et al., 2015)	Turkey	Cyclic, Chronic	34	Testis
24	(Turk et al., 2016)	Turkey	Cyclic, Chronic	34	Testis

This review did not identify systematic or scoping reviews, theses, or dissertations. Nine studies were conducted in Turkey, the remaining in Japan – 4, Brazil – 3, Egypt – 3, United States of America – 2, Iraq – 2, and Iran – 1. The first study identified was conducted in year 2002 (**figure 8**), which means in 20 years, few experiments (24 included studies) evaluated gross or microscopic features of heat stress in Japanese quail organs. The experiment design aimed to study morphology in 15 included studies, which was not explicitly stated in nine studies. The Japanese quail used in the experiments were sex unspecified in 11 records, five records reported using mixed samples, four reported using females only, and three reported using males only. The age of Japanese quail at the start of experiment is different for each experiment, while only four records reported weight range of the birds. Chronic heat

exposure was the format in 22 records, while only two studies were acute heat stress experiments. However, heat exposure was continuous in 13 records and cyclic in 11 records. The average heat exposure hours during cyclic experiments are 7.6 hours (min=4, max=12). The experiment temperature levels for thermoneutral groups averaged 23.4°C (min=22, max=26) and heat stress temperatures averaged 35.3°C (min=32, max=42). None of the records describes method of the birds' body temperature check, which could have been either cloacal or skin temperature measurement.

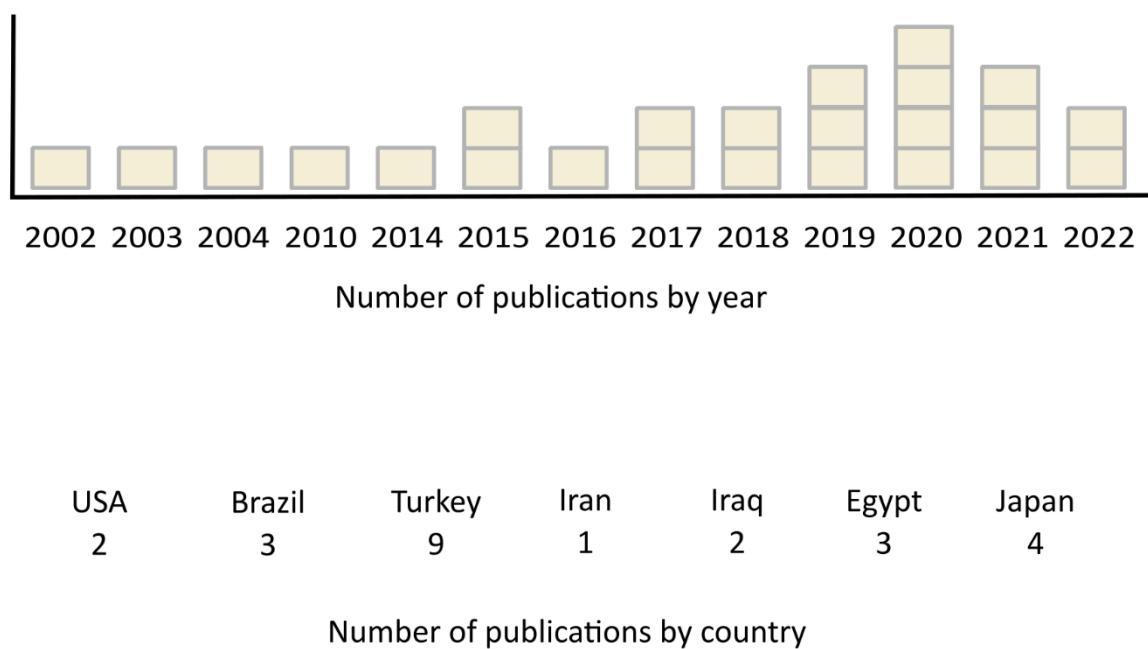


Figure 8: The number of included studies categorised based on country and year.

2.3.3 Review findings

Heat stress increased gizzard weight (Furtado et al., 2022), but other organs remained unaffected, as Rodrigues et al. (2022) reported. In intestines; heat stress decreased villus height and area of small intestines; reduced number of goblet cells; caused crypt epithelium hyperplasia; and decreased Calbindin-D28k (epithelial calcium transporter channel) activity in duodenum (Sandikci et al., 2004; Mehaisen et al., 2017; de Moraes et al., 2021). In addition, heat stress decreased villus width in a report by Mehaisen et al. (2017) but remained the same in a report by (Sandikci et al., 2004). Furthermore, heat stress activates Kupffer cells; causes macrovesicular steatosis, severe congestion, bile accumulation, extramedullary haematopoiesis, hepatocyte degeneration, and necrosis in liver (Sritharet et al., 2002; Ozcelik et al., 2014; Mohamed et al., 2015; Pu et al., 2019b; Pu et al., 2020; de Moraes et al., 2021) (**figure 9**). In kidneys, heat stress causes fatty change, hydropic degeneration, congestion and necrosis of renal tubular cells; enhances mononuclear cell infiltration; increases calbindin-D28k activity in proximal and distal tubules (Mohamed et al., 2015; de Moraes et al., 2021).

Heat stress reduces ejaculate volume, sperm motility, and concentration; kills or distorts sperm cells; and causes disorganization and degeneration of germinal cells layer in testis. It increased immature spermatids and spermatogonia production and dilate seminiferous tubule (Turk et al., 2015; Turk et al., 2016; Razooqi et al., 2019). Furthermore, heat stress reduced the number of primary and secondary uterine folds and lowered Calbindin-D28k activity in uterus (de Moraes et al., 2021). Heat-stressed quail showed low oviduct and ovarian weight; reduced follicle number; and empty oviduct uterovaginal junctions (Hassan et al., 2003; Pu et al., 2019a).

Heat stress decreased nuclei per millimetre of muscle fibre in fast-growing Japanese quail but increased mean muscle fibre diameter. Authors reported varied results on muscle colour, Tekce et al. (2020b) reported lower lightness L* values but subsequently reported no changes in values (Tekce et al., 2020a). The remaining authors reported high lightness L* value, low redness a* and yellowness b* values (Imik et al., 2010; Jimenez et al., 2018; Jimenez and De Jesus, 2021; Kaplan and Koksai, 2021).

Heat-stressed quails showed signs of brain damage, including enlargement of blood sinusoids, vacuolation, necrosis and degeneration of neurons (Mohamed et al., 2015; Shahri, 2020). In spleen, heat stress caused lymphocyte infiltration; promoted necrosis, degeneration, disintegration, congestion and bleeding of lymphatic nodules and pulp tissue (red and white); and dilated sinusoids (Al-Ali et al., 2017). In addition, heat stress caused lung congestion (Mohamed et al., 2015) and degenerated pituitary gland cells (Sritharet et al., 2002). Finally, heat stress increased tonic immobility and asymmetric lengths of beak, outer toe, eye, and nostril (Sarica and Ozdemir, 2018).

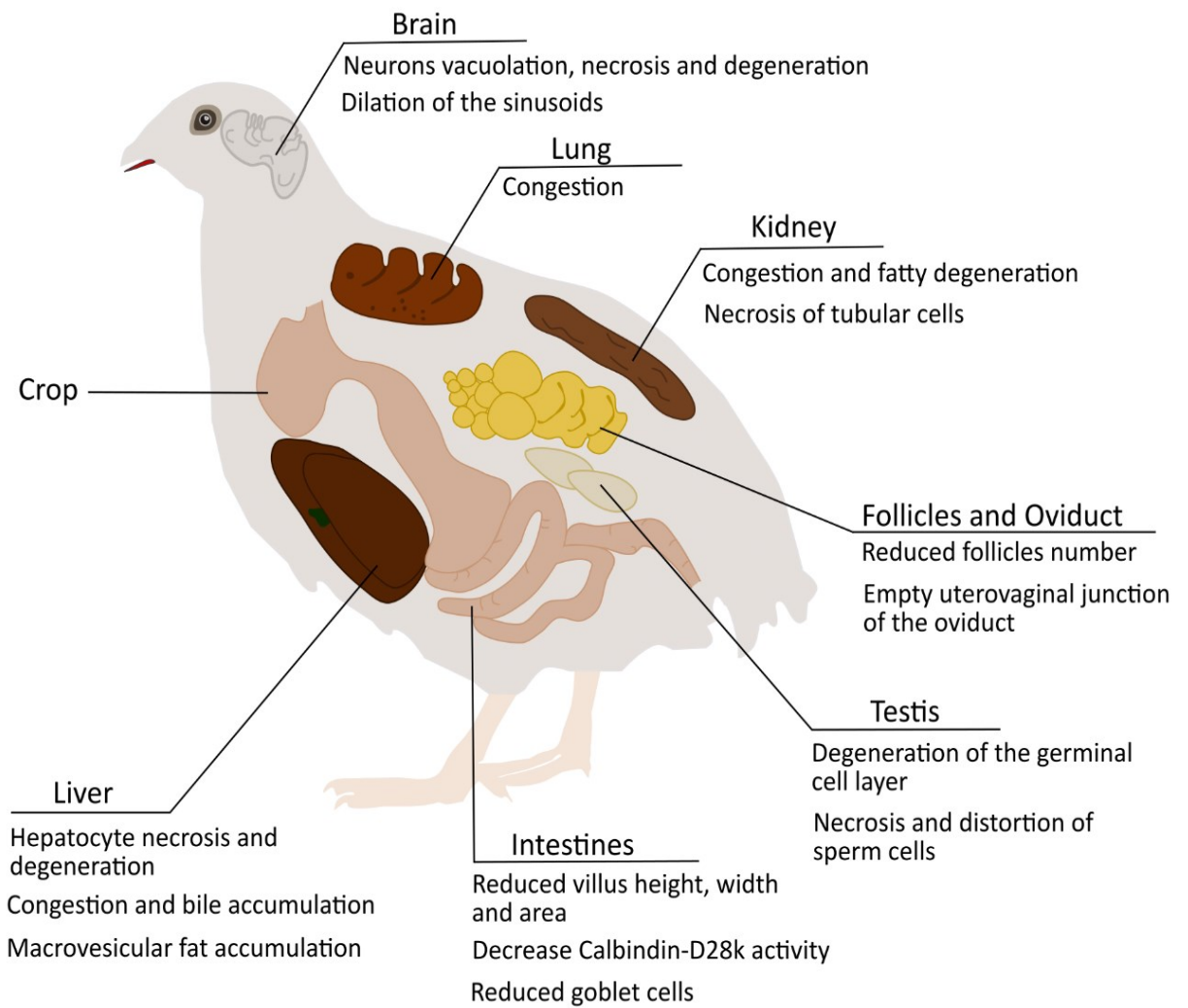


Figure 9: A diagram of the impact of heat stress on Japanese quail.

2.4 Discussion

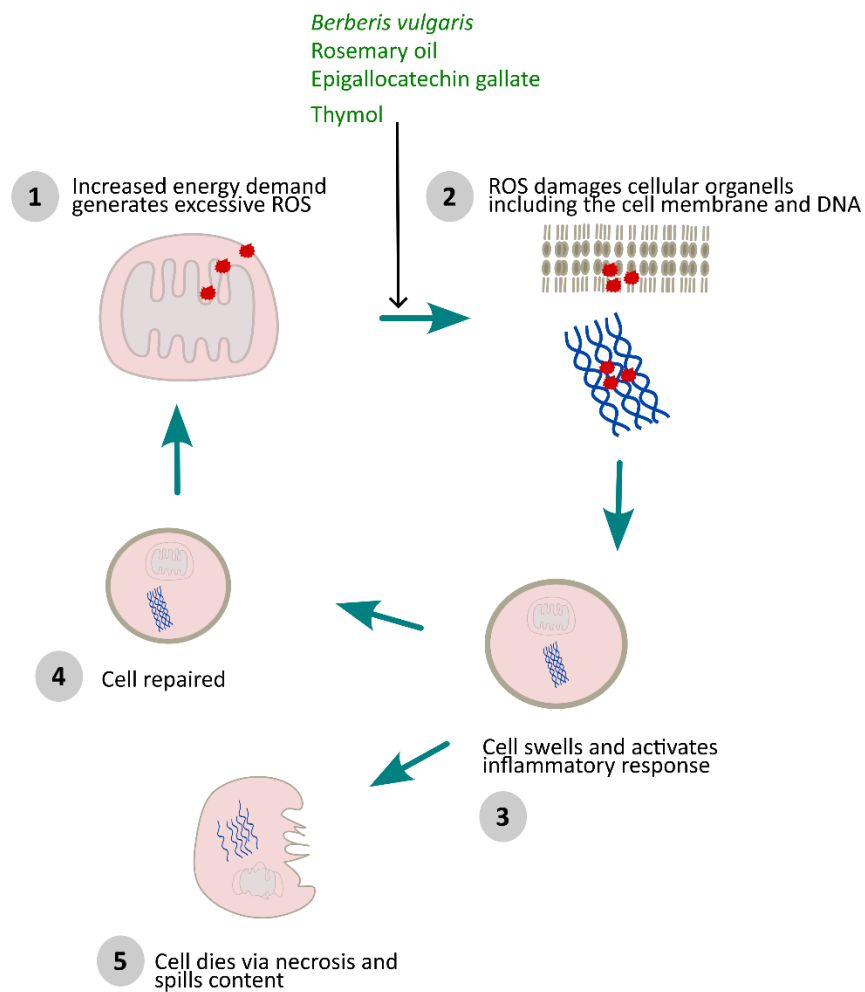
This scoping review found that heat stress affects the meat colour of Japanese quail and causes histological damage to intestines, liver, spleen, kidney, male and female reproductive organs, lungs, and brain. These negative impacts of heat stress on several organs of Japanese quail affect its survival, reproduction, and productive performance.

The decreased villus height and width of intestines induced by heat stress (de Moraes et al., 2021) will create a problem with feed efficiency (Marchini et al., 2016), thus affecting body weight and quality of the product. In addition, reduced Calbindin-D28k in intestines (de Moraes et al., 2021) signifies reduced calcium uptake, which is required for egg production. Furthermore, implications of reduced feed efficiency, electrolyte imbalance from respiratory alkalosis, and reduced intestinal calcium uptake will cause reduced eggshell quality and low egg production. As expected, low egg quality (Akdemir et al., 2019), number of eggs produced, and delay in egg lay were observed in heat-stressed Japanese quail (Mehaisen et al., 2019).

Histologically, liver showed steatosis, bile accumulation, hepatocyte degeneration and necrosis (de Moraes et al., 2021), indicating liver function impairment. Hepatocyte necrosis caused by oxidative stress will cause a widespread inflammatory response causing fibrosis and fat accumulation. In addition, Mohamed et al. (2015) reported kidney tubular cell necrosis; similarly, the kidney is likely to fail. The congestion of blood in lungs might suggest increased shunting of blood to lungs to extract oxygen for metabolism, or it could result from increased respiratory rate. However, degeneration and necrosis of neurons in brain (Mohamed et al., 2015; Shahri, 2020) indicates cell death in organ and can lead to mortality

if stressor is not removed. Furthermore, dead and abnormal sperm cells in testis (Razooqi et al., 2019) and decreased hierarchical follicle number in ovary (Pu et al., 2019a) will affect the reproduction and survival of species.

At molecular level of heat stress, oxidative tissue damage occurs due to excess ROS and depletion of antioxidant enzymes and minerals in blood and tissues (Sahin et al., 2017), as mentioned in the background section of this review. For example, Sahin et al. (2003a) reported increased malondialdehyde (MDA) in serum, heart, kidney and liver tissues and a simultaneous decrease in serum vitamins A, C, E, B12 and folic acid in quails exposed to heat stress (Sahin et al., 2003a). In another study, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were reduced in Japanese quail exposed to heat stress with concurrent increased hepatic MDA (Sahin et al., 2013). Thus, if heat stress continues unabated, it will lead to catastrophic consequences such as low-quality products and increased mortality of Japanese quail. However, on positive side, several strategies are recommended to mitigate heat stress's effect on Japanese quail, such as *Berberis vulgaris*, rosemary oil, *Epigallocatechin gallate* and thymol (**figure 10**).



Key: ● ROS - Reactive oxygen species; DNA - Deoxyribonucleic acid

Figure 10: A diagram of the process of ROS generation and the mechanism of some dietary interventions.

The ROS are generated in the mitochondria, not that the cell can repair and return to normal circle or die. The dietary interventions depicted here act to prevent the damage caused by ROS.

Furthermore, direct effect of heat, especially on the lungs, would require environmental temperature control in addition to dietary supplementation. The reason is that the lungs receive a large volume of direct hot air during exposure because of its involvement in evaporative cooling; hence it will require an additional strategy to cool the air. The downside of microclimate creation to cool the environment is cost, which is a challenge in rural areas.

This scoping review found seven countries where studies were conducted, and Turkey has the highest records. Although several studies can be found from other countries, for objective of this review, few studies were found on morphology of Japanese quail organs. This review found 24 articles directly in tandem with aim, while another 36 articles reported morphological studies but without heat stress exposure. Thus, only 60 articles were identified to report on gross or histological analysis of Japanese quail organ. Studying histopathological changes will elucidate the understanding of underlying physiological and functional derangements caused by heat stress.

For example, histochemical staining of brain will elaborate on the neurons involved in the behavioural changes seen in Japanese quail exposed to heat stress (Soares et al., 2019). But no immunohistochemical study of brain was identified by this scoping review. A detailed ultrastructural study of blood-gas barrier will elucidate the evaporative cooling mechanisms and pathological changes in the lungs of Japanese quail during heat exposure. However, no histological record of the lungs was found in this review, except for lung congestion reported (Mohamed et al., 2015). The statement was unclear whether lung congestion was seen at gross or light microscopy level, and no micrographs were provided. Future experiments could

focus on detailed study of brain, lungs and organs not reported, like pancreas. Furthermore, studies could also focus on cost-effective alternatives to mitigating direct effect of heat stress.

2.5 Limitations of the scoping review

This scoping review was limited to full texts that were written in English language. It is anticipated that valuable information was excluded from one full text written in Brazilian Portuguese (Porto and Fontenele-Neto, 2020). The search query was developed in English, which by default might not identify records in which title, abstract and full text were not in English, as such valuable records might not be identified. In addition, some records might not be indexed in any of the databases and grey literature sources that were searched, hence could be missed. An article was found while searching references of full texts retrieved for second-level screening, which was not identified by database searches. It is recommended that future research should consider employing a language translation for broader coverage of information.

Lastly, findings of this scoping review were mainly gross and light microscopic observations. However, two studies reported immunohistochemical analysis (Turk et al., 2015; de Moraes et al., 2021), but no ultrastructural result was seen in any of the records.

2.6 Conclusions

Heat stress threatened Japanese quail's welfare and survival. Microscopic findings include cellular death (necrosis) in major organs — liver, kidney, brain, testis, ovary, spleen and pituitary gland — affecting normal functioning of the organs. In addition, the review identified

some baseline information which could guide future research related to Japanese quail and heat stress. It means that there is a dearth of microscopic information on effects of heat stress on Japanese quail organs.

3 CHAPTER THREE — MATERIALS & METHODS

3.1 Ethics approval

The experiments and animal handling protocols for this study were approved by animal research ethics committee (AREC) of University of the Witwatersrand. Ethics approval: 2020/10/01/D (Appendix 2).

3.2 Study animals

A total of thirty-eight (38) adult Japanese quails (5 weeks of age) of either sex were acquired from a commercial farm through Wits Research Animal Facility (WRAF). Following an interim approval by the AREC for a pilot study, eight (8) birds were used for pilot study (see appendix 3 for the pilot study report).

After one week of acclimatization, thirty (30) birds were randomly allocated to five (5) groups based on their initial body weight. Thus, there are six (6) birds in each group as follows: control groups (CT1 and CT2), acute heat stress group (AH), and chronic heat stress groups (CH1 and CH2). For the period of the experiment, control groups were maintained at a thermoneutral temperature of 25°C. The AH group were exposed to 38°C for 24 hours and then were euthanised with an intravenous overdose of sodium pentobarbitone (Kyron, South Africa) at 120mg/kg body weight. Chronic heat stress groups were exposed to 35°C. Groups CT1 and CH1 were euthanised after seven days of exposure, while groups CT2 and CH2 were euthanised after 28 days (**figure 11**).

The control group was replicated for 28 days group to account for rapid change in body weight of Japanese quail, which could be a confounding factor for comparison of gross variables such

as body weight, body temperature, and lung size. However, this factor is not anticipated to affect qualitative report of histological and ultrastructural comparison. The birds were provided standard finisher commercial chicken feed and water *ad libitum*. Further, light routine of WRAF 12-hour dark/light cycle was maintained.

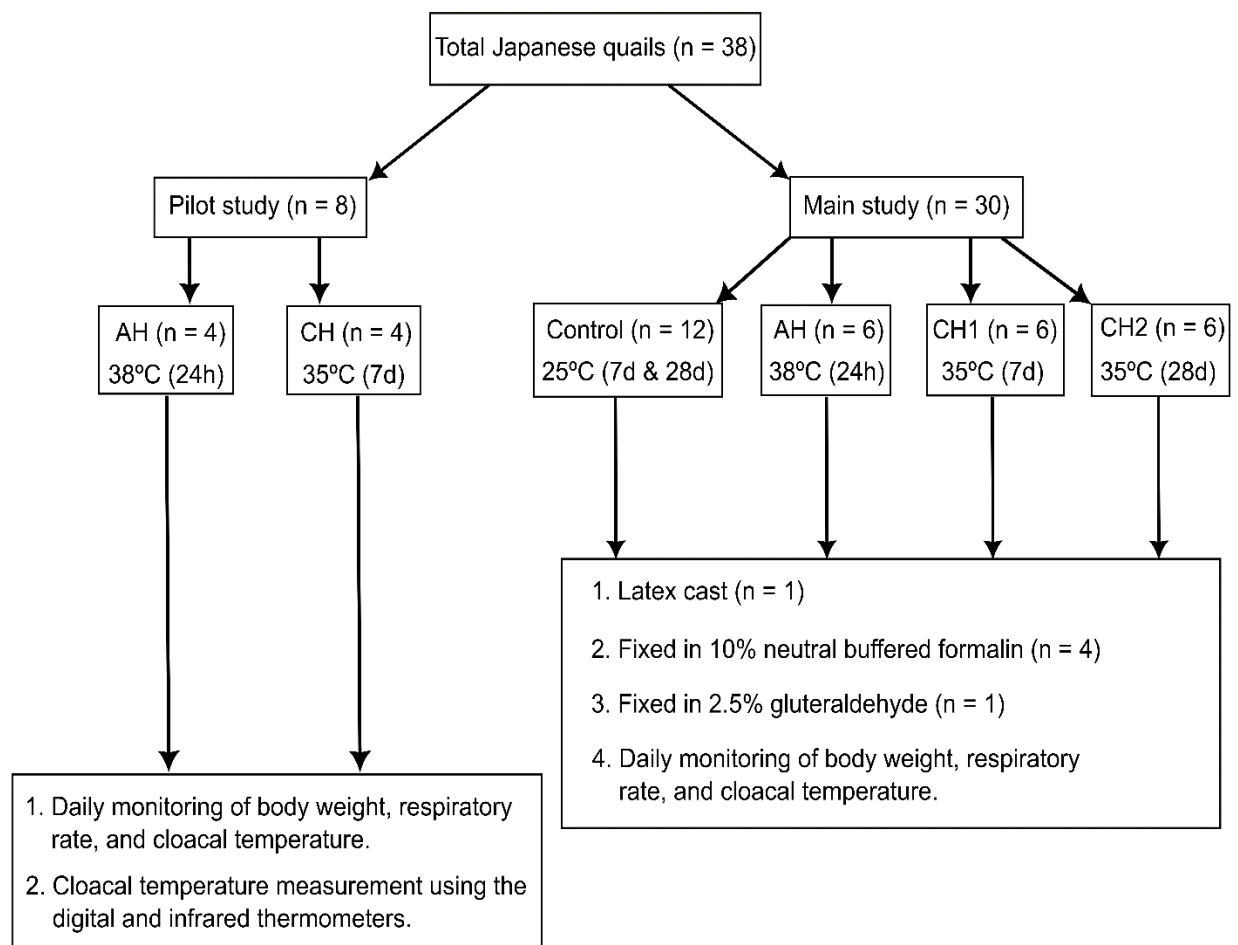


Figure 11: A flow of the animal grouping and experiment procedures.

3.3 Experimental setup

The birds were housed in a perspex cage of 1m x 1m x 0.5m in size (L x W x H), which was divided into compartments with a mesh wire to allow the birds to see each other (**figures 12 & 13**). At the top, it was covered by a wood plank for about a quarter to allow for access, and the remainder was thatched with straw to allow for ventilation while minimising thermal drift. To attain desired temperature exposure, temperature of heating chamber, which is perspex cage, was ramped up using a heater coil and then maintained with an infrared bulb. Individual floor space within the cage was 50 cm x 50 cm with an infrared bulb heat source carefully placed in the middle. These measures ensure that all bird chambers get equal heat distribution and can use the space towards opposite end to escape the heat. The heat source is connected to a programmable thermocouple that can easily switch off the heat once desired temperature level is reached. In addition, there is a thermostat attached to a thermocouple, which can detect a drop in temperature and signal the heat source to be switched on automatically. Appropriate humidity was maintained in the chamber with the aid of wet nestlets. Also, the chamber is equipped with a hygrometer to monitor the humidity level. In addition to the thermocouple, a Rotronic HW4 v.3 data logger (Rotronic AG, Switzerland) was also placed in the chamber to monitor temperature and humidity levels. Respiratory rate, body temperature, body weight, and water consumption were measured daily to monitor health and welfare of the birds throughout heat exposure. Respiratory rate was counted by holding the quail and exposing the left wing while cloacal temperature was measured using digital thermometer as described in the pilot study (**Appendix 3**). Water consumption was measured by estimating the amount of water remaining in the water

container on the following day. However, because of the different temperature level in the holding chambers of the quails, water will evaporate faster in the heat-stressed group and give a false estimate of increased water intake in the group. Although increased water intake was expected in the heat stressed quails, the results were omitted in the results section of this thesis due to uncertainty.

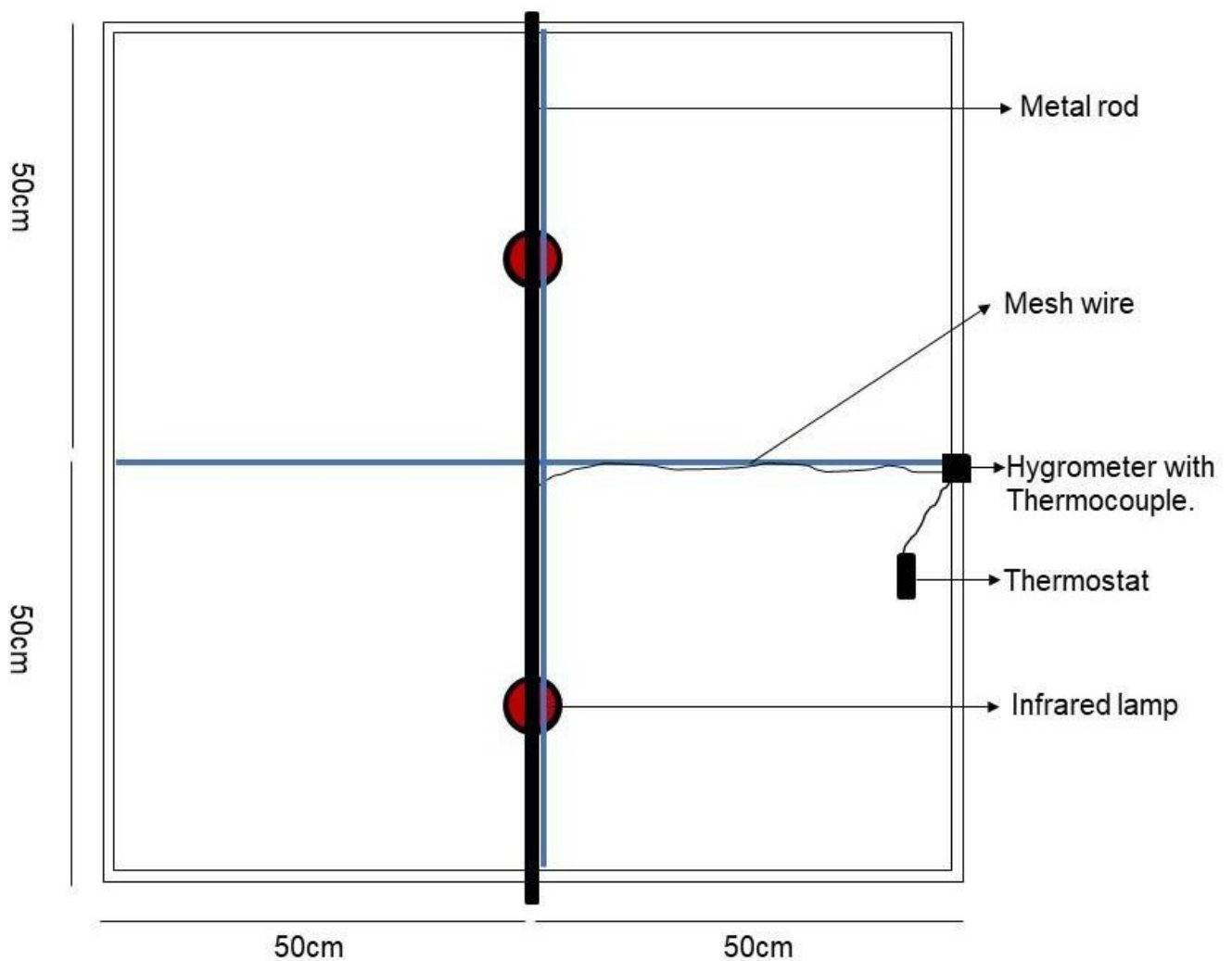


Figure 12: A schematic floor plan of the housing used for the experiment.



Figure 13: The photographs of the cage used for the experiment.

In picture A, the mesh wire used to divide the cage and the quails are seen. In picture B, the roof of the cage with wood plank and straw is seen.

3.4 Terminal procedures

At the end of heat exposure for each group, the birds were euthanised. Their respiratory systems were then intratracheally instilled with fixative or latex (to preserve lung tissue before dissection) and then dissected out from birds through their thoracic cavities.

3.5 Latex casting

The latex (33% latex, 66% water, and 1% ammonia) casting procedure involves intratracheal instillation of water-soluble liquid latex (NJC Adhesive and Sealant Distributors, Industria West, South Africa) using an apparatus which has two independent closure valves (**figure 14**). One valve is for air aspiration, which is used to aspirate air from respiratory system to ensure that the liquid latex occupies the whole tissue space for better results. The second valve was used to inject liquid latex into the tissue. The birds were kept in a freezer for 24 hours to allow latex to solidify (Reddy, 2013). However, for reasons beyond our control, we could not achieve the desired outcome for all treatments. Only casts for CH2 and CT2 were retrieved successfully, which is not enough for comparison across all groups, hence intended analyses from the latex cast were deliberately omitted from the results section of this thesis.



Figure 14: A picture of the latex cast apparatus.

The valves are indicated with letters A and B. First, an empty syringe for air aspiration will be attached to valve position A closest to the insertion of the cannula. Next, another syringe containing the liquid latex will be attached to valve position B. With valve B closed and valve A opened, air will repeatedly be aspirated as much as possible. Then with valve, A closed and valve B opened, liquid latex will be injected into the lungs until there is a backflow of the liquid latex and an increased resistance in the syringe plunger.

For instillation of fixatives, a cannula was attached to a tube and connected to a funnel. The cannula was inserted into an incision made at the level of syrinx and fixative was poured from top at about 30cm water pressure to fill the lungs and air sacs then trachea was ligated. Post ligation of trachea, tissue was allowed to rest for at least five (5) minutes (to allow for fixative infiltration to begin) and then dissected out of the bird and immersed into same fixative that was instilled. All attempts to preserve air sacs were unsuccessful due to their fragility. After at least a month of being immersed in fixative, the lung was removed and cleared of surrounding tissue and volume was measured using Scherle's Method. Subsequently, tracheal

ligature was released to clear all fixatives in the lungs, then size and weight of the lungs were measured.

The lungs were weighed individually using Radwag AS 220.R2 plus scale (Radwag, Poland). This was done 3 times and an average of left and right lungs were taken for each bird ($n = 5$). Similarly, lung size was estimated using a digital vernier calliper by measuring and taking the product of length, width, and thickness (**figure 15**).

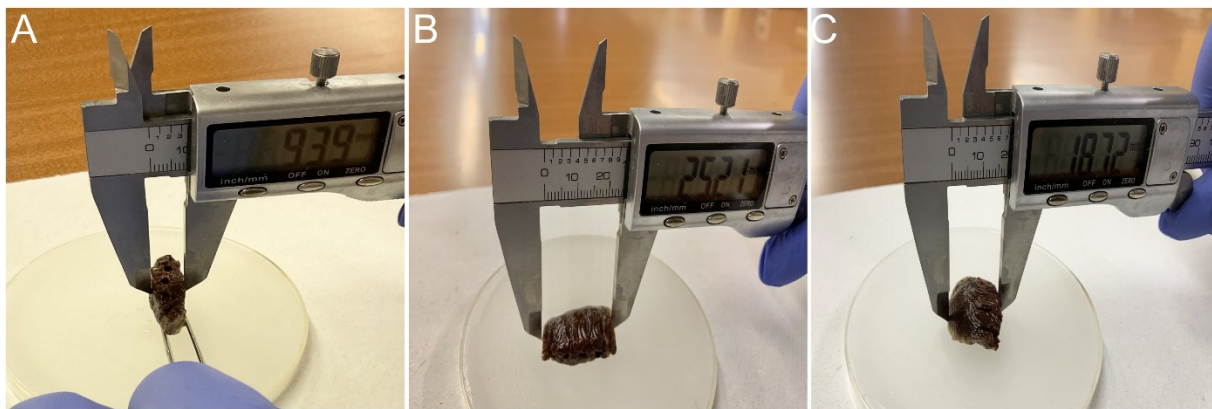


Figure 15: The photographs of the method used to estimate the lung size using a vernier calliper.

3.6 Lung volume estimation using Scherle's method

Volume estimation was done using Scherle's Method based on Archimedes principle (Scherle, 1970). An individual lung filled with a fixative was carefully ligated at the extrapulmonary bronchus to prevent leakage. With this, all air spaces are filled, and lungs' structural integrity is maintained. A beaker with a fixative in it was placed on a weighing scale and calibrated to

0. A coil attached to a string was used to keep lung tissue in the middle of beaker to avoid unnecessary movement. The weight of the coil and string was also considered in the calibration. The volume of lungs is the weight of volume displaced. The diagram in **figure 16** is representation of general principle of Scherle's method, a representative picture of the procedure used in this study is shown in **figure 17**. The volume of each lung was measured repeatedly thrice, and then the average was calculated.

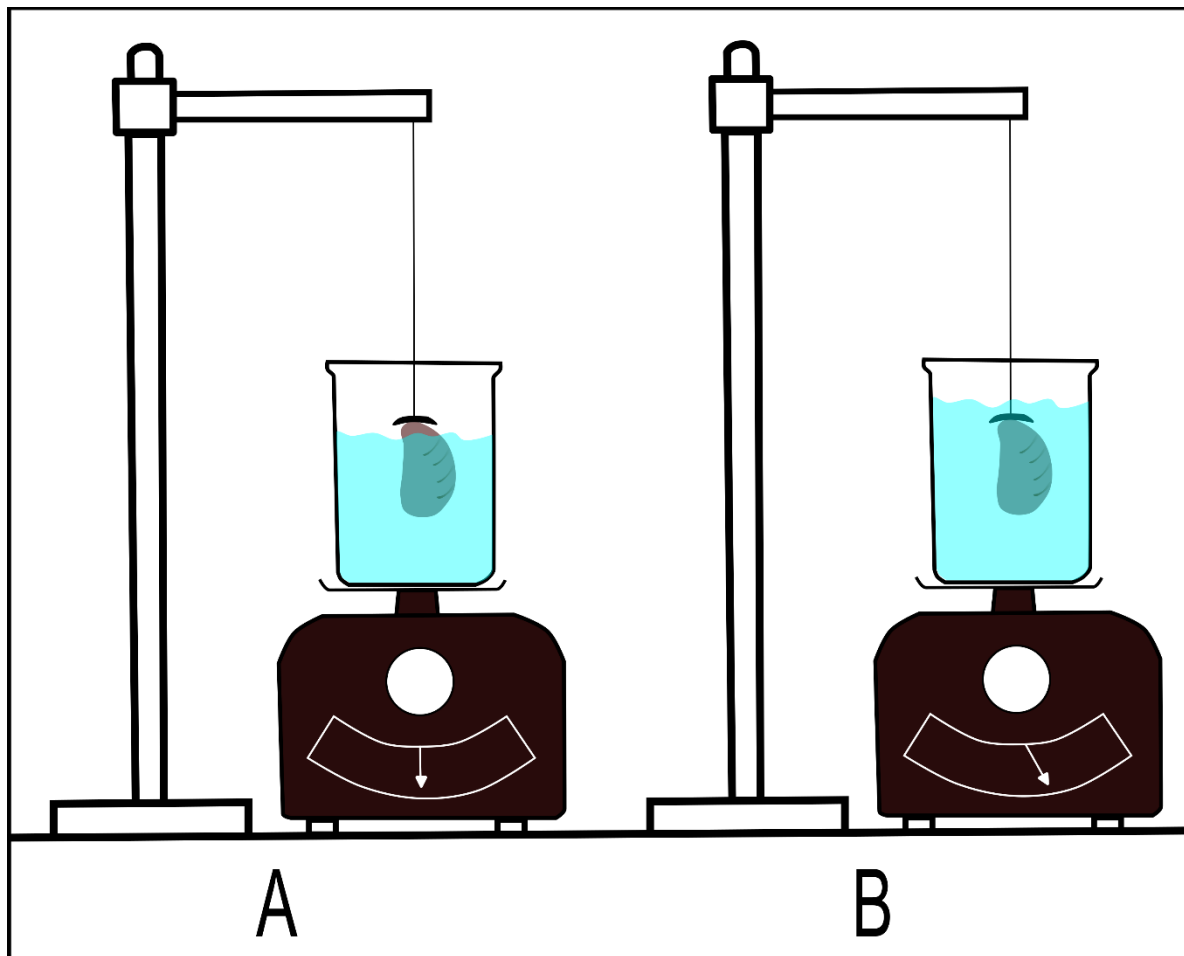


Figure 16: A diagram showing Scherle's method of lung volume measurement.

(A) The calibration procedure where the weight of the beaker, the immersion fixative and the coil with the string used to submerge the lung pair were tared to 0. (B) Shows the weight displacement (volume of the lung pair) following the immersion of the lung pair into the fixative.

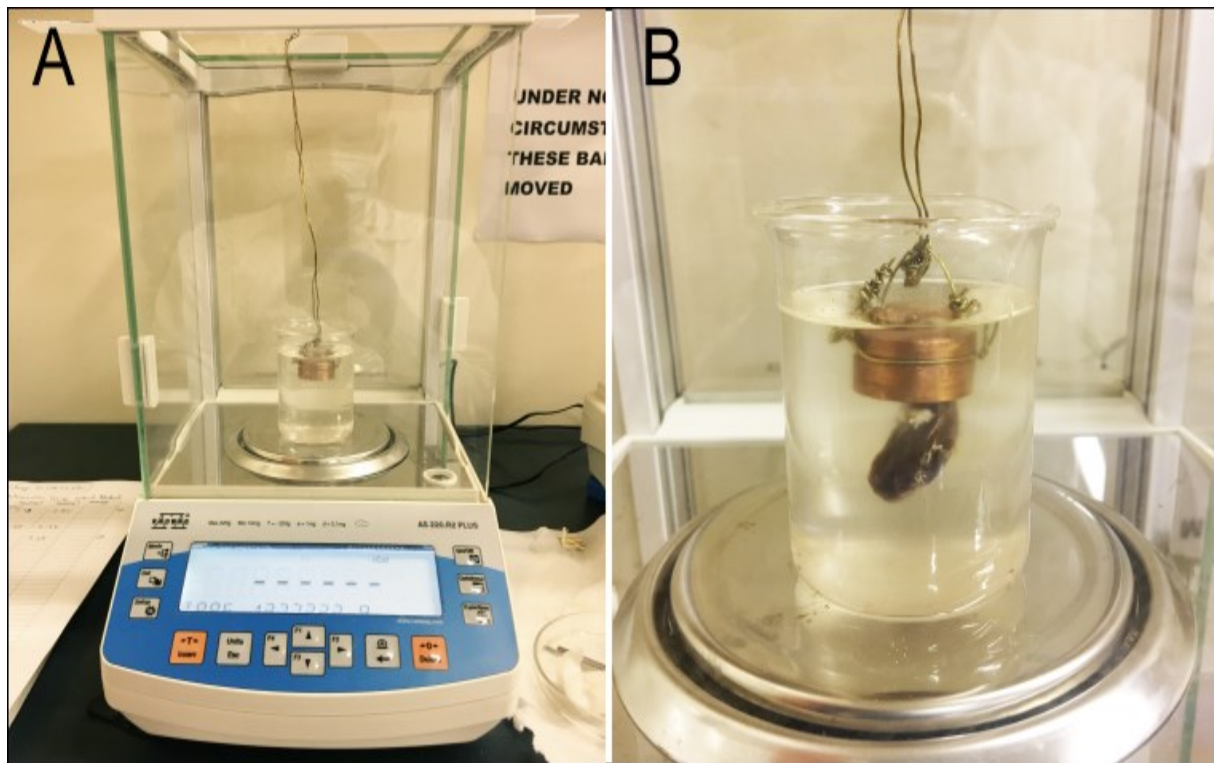


Figure 17: The photographs of modified Scherle's method used in lung volume estimation.

Picture A shows the setup before the measurements while picture B shows the quail lung in situ during volume estimation.

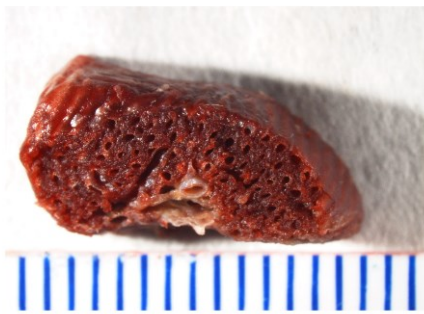
3.7 Sampling

The lungs fixed in 10% neutral buffered formalin were sectioned along costal sulci, and then two alternate blocks were selected for processing (**figure 18**). The selected tissue blocks were processed using Thermo Fisher Shandon Citadel 1000 automatic tissue processor (Shandon, England). Subsequently, 5µm serial sections were cut using rotary microtome Leica RM2125 RTS (Leica Biosystems, Germany). Sections for haematoxylin and eosin (H&E), new pentachrome, and Gomori's one-step trichrome stains were picked on silane-coated slides. While sections for immunofluorescence labelling were picked on *Histobond+* slides

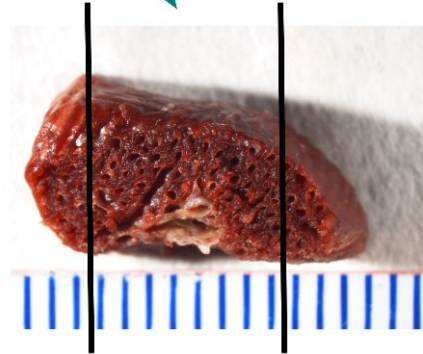
(Marienfeld, Germany). The serial sections were distributed by picking two sections per slide and allocated to a stain until 10 sections were allocated per stain from each tissue block. For SEM, left lung of pair fixed in 2.5% glutaraldehyde was removed and dissected along costal sulci and then one of four tissue blocks is selected (**figure 18**).



A lung cut along the costal sulci as the first step of the sampling process.



A block of tissue to be processed for light microscopy or electron microscopy.



The edges are trimmed to make a rectangular block for SEM processing and viewing.

Figure 18: The sampling processes for light and scanning electron microscopy analyses.

The sampling for paraffin embedding and step 3 shows further trimming necessary for SEM processing.

3.8 Light microscopic procedures

3.8.1 Mayer's H&E staining

The selected sections for Mayer's H&E staining (Bancroft and Layton, 2019) were dewaxed and rehydrated, then immersed into Mayer's haematoxylin for five (5) minutes. The tissue sections were washed in running tap water for five (5) minutes, then dipped thrice into 1% acid alcohol. Then again washed in running tap water and then immersed in scotch tap water for two (2) minutes. The sections were washed again in tap water and immersed in eosin for one (1) minute. Finally, slides were washed in running tap water, dehydrated and coverslipped with entellan.

3.8.2 The new pentachrome staining

The sections selected for new pentachrome staining method (Doello, 2014) were dewaxed and rehydrated, then pre-treated in 6% nitric acid for five (5) minutes. The tissue sections were washed in distilled water and immersed serially in 0.5% toluidine blue and picrosirius solution each for five (5) minutes and a wash with distilled water in between. Finally, tissue sections were dehydrated and coverslipped with entellan.

3.8.3 Gomori's one-step trichrome staining

The sections selected for Gomori's one-step trichrome staining (Gomori, 1950) were dewaxed and rehydrated, then rinsed in distilled water. The tissue sections were immersed in Bouin's solution for one (1) hour at 56°C to mordant. Subsequently, tissue sections were removed from oven and allowed to cool to room temperature and then washed in running water until

yellow colour of Bouin's disappeared. The tissue sections were rinsed in distilled water and then immersed in Wiegert's haematoxylin for ten (10) minutes. Again, tissue sections were washed in running water for ten (10) minutes and then immersed in Gomori's one-step stain for twenty (20) minutes. Finally, a differentiation step was done by immersing tissue sections into 1% acetic acid for one minute and then were dehydrated and coverslipped with entellan.

3.9 Immunofluorescence labelling procedures

3.9.1 Optimisation of antibodies

The selected tissue sections were triple labelled with anti- α -smooth muscle actin (SMA) antibody (ab150301, Abcam UK), anti-collagen 1 (COL1) antibody (ab88147, Abcam UK), and 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI). The SMA was specified to react with the chicken, hence, most probably to work with the Japanese quail tissues. However, the COL1 was unspecified, so the optimisation for COL1 was both for reactivity and dilution factor.

For optimisation procedures, immunohistochemistry (IHC) using diaminobenzidine (DAB) chromogen (Sigma Aldrich, Germany) was used to elicit reactivity and determination of appropriate dilution factor (**figure 19**). Sections selected for IHC step were dewaxed, rehydrated, and washed in running tap water. Then sections were immersed in citrate buffer pH 6.0 and placed in a water bath at 60°C overnight for antigen retrieval. The following morning, sections were removed from water bath and allowed to cool, then rinsed in phosphate-buffered saline (PBS). A circle was drawn around sections with an ImmEdge® Pen (Vector Labs, USA) to restrict solutions overflow. Then, endogenous peroxidases were blocked using 1% hydrogen peroxide in methanol to cover the sections for thirty (30) minutes.

Subsequently, sections were washed in PBS and covered with 5% normal horse serum from Vectastain® Elite ABC Universal Kit PK-6200 (Vector Labs, USA) for another thirty (30) minutes. The sections were then drained of normal horse serum and covered with primary antibody overnight at 4°C except for negative control sections which were covered with PBS. For SMA; 1:200, 1:400 and 1:500 dilution factors were mixed while for COL1; 1:100, 1:200 and 1:300 dilutions were mixed up.

The following morning, sections were washed in PBS and incubated with secondary Biotinylated Horse Anti-Mouse/Rabbit antibody (1:1000) (Vector Labs, USA) and ABC (solution of equal amounts of Biotinylated HRP and Avidin, Vector Labs, USA) each for thirty (30) minutes and PBS wash in between. The sections were incubated with DAB solution for five (5) minutes, washed in running tap water and counterstained with Mayer's haematoxylin for one minute. Finally, sections were washed in running tap water and coverslipped with entellan mounting medium (Merck, Germany) until viewing.

Following analyses of sections under the microscope and comparing them with negative sections, 1:300 dilution was chosen for COL1 and 1:500 for SMA.

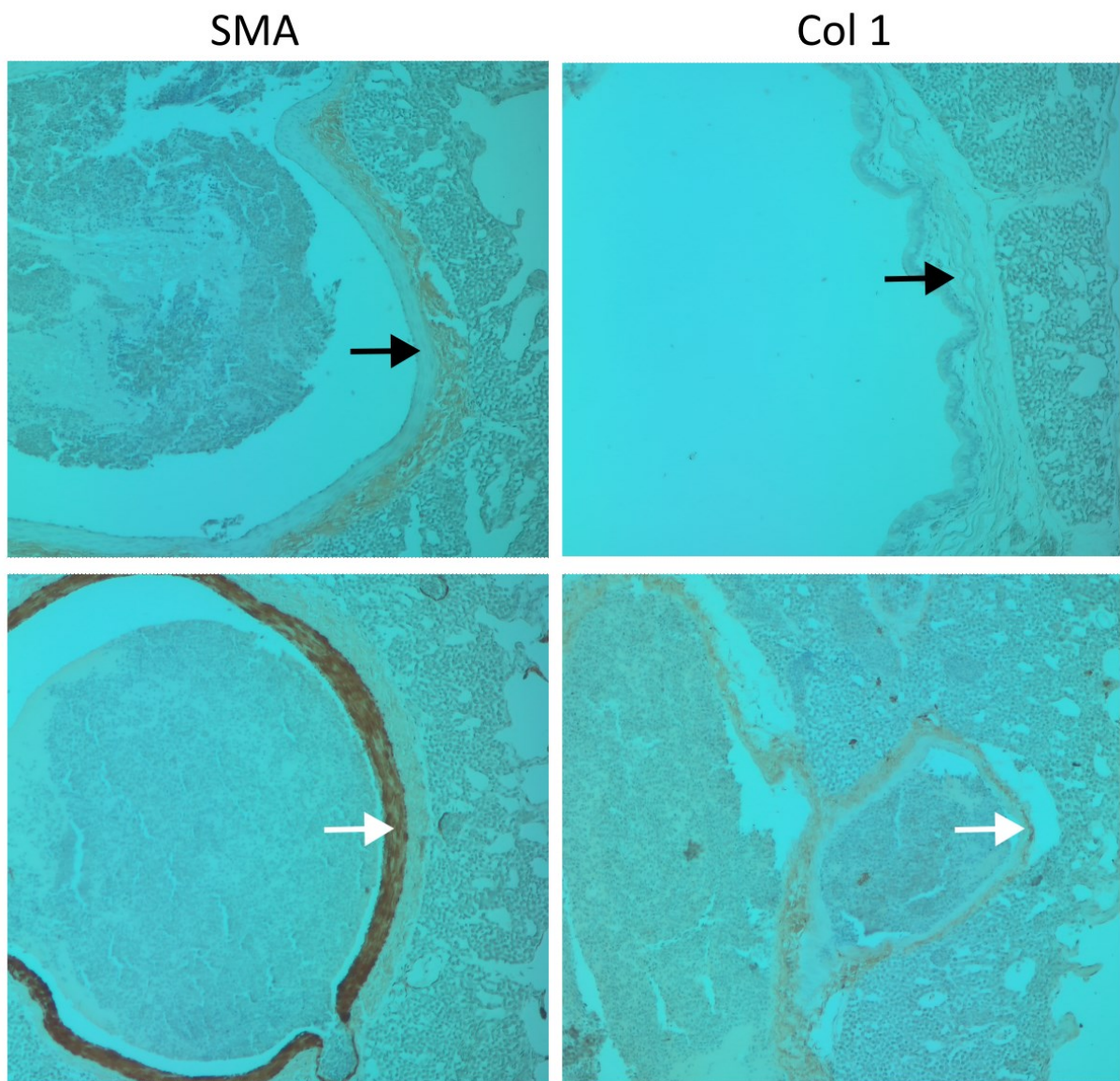


Figure 19: The representative micrographs of the optimisation procedures for α -SMA and Col1 antibodies.

In the negative control micrographs, black arrow indicates the areas of smooth muscle and collagen fibres without antibodies uptake. In the positive control white arrows indicate areas of antibodies uptake at the appropriate dilution factors. Scale bar = 50μm.

3.9.2 Immunofluorescence triple labelling

The sections selected for triple labelling were dewaxed and rehydrated and antigen retrieval was done at 50°C. The following morning, the section slides were removed from the water bath, allowed to cool down and then washed in PBS. Subsequently, a circle is drawn around sections with ImmEdge® Pen and then were covered with 10% normal goat serum for one (1) hour and then incubated with anti- α -SMA antibody (ab150301, Abcam, UK) overnight at 4°C. The following morning, sections were washed in PBS and while working in dark environment, sections were incubated with goat anti-rabbit Alexa fluor 488 (Life Technologies, NL) (1:500) for one (1) hour. Subsequently, sections were incubated with anti-COL1 (ab88147, Abcam, UK) antibody (1:300) overnight at 4°C. Finally, on the day that follows, sections were incubated with goat anti-mouse Alexa fluor 594 (Life Technologies, NL) (1:500) for one (1) hour, followed by 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, NL) (1:8000) for five (5) minutes with PBS wash in between. The sections were coverslipped with fluoromount (Sigma Aldrich, USA) and kept at 4°C until viewing.

3.10 Scanning electron microscopy procedures

The sampled tissue blocks were washed in 0.1M sodium phosphate buffer and then rehydrated through graded alcohol series (100%, 95% and 70%) and then incubated in 1% osmium tetroxide for thirty (30) minutes. After that tissue blocks were incubated in 1% thiocarbazine (Sigma Aldrich, Germany) for ten (10) minutes followed by 1% osmium tetroxide (SPI, USA) for thirty (30) minutes with 0.1M sodium phosphate buffer wash in between. Subsequently, tissue blocks were washed in 0.1M sodium phosphate buffer,

dehydrated in alcohol series (70%, 95%, 100%) and immersed in 100% hexamethyldisilazane (Sigma Aldrich, Germany) for one (1) minute. Finally, tissue blocks were mounted onto aluminium stubs using carbon tape and placed in a desiccator to dry over twenty-four (24) hours. The mounted tissue blocks were coated with 10nm of carbon using an Emitec K950x (Quorum, UK) carbon evaporator and then sputter coated with 5nm of gold palladium using a Emitec K550x sputter coater. They were then returned to desiccator and only removed until viewing them under the SEM (Reddy, 2013).

3.11 Microscopy

Micrographs of tissue sections were taken on an Olympus BX 63 optical fluorescence microscope (Olympus, Japan) fitted with an Olympus DP80 digital camera (Olympus, Japan) using Olyvia CellSens v1.12 imaging software (Olympus, Japan).

Scanning electron micrographs were taken using Tescan Vega scanning electron microscope (TESCAN, Czech Republic) using secondary electron detector.

3.12 Morphometry

The cell area, nuclei area and nuclei mean grey intensity was measured using Fiji version 1.53t image analysis software (Schindelin et al., 2012). Cell area on imported H&E images taken at 1000x magnification was measured using freehand tool after the scale was set properly. Then image is converted to 8-bit grayscale and mean grey intensity was measured by tracing around nuclei using the freehand tool. Both measurements were saved on separate sheets

and measurements were repeated. For all parameters, ten cells and nuclei were measured, the measurements were repeated twice, and mean was taken.

3.13 Statistical analyses

Data from this study were collected and cleaned in Microsoft Excel 365 (Microsoft, USA). GraphPad Prism 8 (GraphPad Software, USA) was used for statistical analyses at a 95% confidence interval. Two-way ANOVA (95% CI) was used to compare results of initial measurements of body weight, cloacal temperature, and respiratory rate to final measurements. One-way ANOVA (95% CI) was used to compare results of lung parameters and other morphometric parameters. Data were assessed for normality using Shapiro-Wilk test and all non-parametric results were compared with Kruskal-Wallis test.

4 CHAPTER FOUR — RESULTS

4.1 Body vitals measurements

The results of body weight, temperature and respiratory rate were not statistically different in heat exposure groups – AH, CH1 and CH2 – compared with control group ($p = 0.2873$). However, 24-hour heat exposure in the AH group reduces mean body weight and raises body temperature by 0.5°C . The respiratory rate (breaths/min) slightly increases like control group (**table 11**). The CH1 group maintained a higher body weight compared with control group throughout the seven (7) days of heat exposure. Similarly, body temperature remained higher in CH1 group, but respiration fluctuated compared with control group (**figure 20**). The mean body weight of CH2 group was lower than control group during the initial 14 days, then fluctuated to the end. The CH2 group had a higher mean body temperature in the initial five (5) days, then fluctuated afterwards, while respiration fluctuated throughout the 28 days (**figure 21**).

Table 11: The table of vital parameters of Japanese quails in AH and control group.

The results are shown as mean±SD. No significant statistical difference was detected using the Two-way ANOVA ($p = 0.2873$).

Time	Body weight (g)		Body temperature (°C)		Respiratory rate (breath/min)	
	0 hour	24 hours	0 hour	24 hours	0 hour	24 hours
Control	223.1±20.4	222.8±20.9	41.9±0.3	42.0±0.4	102±16	103±20
AH	223.8±27	218.5±25.1	42.0±0.6	42.5±0.4	96±4	98±15

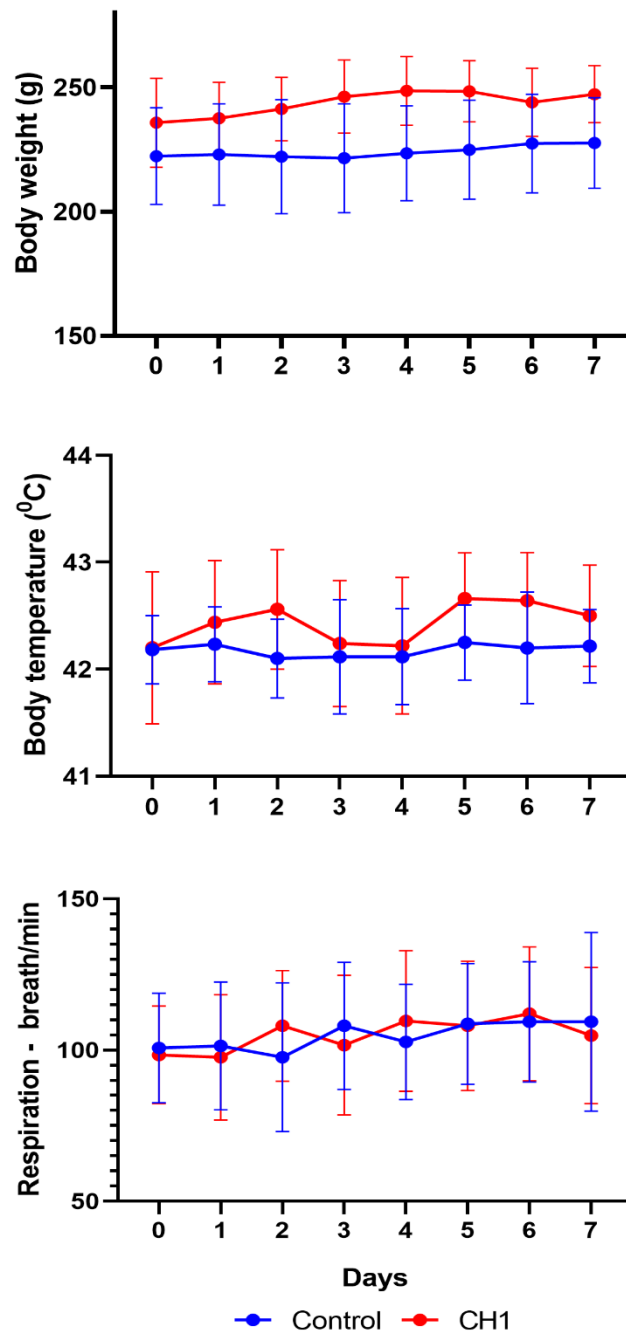


Figure 20: The line graphs of vital parameters of Japanese quails in CH1 and control group over seven days.

The error bar indicates standard deviation. No significant statistical difference was detected using Two-way ANOVA ($p = 0.8630$).

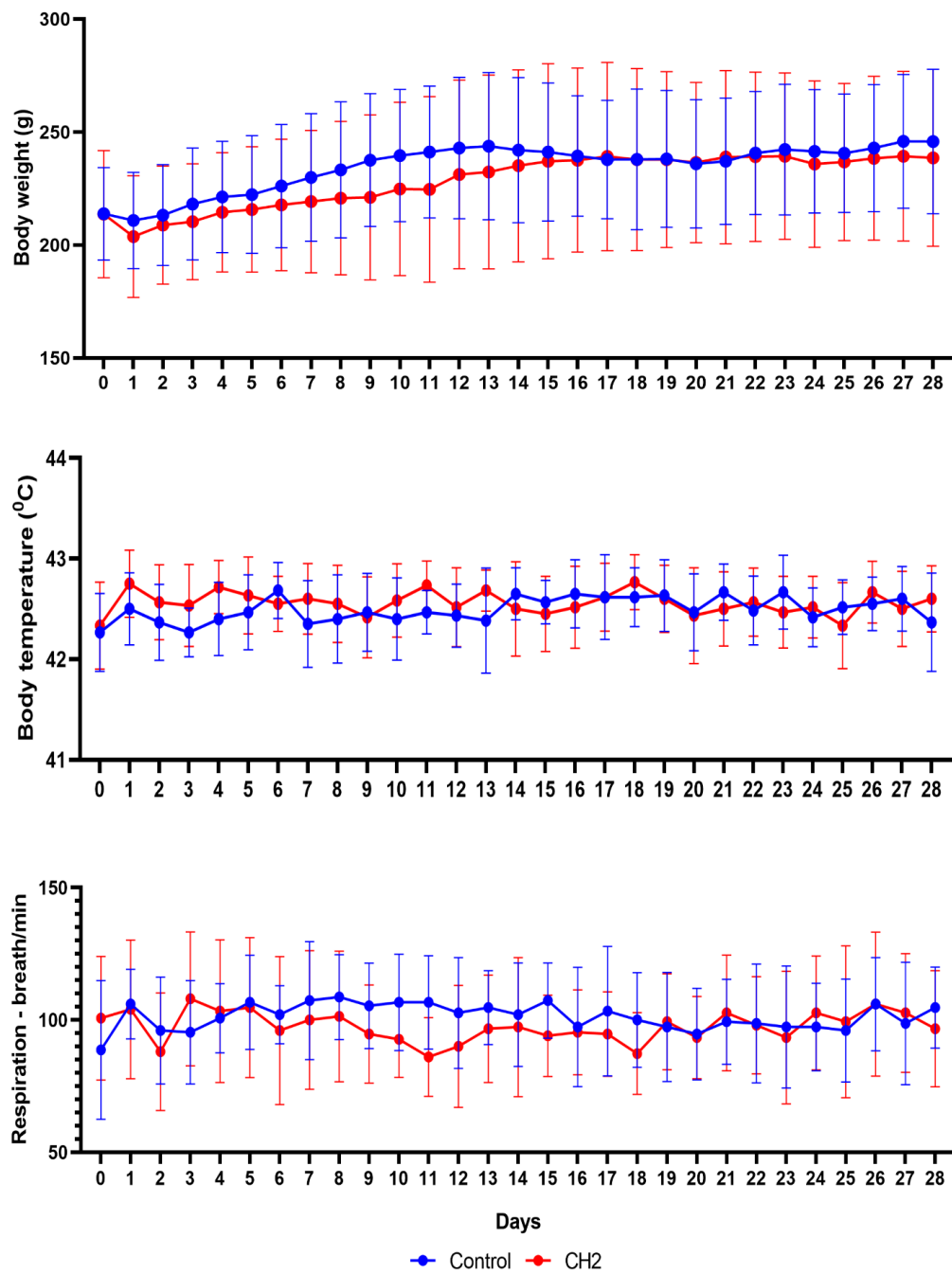


Figure 21: The line graphs of vital parameters of Japanese quails in CH2 and control groups over 28 days.

The error bar indicates the standard deviation. No significant statistical difference was detected using Two-way ANOVA ($p = >0.9999$).

4.2 Lung parameters

Table 12: The table of the gross measurements on the Japanese quail lungs.

The results are shown in mean±SD. No significant statistical difference.

	CT	AH	CH1	CT2	CH2	One-way ANOVA
Lung weight (g)	4.7±0.43	4.0±0.94	4.4±0.39	4.1±0.49	4.3±0.36	P = 0.2936
						F (4, 20) = 1.3
Lung volume	2.52±0.16	2.18±0.3	2.47±0.29	2.41±0.23	2.52±0.085	P = 0.6720
(cm ³)						KW Statistics = 2.678
Lung size (cm ³)	3866±298	3516±801	3562±590	3398±654	3631±207	P = 0.7438
						F (4, 20) = 0.49

The mean lung weight, lung volume and lung size were not statistically different in heat stress groups – AH, CH1 and CH2 – compared with the control (**table 12**). However, AH and CH1 groups had lower mean lung weight, volume and size compared with CT1. In contrast, CH2 had higher values compared with CT2.

4.3 Light microscopy analyses

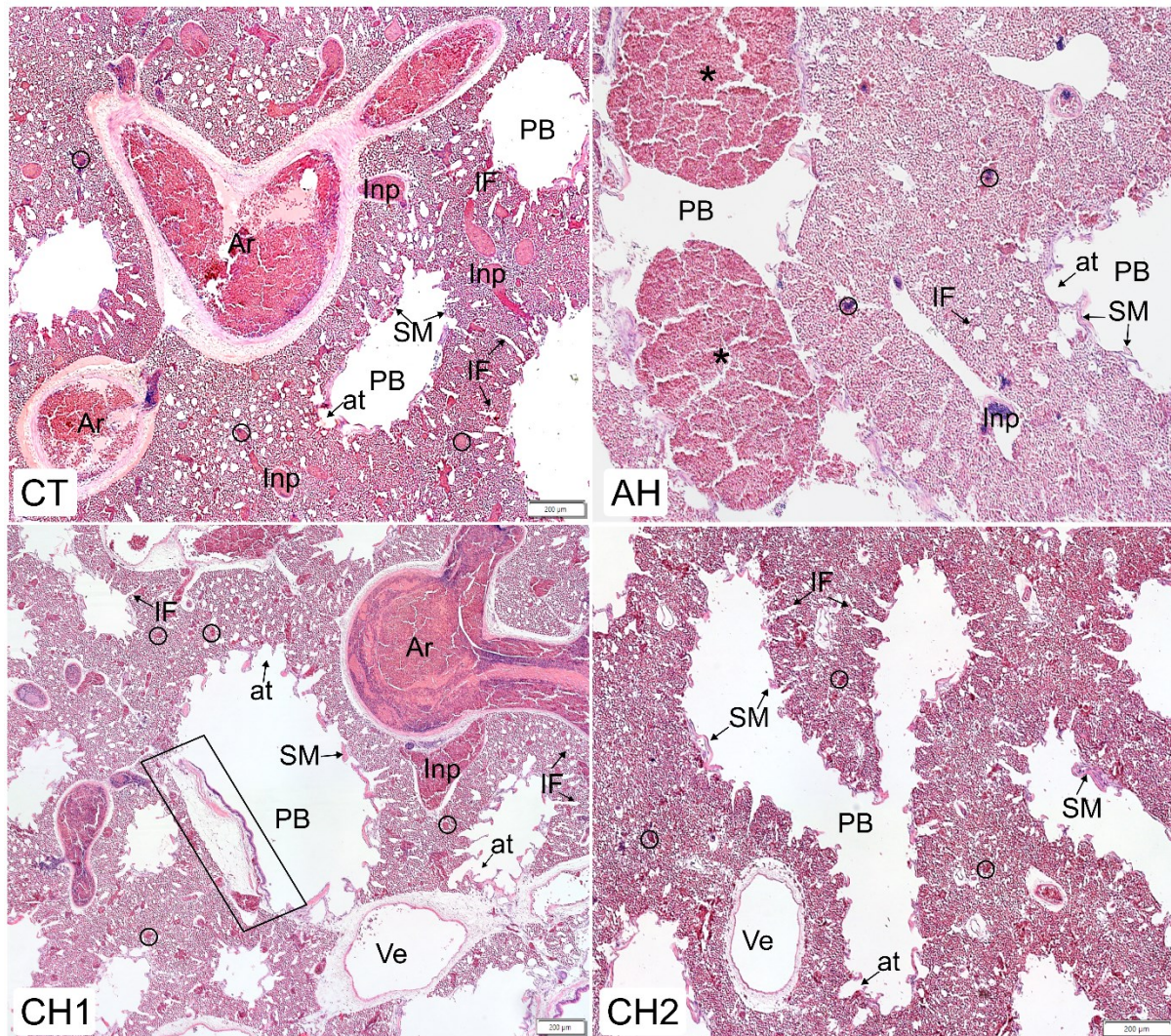


Figure 22: The representative micrographs of the H&E-stained Japanese quail lungs of the control and heat-stress groups.

Asterisks indicate coagulated blood within the parabronchial lumen (PB). Ar — major artery branch; Ve — major venous branch; SM — smooth muscle; at — atria; IF — infundibulae; Inp — inter-parabronchial blood vessel; black circles — intra-parabronchial blood vessel. The rectangle indicates an area of intersection between secondary bronchi and parabronchi. Note that the blood capillaries, air capillaries and the blood-gas barrier are not distinguishable at this magnification. Scale bar = 200 μ m.

In the representative H&E-stained micrograph (**figure 22**) of Japanese quail lungs, parabronchial wall forms exchange tissue mantle. Embedded within the exchange tissue mantle are inter-parabronchial and intra-parabronchial blood vessels which eventually branched into blood capillaries. The exchange tissue mantle separates parabronchial lumen from major blood vessels. Within the wall of parabronchi, atria are seen as the opening into airway, which is the primary entrance into exchange tissue mantle. The atria are guarded by smooth muscle. The infundibulae arise from atria, leading deeper into exchange tissue mantle, and branch into air capillaries, intermingling with blood capillaries at blood-gas barrier for gas exchange. In the control group, blood within the vessels contains more eosinophilic-stained cells indicating a predominance of erythrocytes. In AH group, blood contains more basophilic-stained cells, but more remarkable is presence of a significant amount of coagulated blood within parabronchial airway. In addition, there are tissue breakages within the parenchyma of the AH group, which might be from processing or the direct effect of heat. In CH1 and CH2 groups, blood within arterioles showed more basophilic-stained cells than the control.

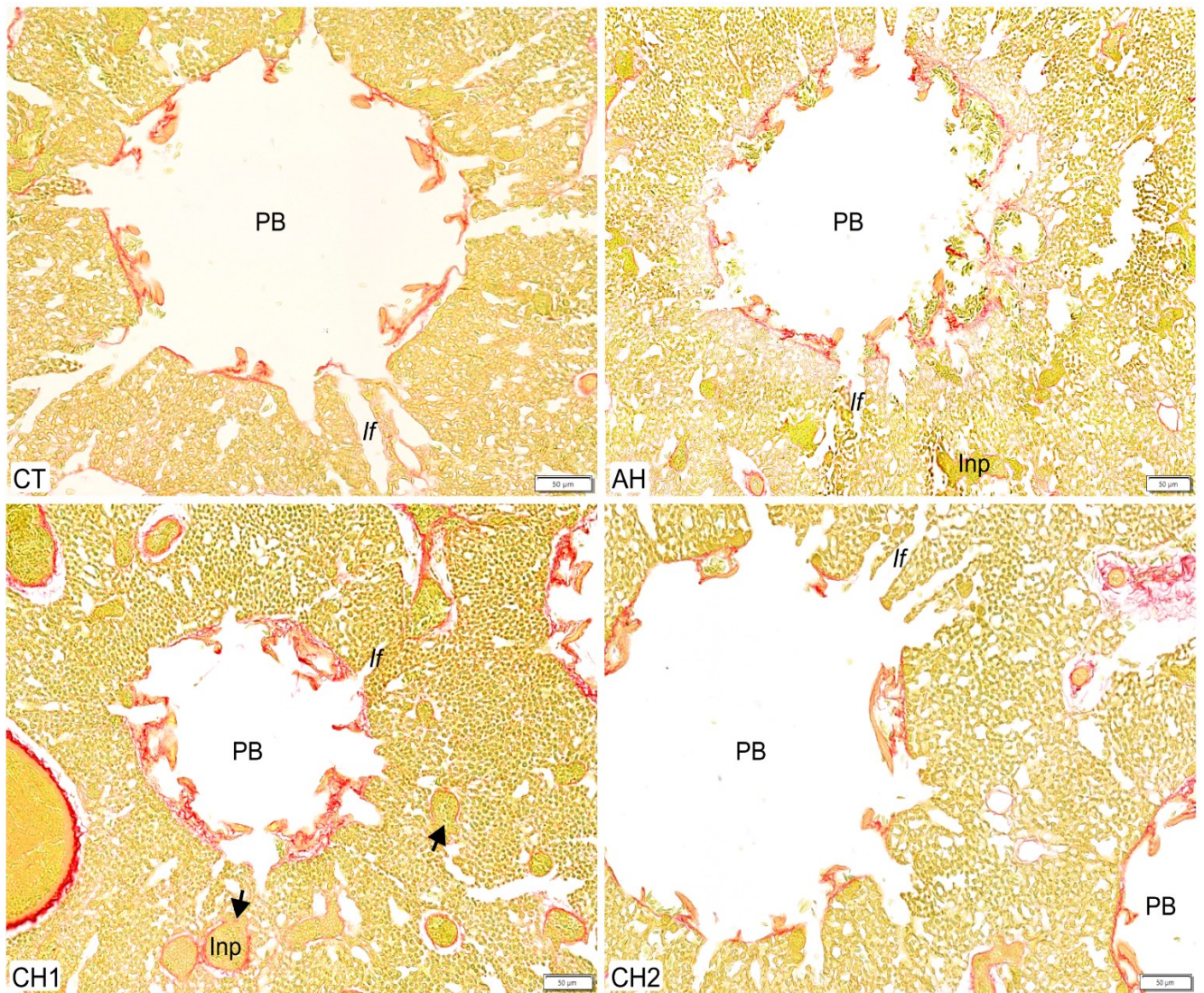


Figure 23: The representative micrographs of new pentachrome-stained Japanese quail lungs of control and heat-stressed groups.

Note the ruptured blood vessel in CH1 group indicated with black arrow. The other blood vessel shows blobs but has not ruptured yet. In addition, there are erythrocytes within the airway in control group. The collagen fibres show an irregular pattern in AH and CH1 groups. PB — Parabronchial lumen; If — Infundibulae; Inp — Interparabronchial artery. Scale bar = 50μm.

In new pentachrome-stained Japanese quail lungs (**figure 23**), there are ruptured blood vessels and increased deposition of collagen fibres in CH1 group. The arrangement of collagen fibres in AH and CH1 was irregular compared with the nicely arranged collagen fibres in CT and CH2, especially at the base of interatrial septa.

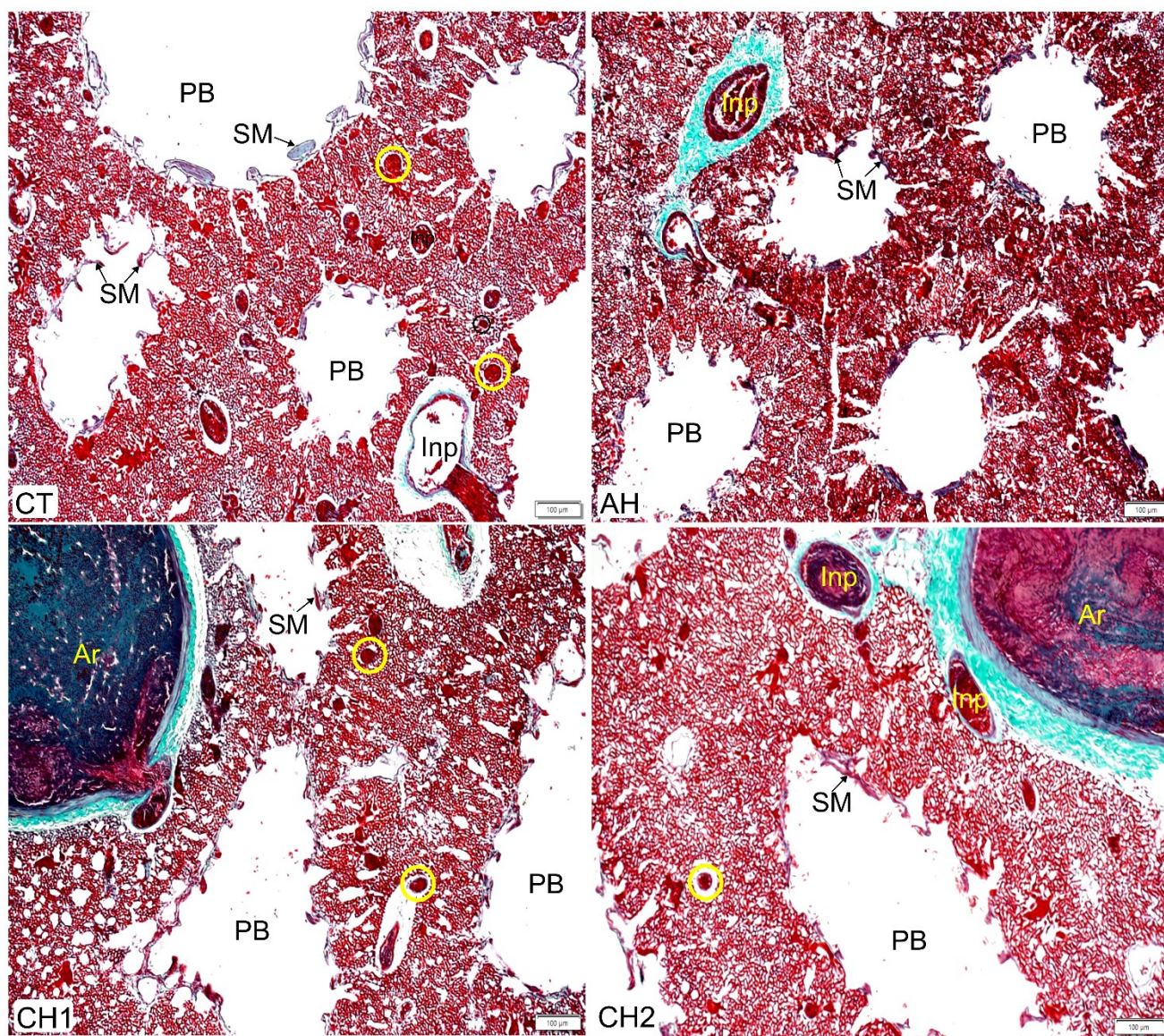


Figure 24: The representative micrographs of Gomori's trichrome stained Japanese quail lung of control and heat-stressed groups (AH, CH1 and CH2).

PB — the parabronchial lumen; SM — smooth muscle; Ar — Artery; Inp — Interparabronchial vessels; Circled — Intrapara-bronchial artery. Scale bar = 100μm.

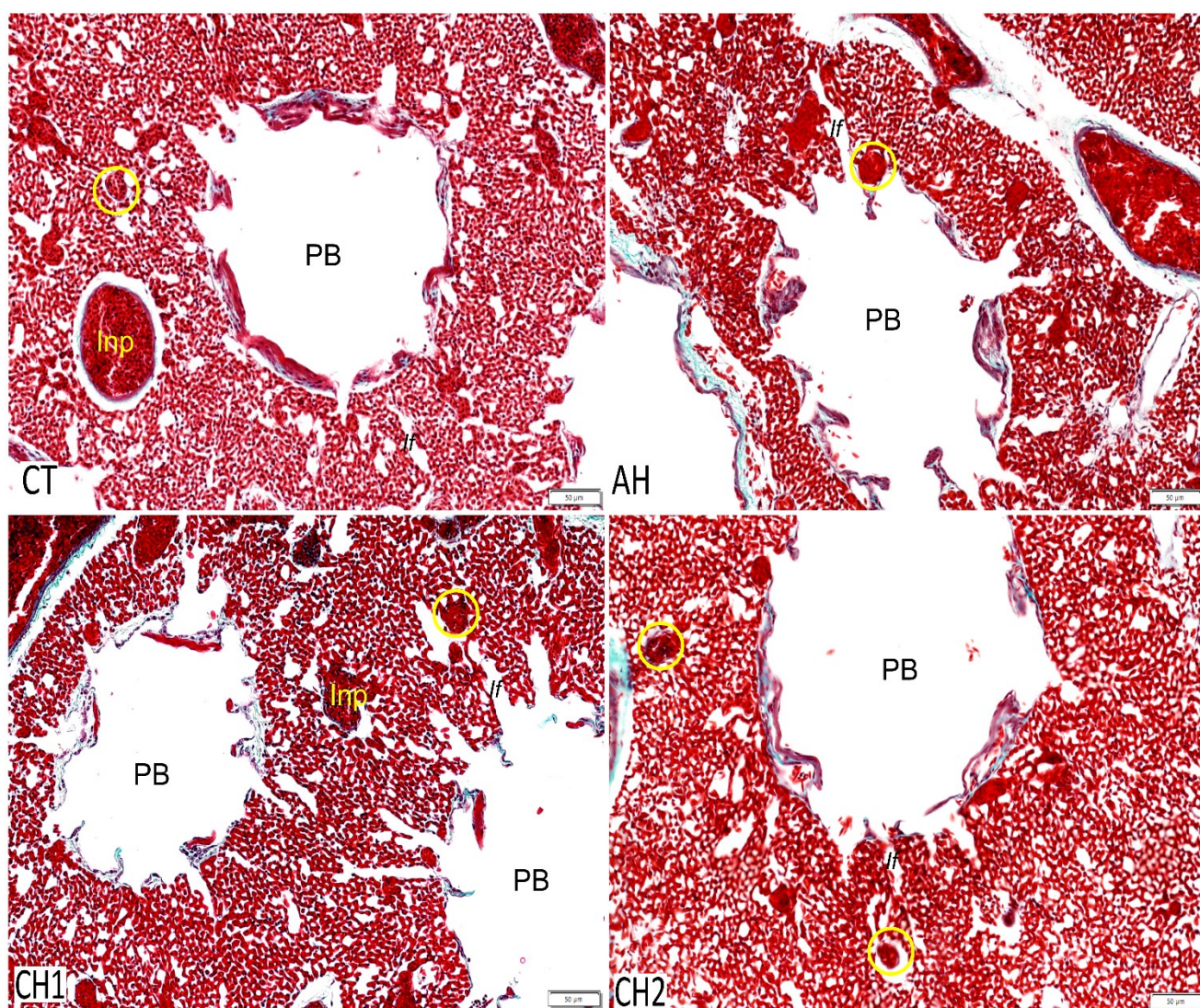


Figure 25: The representative micrographs of Gomori's trichrome stained Japanese quail lung of control and heat-stressed groups.

Note the blood congestion around the parabronchial lumen (PB) in the AH, CH1 and CH2 groups compared with the control. In addition, the smooth muscles of the interatrial septa show thinning in the CH1 group. Imp — Interparabronchial artery; Circled — intrapara-bronchial artery. Scale bar = 50 μ m.

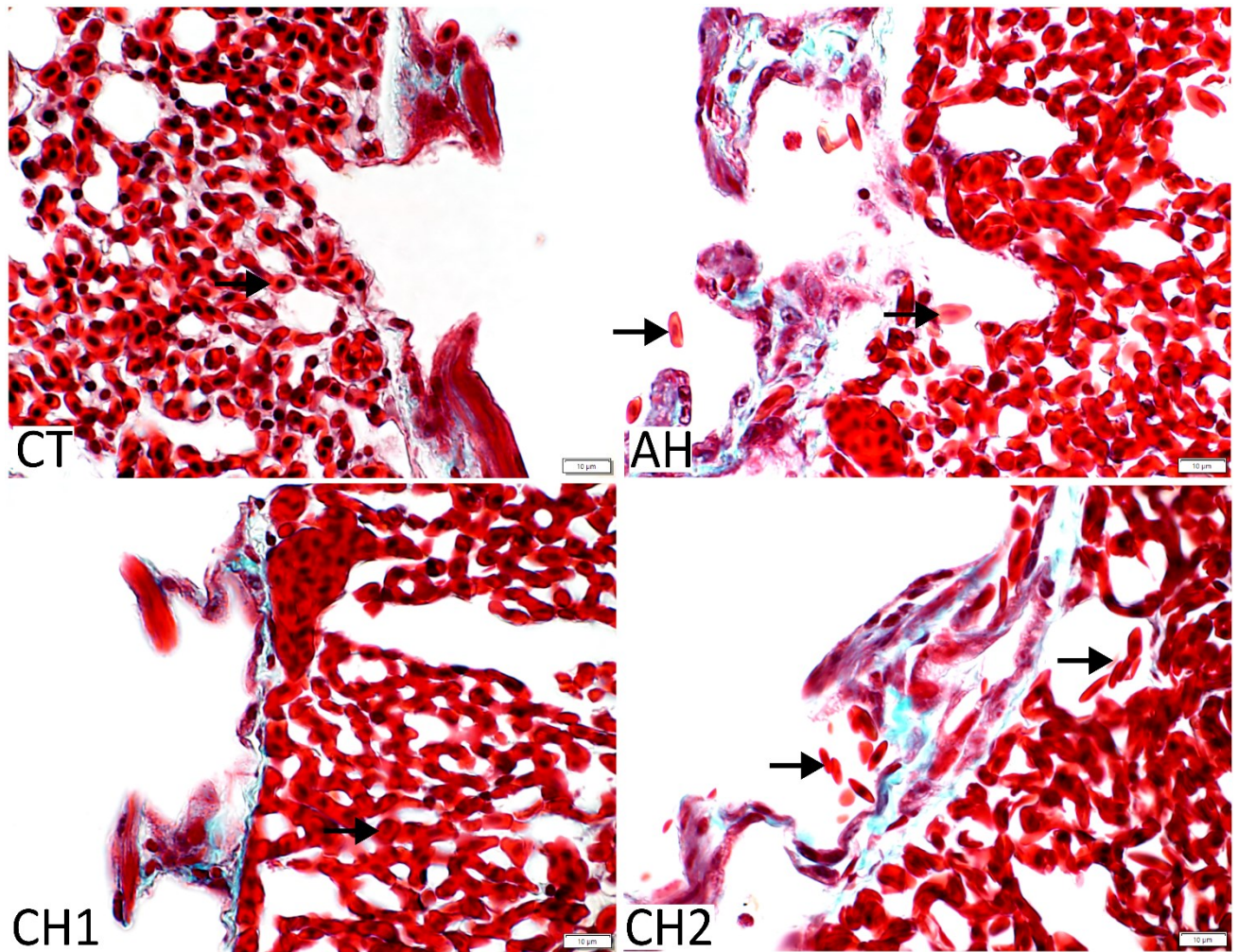


Figure 26: A representative micrograph of Gomori's trichrome stained Japanese quail lung of control and heat-stressed groups.

The black arrows show normal erythrocytes in the CT group. Note the decreased nuclei stain in the AH, CH1 and CH2 groups. Some cells in AH and CH1 groups are swollen, in the CH2 the cells within the infundibulae are spherical but the nuclei stain intensity has improved compared with the control. Scale bar = 10 μ m.

In Gomori's one-step trichrome stain (**figures 24 - 26**), control group showed uniform blood distribution within exchange tissue mantle. There are a few erythrocytes within the parabronchial lumen, and smooth muscle appears within the colour profile of stain (orange). The blood contains mostly eosinophilic-stained cells indicating a predominance of erythrocytes. In AH and CH1 groups, most parenchyma regions show blood congestion. The parabronchial lumen has an excessive amount of erythrocytes, and smooth muscle appears darker than control group. In addition, blood shows more basophilic-stained cells indicating predominance of white blood cells. In CH2 group, parenchyma shows uniform blood distribution like control group. However, there is a significant amount of erythrocytes within the lumen, and the blood contains basophilic-stained cells. Interestingly, the smooth muscle profile appears orange compared with control group. At a higher magnification, cells in AH and CH1 groups show fewer nuclei stain intensity, some cells are swollen and there is an increased number of spherical erythrocytes in CH2 group compared to the control group. The darker muscle knobs in AH, CH1 and CH2 became brighter at higher magnification, but green stain of the collagen increased indicating more collagen deposition. Hence it is the increased collagen deposition causing darker appearance of muscle knobs at lower magnification.

Table 13: The morphometric values of cell and nuclei in Japanese quail lungs.

Results are shown as mean±SD. *indicates a significant difference compared with control. KW superscript indicates that Kruskal-Wallis test was used. MGI – Mean grey intensity.

	CT	AH	CH1	CH2	One-way ANOVA
Cell area	18±1.5	27±5.0*	21±2.2	19±4.0	P = 0.0001 F (3, 36) = 14
Nuclei MGI	47±5.4	71±9.7*	58±8.7*	57±8.5*	P = 0.0001 F (3, 36) = 15
Nuclei area ^{KW}	4.8±0.9	5.3±2.1	4.4±1.0	4.9±1.1	P = 0.6688 KW statistic = 1.6

4.4 Fluorescence microscopy analysis

In the immunofluorescence labelling representative micrographs, α -SMA intensity of blood vessels is similar in the experimental groups — AH, CH1 and CH1 — compared with control. However, α -SMA of smooth muscle around atria showed more bulk and intensity in AH group compared with control. The collagen 1 (Col1) plates did not show significant differences except for abundance of bright collagen-producing cells seen in AH group compared with control. In merged/DAPI plates, collagen-producing cells are seen within parabronchi in AH group, which is absent in control and CH groups. In addition, some regions of parenchyma in AH group exhibit a high DAPI intensity compared to rest of the tissue (**figures 27 & 28**). However, at higher magnification, Col1 showed broader distribution in CT and CH2 groups compared with AH and CH1 groups, and distribution is less in AH than in CH1. The Col1 distribution in **figures 28 – 30** showed reduced collagen 1 intensity in AH and CH2 groups compared with the control group. In **figure 31**, some erythrocytes in AH and CH1 are spherical compared to other groups indicating an increased presence of immature red blood cells in circulation.

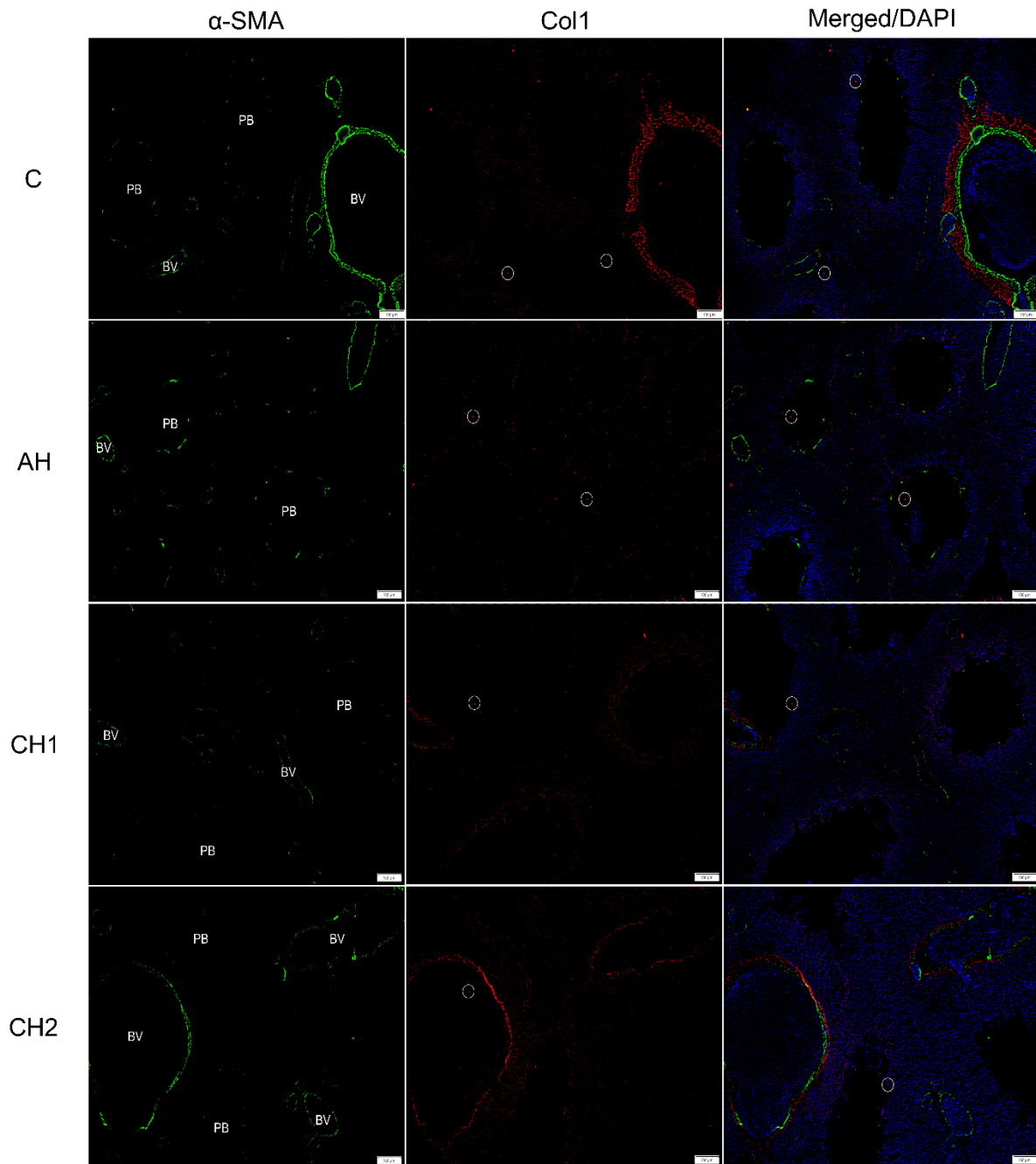


Figure 27: A representative micrograph of the immunolabelled Japanese quail lungs of the control and heat-stressed groups.

Left plates — the anti- α -smooth muscle actin (α -SMA) antibody (1:500). Middle plates — the anti-collagen 1 (Col1) antibody (1:300). Right plates — the merged micrographs with DAPI (1:8000). BV — blood vessel; PB — parabronchial lumen; white circles — collagen-producing cells. Scale bar = 100 μ m.

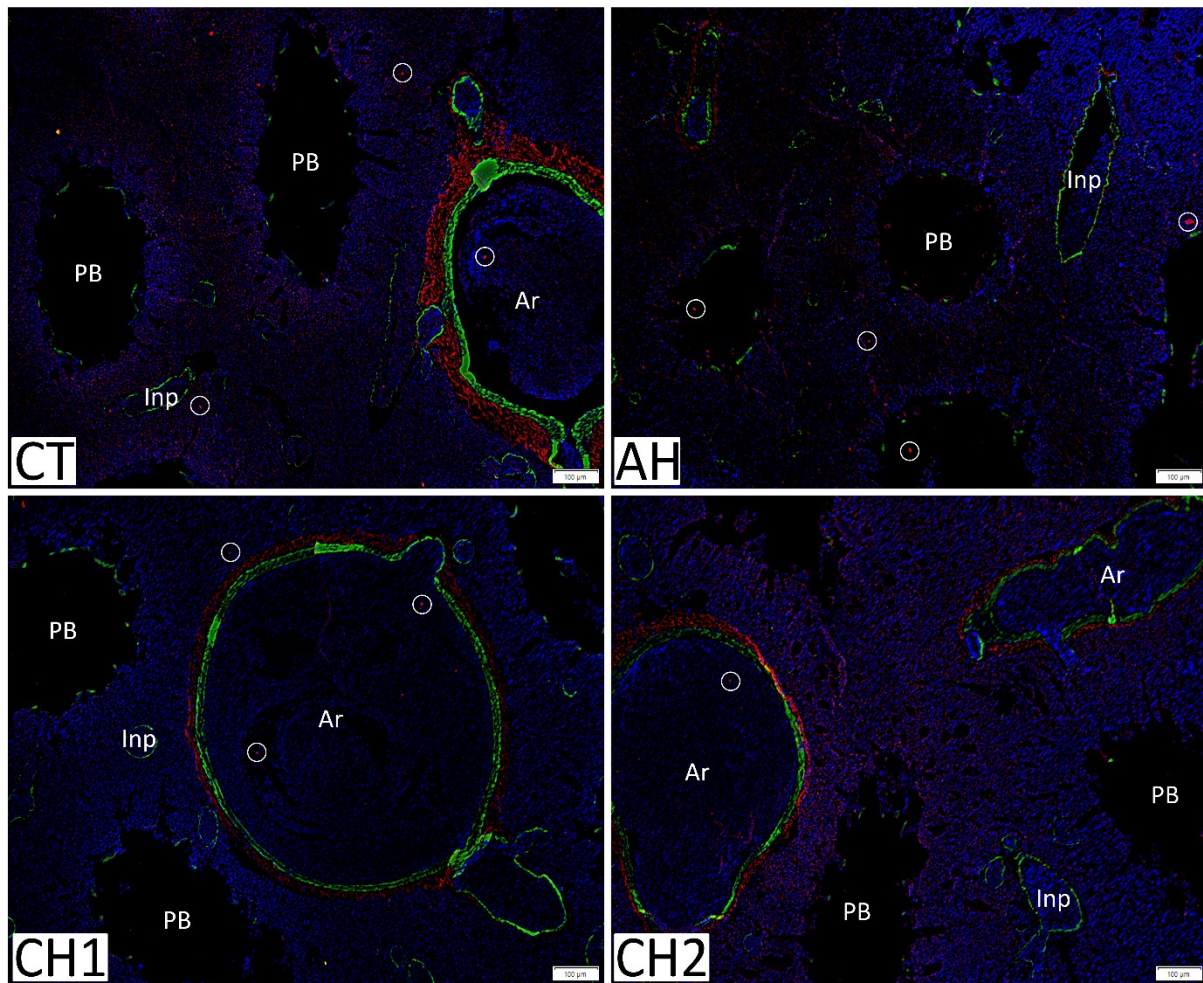


Figure 28: A representative micrograph of the immunolabelled (merged) Japanese quail lungs of the control and heat-stressed groups.

Note the reduced thickness of the collagen layer in the artery (Ar) of the CH1 and CH2 groups compared with the control. In addition, the collagen-producing cells (circled) are increased in the AH group. PB — parabronchial lumen; Inp — Interparabronchial blood vessels. Scale bar = 100 μm.

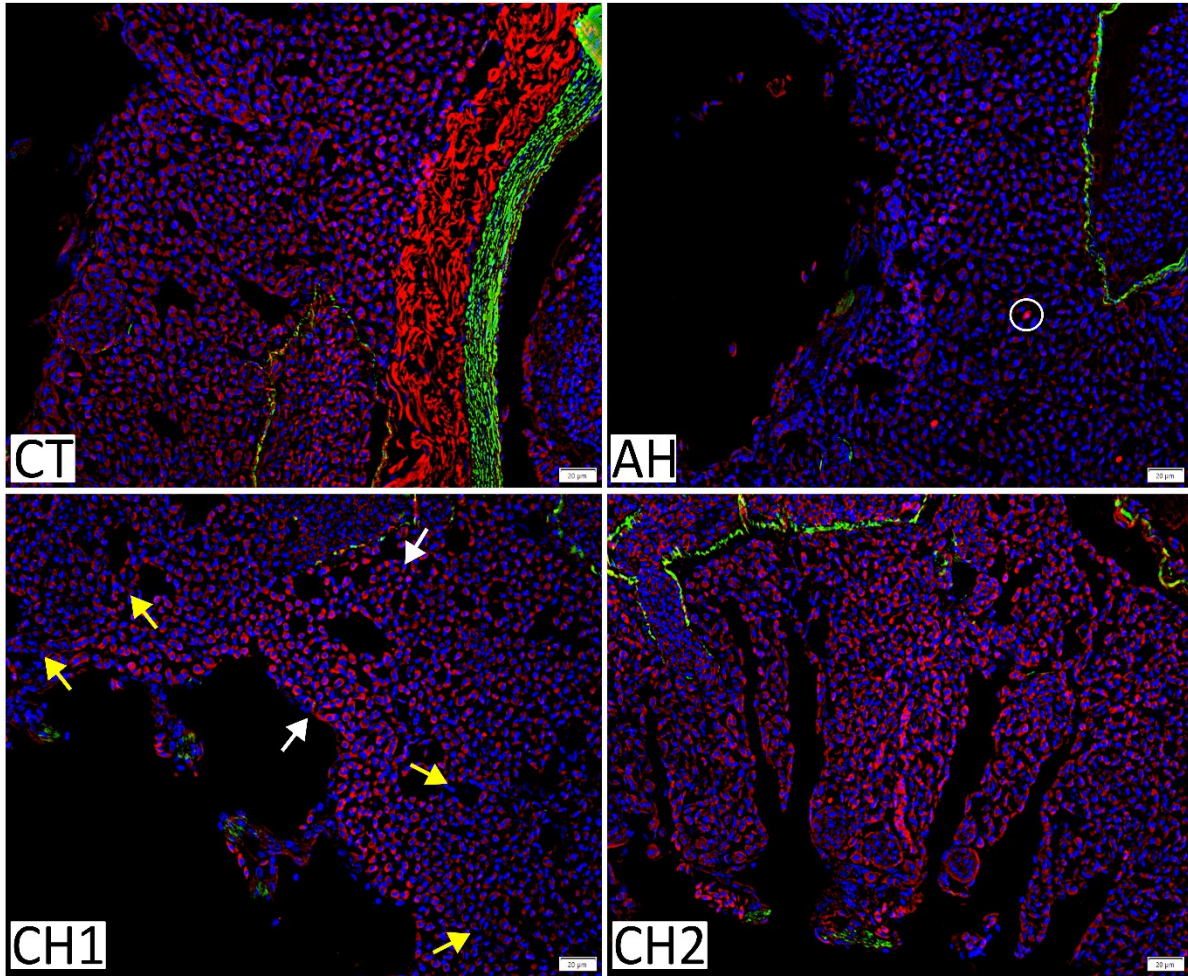


Figure 29: A representative micrograph of the immunolabelled (merged) Japanese quail lungs of the control and heat-stressed groups.

White arrows — regions with high Col1 distribution; Yellow arrows — regions with low Col1 distribution; white circle — collagen-producing cell. In CT and CH2, Col1 is uniformly distributed, while AH and CH1 showed areas of low distribution. Scale bar = 20 μm.

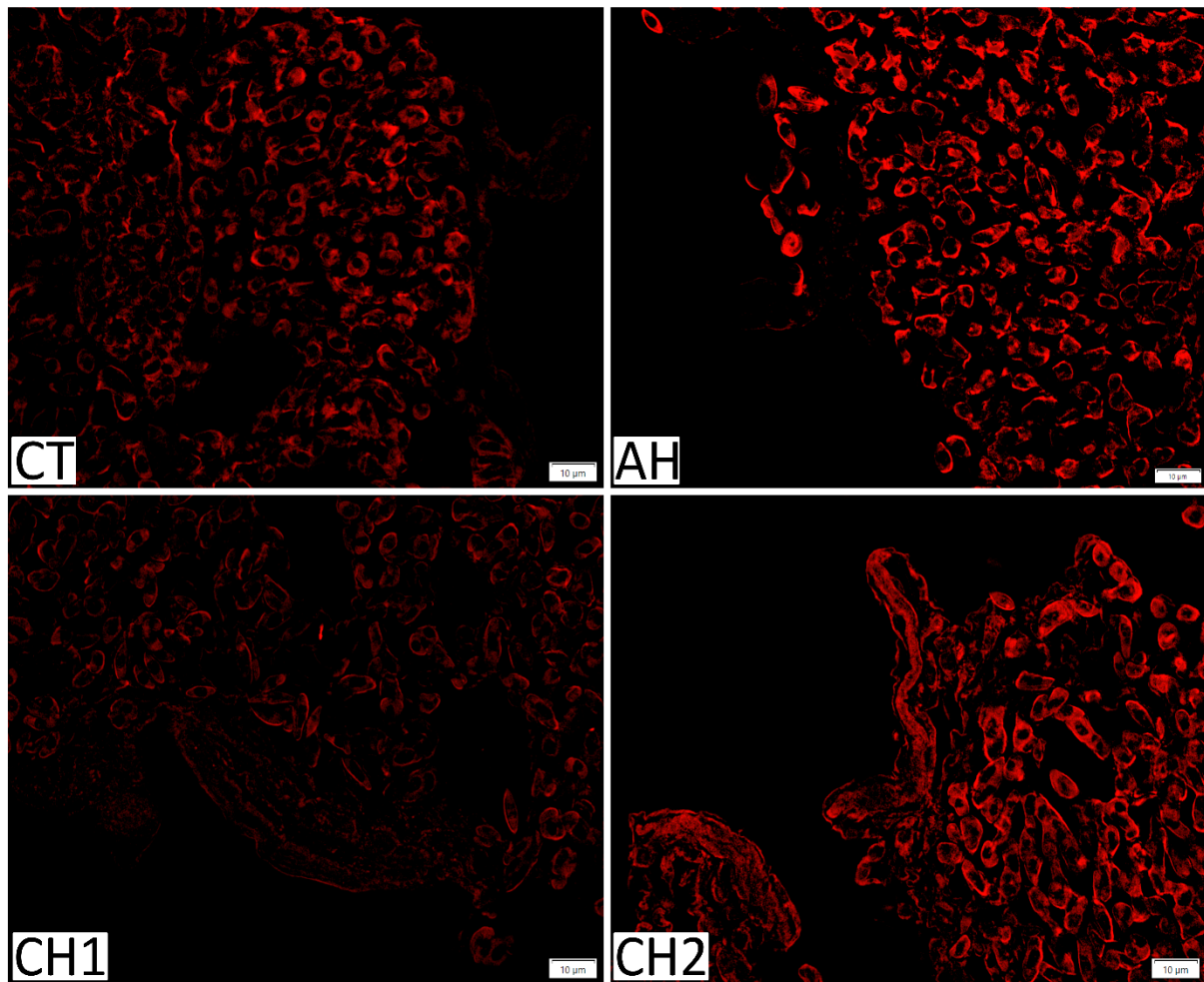


Figure 30: A representative micrograph of the immunolabelled (Col1) Japanese quail lungs of the control and heat-stressed groups.

Note the low fluorescence intensity in the CH1 compared with the rest of the micrographs. Scale bar = 10 μ m.

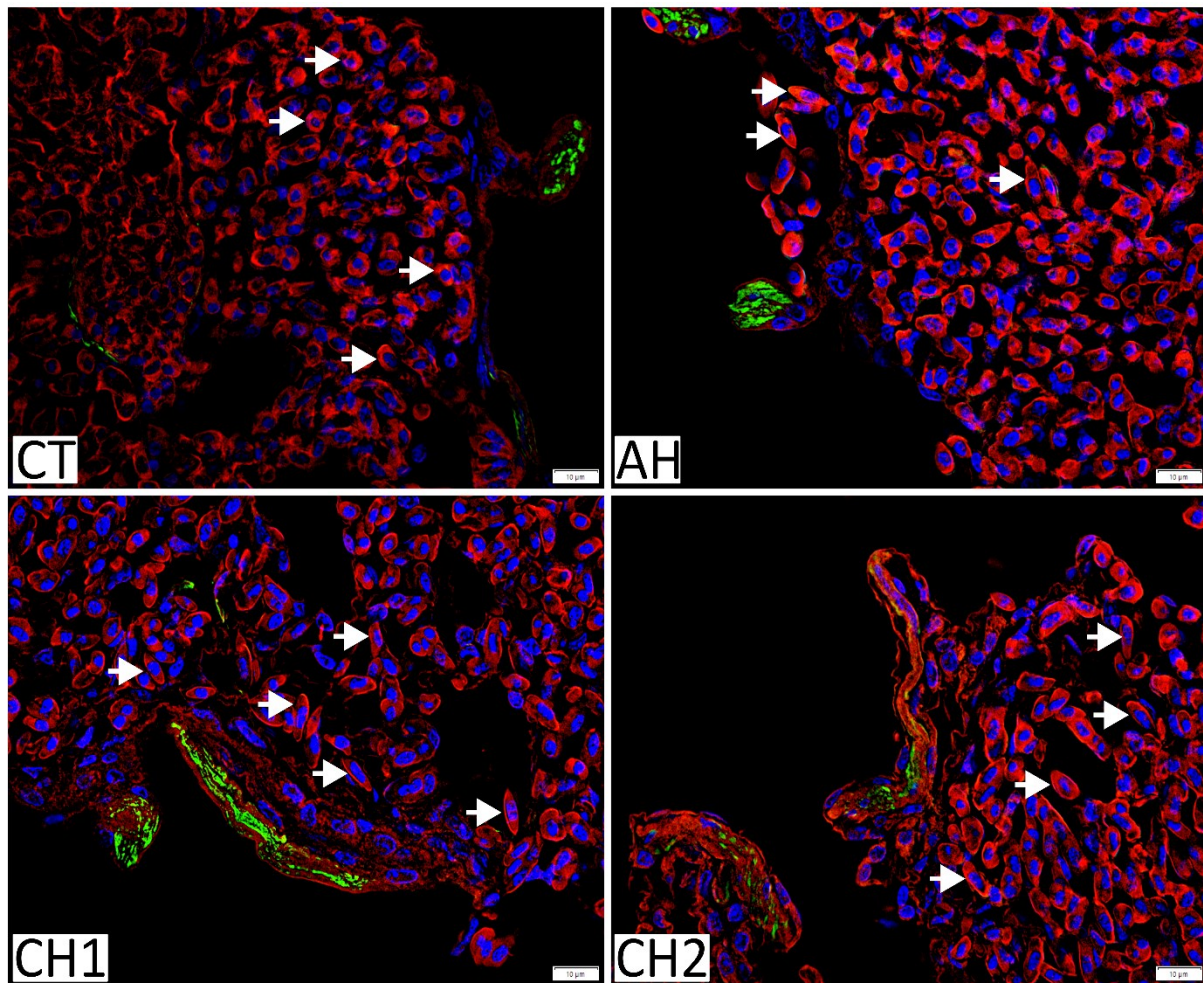


Figure 31: The representative micrographs of the immunolabelled (merged) Japanese quail lungs of the control and heat-stressed groups.

White arrows — erythrocytes. Note the spheric erythrocytes in the AH, CH1 and CH2 compared with ovoid erythrocytes in control (CT). Erythrocytes are seen within the airway in the AH, CH1 and CH2 compared with the control. Scale bar = 10 μ m.

4.5 Electron microscopy analyses

In the SEM micrographs (**figures 32 – 34**), depth of atria became shallow, and interatrial septa flatten to expose infundibulae openings directly to airflow in CH1 group. Furthermore, surfactants reduced significantly, surface appear smoother and some of blood capillaries are ruptured in CH1 group compared with the control group. These observations did not occur in AH and CH2 heat-stressed groups compared with the control group.

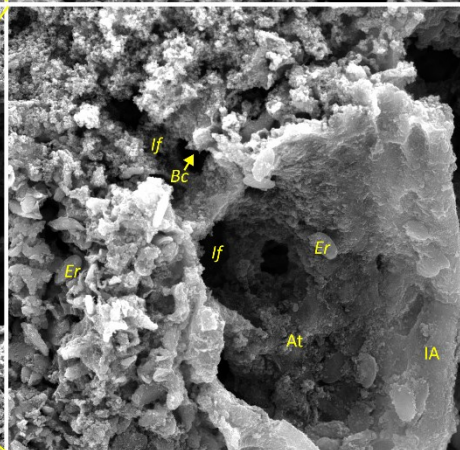
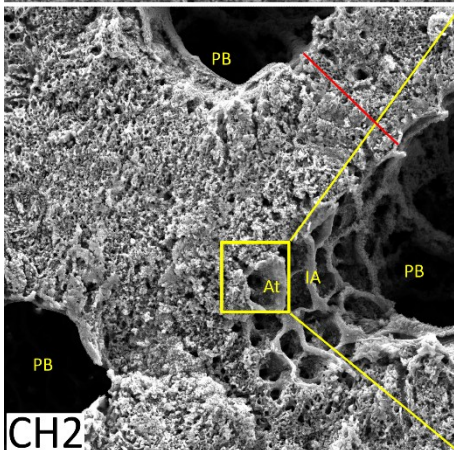
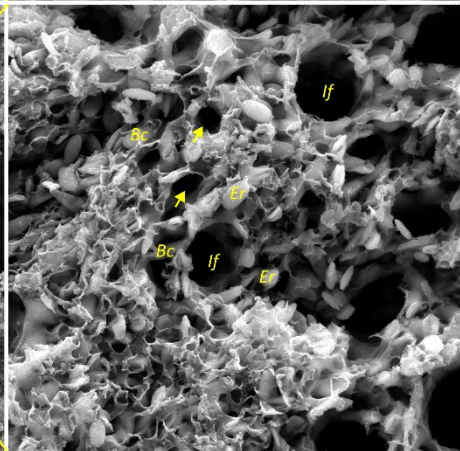
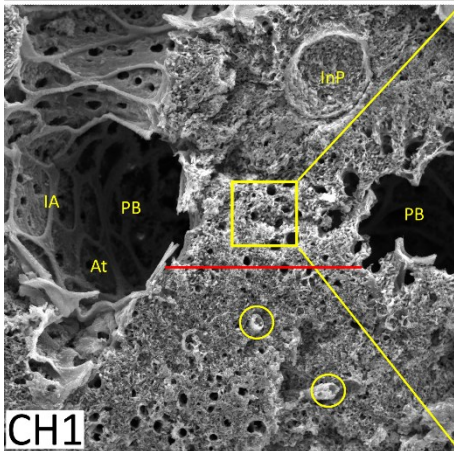
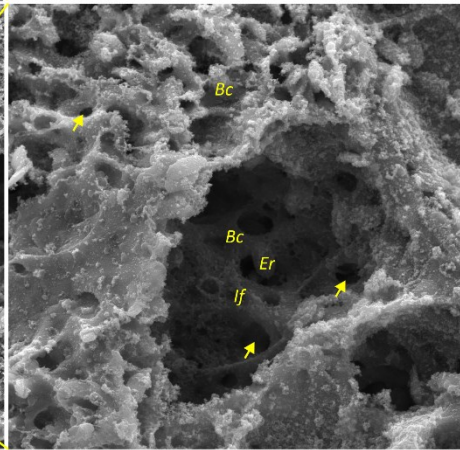
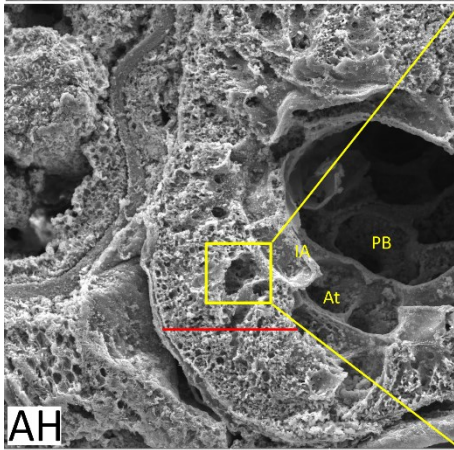
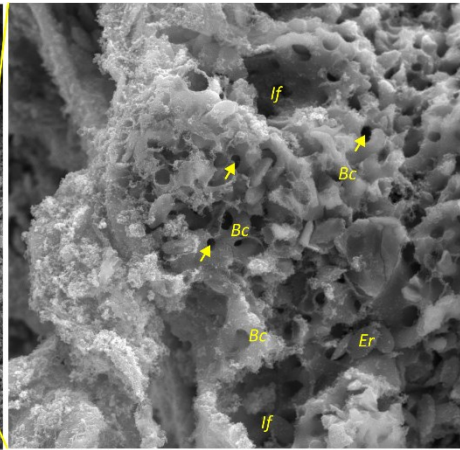
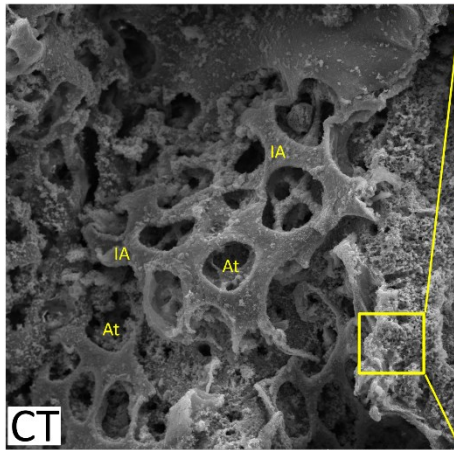


Figure 32: The representative scanning electron micrographs of the Japanese quail lungs of the control and heat-stressed groups.

The exchange tissue mantle extends the span of the red line as indicated. PB — the parabronchial lumen; At — Atria; IA — Interatrial septa; Bc — Blood capillary; If — Infundibulae; Er — Erythrocyte. The air capillaries are shown with yellow arrows. The intraprabronchial arterioles are indicated with a yellow circle. Note the thinning of the interatrial septa, reduced depth of the atria and the ruptured blood capillaries in the CH1 group. In addition, the surface of the parabronchi is smooth and has few surfactants available. Scale bar = 200µm (left panel) and 20µm (right panel).

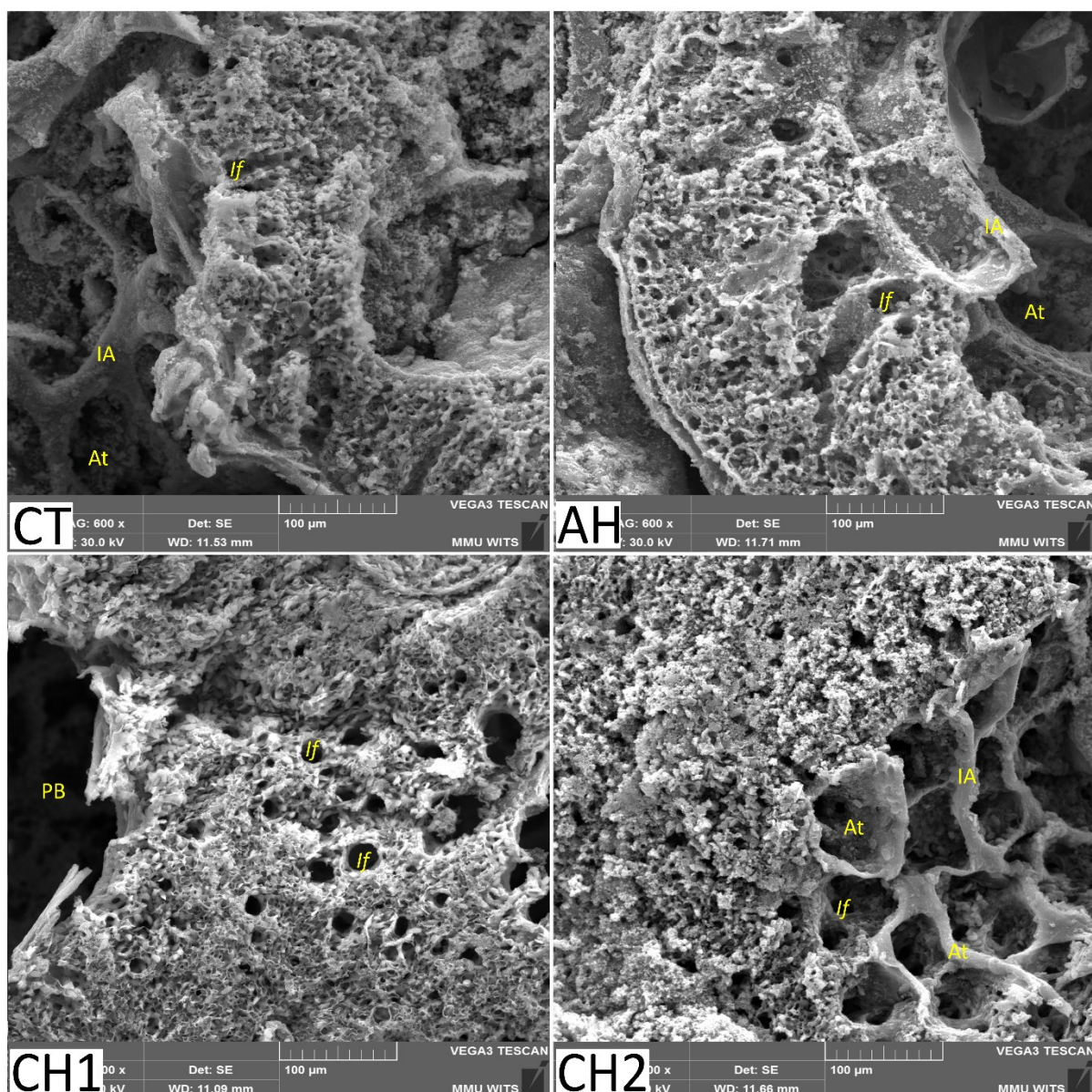


Figure 33: The representative scanning electron micrographs of the Japanese quail lungs of the control and heat-stressed groups. Refer to figure 32 for more details.

PB — Parabronchial lumen; IA — Interatrial septa; If — Infundibulae. Scale bar = 100µm

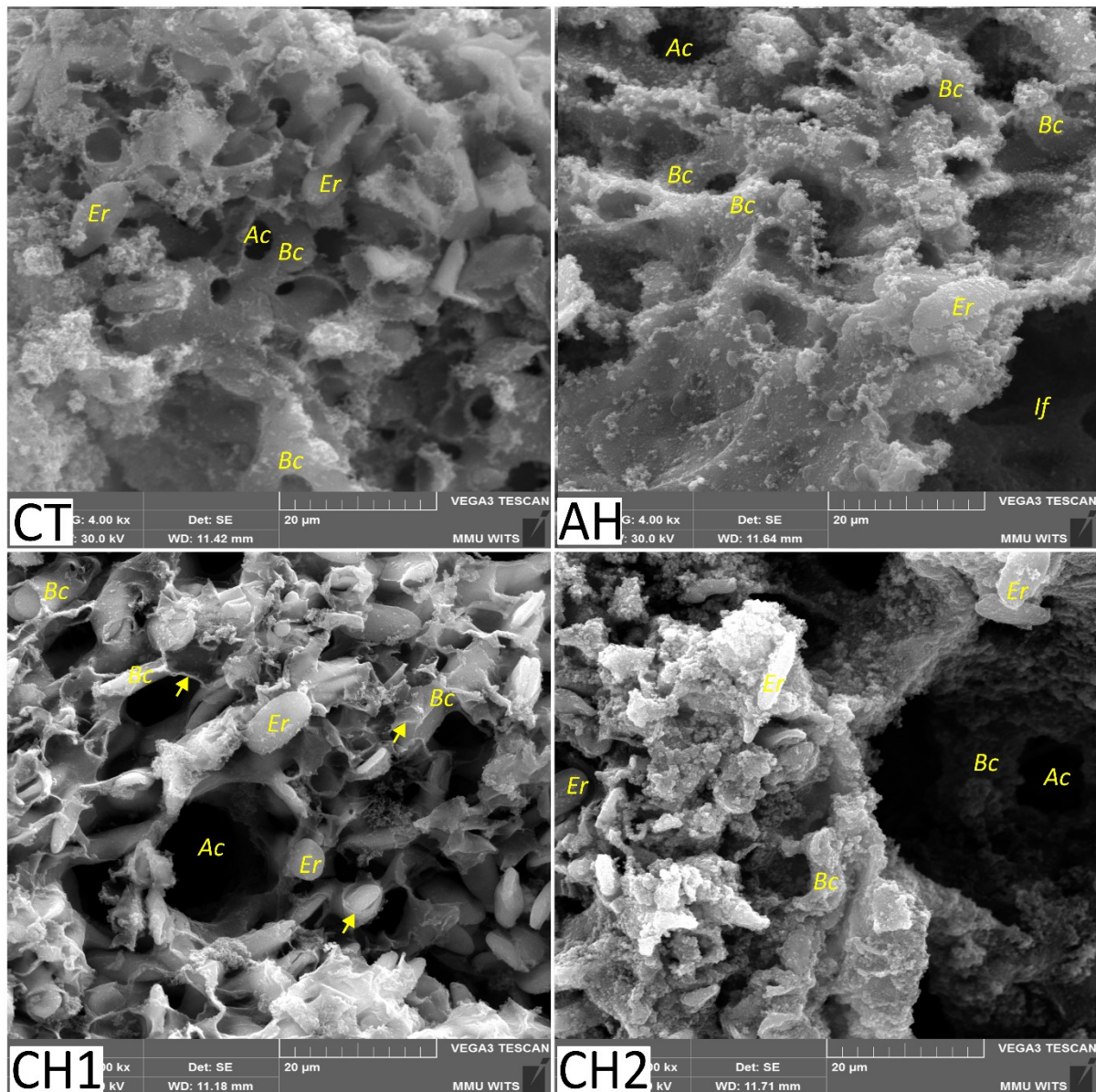


Figure 34: The representative scanning electron micrographs of the Japanese quail lungs of the control and heat-stressed groups. Refer to figure 32 for more details.

Bc — Blood capillaries; Ac — Air capillaries; Er — Erythrocytes; If — Infundibulae. The blood-gas barrier is indicated with arrow. Scale bar = 20μm

5 CHAPTER FIVE — DISCUSSION & CONCLUSION

“In heat stress, the lung is one of the most affected and injured organs, as its unique structure and impaired functions promote damages in other organs and tissues, as anoxia”.

(Rebez et al., 2023)

5.1 Discussion

In this study, heat stress negatively impacts morphology of Japanese quail lung. These impacts include increased blood congestion and excessive leakage of blood into the airway. Increased circulatory white blood cells indicating inflammatory response were noted. Nuclei swelling accompanies cellular swelling signalling cellular injury which could be irreversible and lead to widespread necrosis. Furthermore, heat stress affected structural integrity of collagen framework and smooth muscle activity in the lungs of the Japanese quail. The three-dimensional (3D) images revealed flattened interatrial septa and reduced surfactants on the airway surface. These microscopic effects translated to macroscopic lung mass, size and volume changes in an interesting pattern. Similarly, the birds' body mass, cloacal temperature and respiratory rate were changed by heat stress. In this section, a detailed implication of the study's results is presented.

Body weight changes in Japanese quail exposed to heat stress vary with duration of exposure. In an acute situation, immediate response of the bird to reduce feed intake instantly led to weight loss. However, as the condition progresses to a chronic situation, the bird begins to adapt and regain body mass. The recovery is not the same as birds reared in comfort ambient temperature conditions. However, feed intake is generally reduced during both acute and chronic heat conditions — the reason for body mass loss.

Mehaisen et al. (2019) reported significantly less weight gain and feed intake in Japanese quail, during six weeks of heat exposure at 35°C. Similarly, Japanese quail exposed to 35 days of 37°C heat showed reduced feed intake and significantly less weight gain than the bird

reared in thermoneutral comfort (Tekce et al., 2020a). In these studies, heat-stressed quails did not lose body mass but gained weight slower than comfortable birds. The birds expend their energy to supply the increased cellular energy demand and maintain evaporative cooling. Thus, energy is diverted and utilised to adapt to the heat stress conditions, instead of being utilised for growth. In addition, heat intensity and duration of heat exposure is not directly correlated to its overall effect on body mass and feed intake when assessed over a long duration. However, the effects are rather similar in exposed Japanese quail, once temperature is above thermoneutral comfort. Other studies with similar findings include Orhan et al. (2012) and Sahin et al. (2004).

In contrast, Japanese quail assessed during 24-hour acute heat exposure shows outright body mass loss. In the experiments conducted by Del Vesco and Gasparino (2013), Japanese quails exposed to 34°C for 24 hours showed weight loss and decrease feed intake. Similar outcomes were recorded in subsequent experiments by the same research group, which exposed quails to 38°C for 24 hours (Del Vesco et al., 2014a; Del Vesco et al., 2014b; Del Vesco et al., 2017). Few studies experimented with acute heat stress in Japanese quails, for example, Jimenez and De Jesus (2021). In addition, many of the heat stress studies conducted tested chronic exposure, perhaps because longer duration elicited more effects. For example, in the scoping review presented (chapter 2), only two of the 24 included studies were acute heat stress experiments. The downside of doing just a chronic heat exposure experiment is that it misses the trend from acute to chronic as seen in this study. A chronic heat experiment will always have an acute component at the beginning irrespective of temperature level.

In this study, the mean body weight in AH group reduces, which is however not statistically significant compared with control group. In CH1 group, no changes in body weight were recorded compared with control group. Group composition with more female quails could be the reason for sustained weight increase in CH1 group because female quails are heavier than males. In this experiment, sex of the bird was ignored as a factor during randomisation of the birds into groups because of limited option on the availability of quails from the farm. In the CH2 group, initial body mass loss was noticed until around the 14th day when it begins to normalise compared with the control. The trend is that acute heat stress will cause a negative body mass effect and a significant reduction of feed intake. As the birds continue to adapt, they marginally increase feed intake and regain body weight and in some days equalise with the control groups. The effect of heat stress on Japanese quail body mass is a continuous phenomenon transitioning from acute to chronic exposure irrespective of temperature intensity.

The smaller birds at maturity have a negative impact on business and research because body mass is an important factor for poultry market. Higher body mass in grams or kilograms brings more income. Similarly, body mass is an important factor in assessing research outcome of most experimental animal studies. Hence, quails exposed to heat stress at the farm could bring a confounding factor, which researcher might be unaware of and cannot be controlled.

The effect of heat stress on body mass correlates with reduced feed intake aimed at minimising metabolic heat production. However, reducing feed intake is inefficient to combat heat stress, thus birds increased their respiratory rate as an additional strategy. The

discussion here is in sequence, but the physiological response of evaporative cooling and minimising metabolic heat production occurs concurrently.

Increased respiration helps the bird dissipate heat from the body and cool incoming air through the respiratory surfaces (Weathers and Schoenbaechler, 1976). Weathers and Schoenbaechler (1976) studied evaporative cooling in small birds and found that it contributes significantly to the effort of combatting heat stress. For example, surgically transecting hyoid muscle in a Japanese quail revealed that gular flutter contributes significantly to evaporative cooling (Weathers and Schoenbaechler, 1976). Japanese quail will normally pant and gular flutter during heat stress, and these mechanisms used the respiratory surface efficiently (Weathers and Schoenbaechler, 1976). However, the same is not the case in non-flying large birds, for example, the respiratory effort did not contribute to heat dissipation in some species of cranes despite the dead space created by their long neck (Prange et al., 1985).

In this study, no significant increase in respiration among heat-stressed Japanese quail in acute or chronic phase was seen. Sometimes respiratory rate is higher in the control groups. Although a significant increase in respiratory rate (mean of control group = 44 breath/min; mean of heat-stressed group = 85 breath/min) was reported in Japanese quail exposed to 34°C (Mohamed et al., 2015), our 38°C exposure in acute heat-stress group and 35°C in chronic heat-stress group did not elicit such response. However, quails used in this study had a higher basal respiratory rate prior to heat exposure compared with the experiment by Mohamed et al. (2015). Most quails in this study efficiently used gular flutter mechanism to combat heat thereby relegating the need for increased respiration. Another reason for this

observation could be that specimens in this study had already developed adaptive mechanisms from source, hence the higher basal respiratory rate.

Because researchers found that Japanese quail had poor capacity for cutaneous evaporation (Marder and Ben-Asher, 1983), we only assume the birds employed panting and gular flutter to combat the heat. Even with little respiratory effort, heat-stressed quails in this study maintained cloacal temperature similar to the control.

Non-significant increased cloacal temperature in AH compared with control group is similar to initial response seen in CH1 and CH2 groups. However, in CH2 group, cloacal temperature begins to fluctuate from fifth day of exposure some days slightly higher or lower compared with control group. Meanwhile, cloacal temperature in CH1 remained slightly higher for seven days of exposure compared with control. This finding further supports the assumption that quail in this study might have developed some adaptation from the source. Furthermore, Japanese quail might have higher thermal tolerance because, at 35°C, the CH1 group sustained body mass gain and a fluctuation of respiratory rate compared with the control group despite slight body temperature increase. Maybe, the results could be different if we had extended the 38°C to a chronic phase.

Most of the studies reviewed for writing this thesis did not report body temperature measurements for Japanese quail. For example, in the scoping review (chapter 2) none of the included studies reported body temperature measurement. However, compared with chicken (*Gallus gallus*) exposed to 32°C (Lin et al., 2006), rectal temperatures significantly increased (mean of control group = 42.1°C; mean of heat-stressed group = 42.8°C) after six hours of

exposure. The Japanese quail could adapt better to heat stress than chicken when comparing this study to our results. However, more studies are needed to substantiate that, because if that is the case, Japanese quail could replace chicken as a reliable source of protein food and income in regions with higher environmental temperatures.

The quail organs, as presented in the scoping review, were negatively affected by heat stress, but the lungs were not studied. However, several researchers studied Japanese quail lungs for example, chemical exposure (Rombout et al., 1991) and respiratory infections (Fadhilah et al., 2020), but not heat stress.

The results of lung weight, size and volume follow a similar pattern in AH and CH1 groups. Initial decline of all parameters, as in AH group, then gradual improvement as experiment progresses, as depicted by CH1 group. The AH group were exposed to a higher temperature than CH1, hence we expected an exaggerated initial negative impact of heat stress in that group. The CH2 group shows a slight improvement in lung parameters compared with AH and CH1 groups. Therefore, we hypothesized that firstly, because of the fragility of tissue, fixation will cause shrinkage in the heat-stressed lungs than the control group. Secondly, dead cells will account for the weight and volume decline in the AH group compared with the control and CH1, because dead cells will shrink and be replaced with collagen fibre deposition or new cells. But in acute experiment, animals were terminated before repair could be completed or even started.

Furthermore, CH1 group shows an improvement in lung weight and volume because of the chance to undergo cellular adaptation and possible repair. The lung size is similar in AH and

CH1 group, unlike results of weight and volume, because volume measurement is a derivative of lung weight, considering Scherle's method procedures. However, lung size measurement ignored internal changes within the lungs. Because of these reasons, it is obvious heat stress had little effect on the gross morphology of Japanese quail lungs. However, it also points that a lot has happened to the internal structure while the bird was trying to maintain the overall gross morphology. So far, blood congestion and cell death has been reported as part of the microscopic observations.

Blood congestion was the only observation reported on Japanese quail lungs in the literature (Mohamed et al., 2015) and it was apparent in the lungs of the heat-stressed quails in this study. It represents an increased blood supply to region of high oxygen concentration within the lungs. The reasons could be to extract more oxygen for supply to areas of increased tissue oxygen demand all over the body — caused by heat stress — or it could just be a normal inflammatory response to protect and repair the lung. The second reason — i.e. is just an inflammatory response — was because blood congestion happens in liver and the kidney of the Japanese quail exposed to heat stress (Mohamed et al., 2015).

The reason for inflammation in this study is predominance of basophilic-stained cells in heat-stressed lungs — compared with predominant erythrocytes in control group — representing white cell components of the blood especially thrombocytes to help fix ruptures and leakages caused by congestion and increased pressure. This trend was observed in H&E-stained and the Gomori's trichrome-stained sections of heat-stressed quail lungs. In the fluorescence micrographs, collagen layer thins out indicating stretching of blood vessels due to increased pressure in the lungs of heat-stressed quails compared with the control group.

However, considering the increased cellular energy demand caused by heat stress and requirement of oxygen for ATP production, one could say congestion in the lungs is to extract more oxygen. In addition to blood congestion, Aengwanich and Simaraks (2004) reported oedema in gross observation of chicken lungs, although gross images and micrographs provided were not clear. In this study, no sign of oedema was identified in Japanese quail lungs at gross or microscopic levels. Similarly, lungs of galahs and rock doves exposed to heat stress did not show oedema in gross or microscopic observations (Xie et al., 2019). However, cellular swelling resulting from lipid peroxidation and increased cell membrane permeability caused by oxidative stress was seen in this study. Similarly, endothelial hypertrophy was seen in the lungs of galahs exposed to heat stress (Xie et al., 2019).

Cellular swelling or endothelial hypertrophy would have caused an overall lung size or weight increase but that did not happen in this study. Instead, lung size and weight were decreased in heat-stressed groups. Therefore, blood congestion serves the purpose of lung repair (inflammatory response) and oxygen extraction because other tissues depend on it for energy production.

The function of the lungs is vital to survival of quail in heat-stressed conditions, not only because of evaporative cooling but also because other tissues depend on it, to avoid anoxia (Rebez et al., 2023). In an effort to maintain this function, smaller blood vessels rupture due to increased congestion and pressure. Inevitably leading to excessive leakage of blood into the airway, further disrupting lung function. The mechanism of blood coagulation by surfactants and subsequent removal from lungs seems effective but takes time to accomplish. After twenty-four hours of heat exposure, large amounts of coagulated blood was found in

the lungs of quail in the AH group. The amounts of blood within the airway reduced over weeks as quails adapted to heat as seen in CH1 and CH2 groups, and because the temperature level was lower than in AH group. In addition, immature erythrocytes (Scanes, 2015) were recruited to compensate for blood loss and maintain circulatory homeostasis. The amount of spherical erythrocytes (immature erythrocytes) increased in heat-stressed groups compared with the control group. Initially, one could assume that the plane of the tissue section is the reason spherical erythrocytes were found. While it is possible to find a matured erythrocyte in a perpendicular orientation because of tissue fixation and sectioning, occurrence of sphericity in heat-stressed groups is overwhelming to just be as a result of tissue processing or coincidence. Especially when the same observations cannot be confirmed in the lungs of quails reared under comfortable ambient temperature conditions. In addition, collagen-producing cells increased in heat-stressed groups as part of the inflammatory response.

Collagen production strengthens overall lung structure from the effect of heat stress. In new pentachrome stain, increased irregularly arranged collagen fibres were observed suggesting new fibres deposit at the base of interatrial septa. In the control and CH2 groups, collagen fibres are nicely regularly arranged. The evidence in Gomori's trichrome suggests increased collagen deposition in some regions of interatrial septa, logically collagen fibre deposition and tissue contraction are part of inflammatory response because resident epithelial cells also produce collagen fibres in addition to infiltrating fibroblasts and fibrocytes during inflammatory response (Mack, 2018).

The same is not the case within exchange tissue mantle where different attenuation of collagen 1 fluorescence intensity occurred in heat-stressed group. However, collagen 1 in

control group had a uniform distribution, because there is no need for differential attenuation of connective tissues. In Japanese quail lungs, interparabronchial septa are absent, hence the collagen fibres within the exchange tissue are most likely connected with the interatrial and infundibulae collagen fibres for support. It is unlike the domestic fowl where collagen fibres from the interparabronchial septa directly connect to the thin fibres of exchange tissue mantle (Maina et al., 2010).

While interatrial septa receive collagen deposits for strength, exchange tissue mantle was almost spared. The hypothesis is because airflow is stronger in the parabronchial lumen, structural integrity needs to be maintained, while within the exchange tissue oxygen extraction is more important, hence less impedance is required. The need to add collagen fibres in some of the parabronchi can be due to thinning of the smooth muscle of interatrial septa which was obvious in the light microscopy and scanning electron micrographs. Analyses of tissue sections in this study encountered different levels of inflammatory responses because most of the sections analysed with light microscopy shows some interatrial septal regions with thinning of smooth muscles, while some regions show bulky septa, however, both containing more collagen fibre compared with control group. The exact mechanism of collagen deposition in this study is not clear, but it could be triggered by cell death from necrosis that will spill its contents and incite an inflammatory response and the cycle continues with heat stress at the centre.

Oxidative stress is implicated in the negative effects of heat stress. Excessive reactive oxygen species production in the cell damages the DNA, protein assembly and cell membrane. The resultant effect causes cellular swelling and death. In this study, analyses of cells showed

significant increases in size of cells in the lungs of heat-stressed Japanese quails compared with the control group. In addition, nuclei stain uptake decreases in cells of heat-stressed lungs and possibility is that the denatured proteins of the nuclei material did not interact with the chemical constituents of the dye. Mean grey intensity was measured and it was significantly higher in heat-stressed groups compared with the control group, indicating less uptake of nuclei stain. Another reason could be due to high influx of ions such as sodium and calcium in heat-stressed cells and presence of excessive ions intracellularly could interfere with dye uptake. In essence, these features point towards a cell that is dying but struggling to stay alive through repair mechanisms or could just be undergoing necrosis.

In the scanning electron microscopic analyses of Japanese quail lungs exposed to heat stress; a surface three-dimensional (3D) view was possible. Ruptured blood capillaries and reduced amount of surfactants in CH1 group was only seen in 3D view. One function of surfactants in quail lungs is to reabsorb leaked plasma and coagulate blood in general. Therefore, excessive leakage of blood into airway will diminish the amount of the surfactants present on the airway surface while it is being reproduced, as seen in CH1 group.

5.2 Limitations of the study

Ethics consideration limits the use of Japanese quail to the minimum in this study. The sample size used is adequate for gross and microscopic analyses. However, statistically, it is just the minimum sample size required for an ANOVA comparison. Furthermore, microscopic examination reveals features in different regions of tissue section, which cannot be captured

in one micrograph. Hence, in some cases, certain features were left out in some stains and then captured in a different stain.

Another limitation is the outsourcing of Japanese quails, because we cannot account for environmental conditions the Japanese quails were exposed to at the farm, between hatching and time they were received.

The COVID-19 pandemic affected the initial timeline of the study thereby putting constraints on initially proposed laboratory analyses and objectives because the funding for the student scholarship was only for three years and it was exhausted.

5.3 Conclusion

Heat stress caused significant damage to lungs of Japanese quail. The damaging effects occurred based on intensity and duration of heat exposure, which elicits an initial intense inflammatory response within lung tissue. The bird could die in an instant depending on response or survive and get a chance to adapt and recover over time. Ruptured blood vessels and excessive leakage of blood into airway are some of the commonest features found in this study. Furthermore, differential collagen deposition and improved oxygen extraction efficiency were observed within lung parenchyma.

The birds showed initial body mass loss and increased panting and gular flutter to combat heat stress, which led to decreased performance. Although Japanese quail showed adaptation capacity to increased environmental temperature up to 38°C in this study, the outcome is still not good as bird reared in comfortable temperature conditions (the control group).

Heat stress causes increased cellular energy demand that led to increased oxidative stress-induced cellular damage and necrosis in various tissues of the body. The lung is the most important organ in heat stress because other organs depend on it for oxygen supply. Hence, alleviating heat stress is vital to ensuring the welfare and quality of Japanese quail production for food security and biomedical research, especially in sub-Saharan Africa.

To achieve that, dietary supplements were tested and proved to be helpful, but a significant number of supplements target oxidative stress in alleviating heat stress. Despite improvement in performance and other reported parameters while using the supplements, for example, propolis (Mehaisen et al., 2019), it is comparably still not effective to reverse the effects of heat stress completely. Hence, additional strategies such as cooling and floor space should be considered to improve welfare, especially because the lung receives direct hot air during exposure. However cooling systems are capital intensive and could not be affordable to rural households who are most affected by high environmental temperatures. An early warning system should be developed in future investigations to detect possibility of heat waves and prepare the rearing environment for cooling. Furthermore, capital investment should be encouraged into quail farming to improve the system in order to sustain food security and biomedical research in Africa.

Conclusively, this project presents a unique assessment of the morphology of Japanese quail lungs exposed to heat stress, which was not done before, and this might help improve quail husbandry in African continent and globally.

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7 APPENDICES

7.1 Appendix 1 —Similarity index report

Thesis Draft - Abdul 1332569.docx

ORIGINALITY REPORT

16%	12%	14%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Abdurrahman Abdulkadir, Deran Reddy. "A scoping review of the impact of heat stress on the organs of the Japanese quail (Coturnix japonica)", The Journal of Basic and Applied Zoology, 2023 Publication	2%
2	www.researchgate.net Internet Source	1%
3	www.apec.org Internet Source	1%
4	hdl.handle.net Internet Source	1%
5	www.tandfonline.com Internet Source	<1%
6	www.science.gov Internet Source	<1%
7	www.mdpi.com Internet Source	<1%
8	core.ac.uk Internet Source	

7.2 Appendix 2 — Ethics Approval Certificate

ANIMALS RESEARCH ETHICS COMMITTEE (AREC)



STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2020/10/01/D

APPLICANT: Mr A Abdulkadir

School: School of Anatomical Sciences; Department: N/A; Location: WRAF

PROJECT TITLE: Morphological changes in the lungs and air sacs of the Japanese quail (*Coturnix japonica*) exposed to heat stress.

Category: D; Species and Numbers involved: 8X 100-160g , male or female *Coturnix japonica*

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 27 Oct 2020. This approval remains valid until 17 Feb 2023 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4
Liaise with WRAF concerning the measurement of temperature and the housing of the quails			

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

Signed: _____  _____ Date: __17/02/2021__
(Chairperson of the AREC)

I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

Signed: _____  _____ Date: __19/02/2021__

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Central Animals Service: Dr Kim Jardine

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

- a. Name: Abdurrahman Abdulkadir
- b. Department: School of Anatomical Sciences

c. Experiment to be modified / extended

AESC NO

Original AESC number	2020	10	01/D
Other M&Es : Additional Co-workers			

- d. Project Title: Morphological changes in the lungs and air sacs of the Japanese quail (*Cortunix japonica*) exposed to heat stress.

	No.	Species
e. Number and species of animals originally approved:	8	<i>C. japonica</i>
f. Number of additional animals previously allocated on M&Es:	nil	
g. Total number of animals allocated to the experiment to date:	8	<i>C. japonica</i>
h. Number of animals used to date:	8	<i>C. japonica</i>

- i. Specific modification / extension requested:
1. Additional 30 *Cortunix japonica* for the main study.
 2. Animals grouping
- j. Motivation for modification / extension:
1. Additional animals for the continuation of the study after the pilot was done to confirm the feasibility of exposing the birds to high temperatures.
 2. For the pilot study the birds are grouped as follows: acute heat stress (AH) – 4 birds; chronic heat stress (CH1) – 4 birds.
For the main study the birds were grouped as follows: control group (C) – 10 quails; Acute heat stress group (AH) – 5 quails; acute heat stress recovery group (AR) – 5 quails; chronic heat stress group (CH) – 5 quails; chronic heat stress recovery group (CR) – 5 quails.
 3. However, modification is requested for the main study to replace the recovery groups (AR and CR) with another chronic group (see flow chart on the next page). Firstly, for simplification of the study. Secondly, a chronic group that exposed the birds to 35°C for 7 days (CH1) will enable the study of the adaptation of the birds to chronic heat exposure when compared to another chronic group that exposed the birds to 35°C for 28 days (CH2). This insight was noted during the pilot study.

Date: 08/06/2021

Signature: 

RECOMMENDATIONS

Approval of an additional 30 animals for the main study and modification of the study groups as indicated in the flow diagram below. Researcher is to ensure daily monitoring of all animals and

AESC 2012 M&E

report any severe heat stress symptoms to Dr Kim Jardine (011 717 1301).

Date: 08 June 2021

Signature:


Deputy Chair, AESC

Figure 1: Experimental flow diagram

AH – Acute heat stress group (38°C for 24 hours)
CH1 – Chronic heat stress group 1 (35°C for 7 days)
CH2 – Chronic heat stress group 2 (35°C for 28 days)
Control – replicated for AH and CH

7.3 Appendix 3 — Pilot Study Report

Introduction

The Animal research ethics committee approved the study (2020/10/01/D) - morphological changes in the lungs and air sacs of Japanese quail exposed to heat stress. However, the committee requested a pilot be conducted first to resolve potential experimental paradigm flaws, as follows:

1. That temperature exposure up to 38°C is likely to cause mortality among the birds.
2. That a disparity in measured body temperature may exist when using an infrared thermometer or a digital cloacal thermometer.
3. For the pilot, eight Japanese quail were bought from a commercial farm in East London and randomly distributed (4 each) into an acute heat stress group (AH) and a chronic heat stress group (CH).
4. The AH group were exposed to heat at 38°C for 24 hours after one week of acclimatization.
The CH group were exposed to 35°C for 7 days after one week of acclimatization.

Results

1. The bird groups exposed to 38°C and 35°C, for 24 hours and 7 days respectively were healthy displaying no signs of morbidity.
2. There was evident gular fluttering which is an evaporative cooling mechanism commonly seen in birds (Smit et al., 2018).
3. Routine vital signs measurements were within the expected range (see figure 1).

4. The body temperatures of the birds were measured from the cloaca using a digital thermometer and from the skin using an infrared thermometer (see figure 2). Both instruments measured body temperature with high precision but the variation in the measurements obtained was consistently different. As seen in Figure 1 C&D, higher body temperatures were recorded when using the infrared thermometer compared to those using the digital thermometer.
5. It was also noted that the digital thermometer takes a longer time to record temperature from the cloaca whilst the infrared thermometer gives measurements instantly. However, the digital thermometer most likely provides a more reliable record from the cloaca representing the actual state of the body temperature. This method will therefore be adopted to measure body temperature in this study.

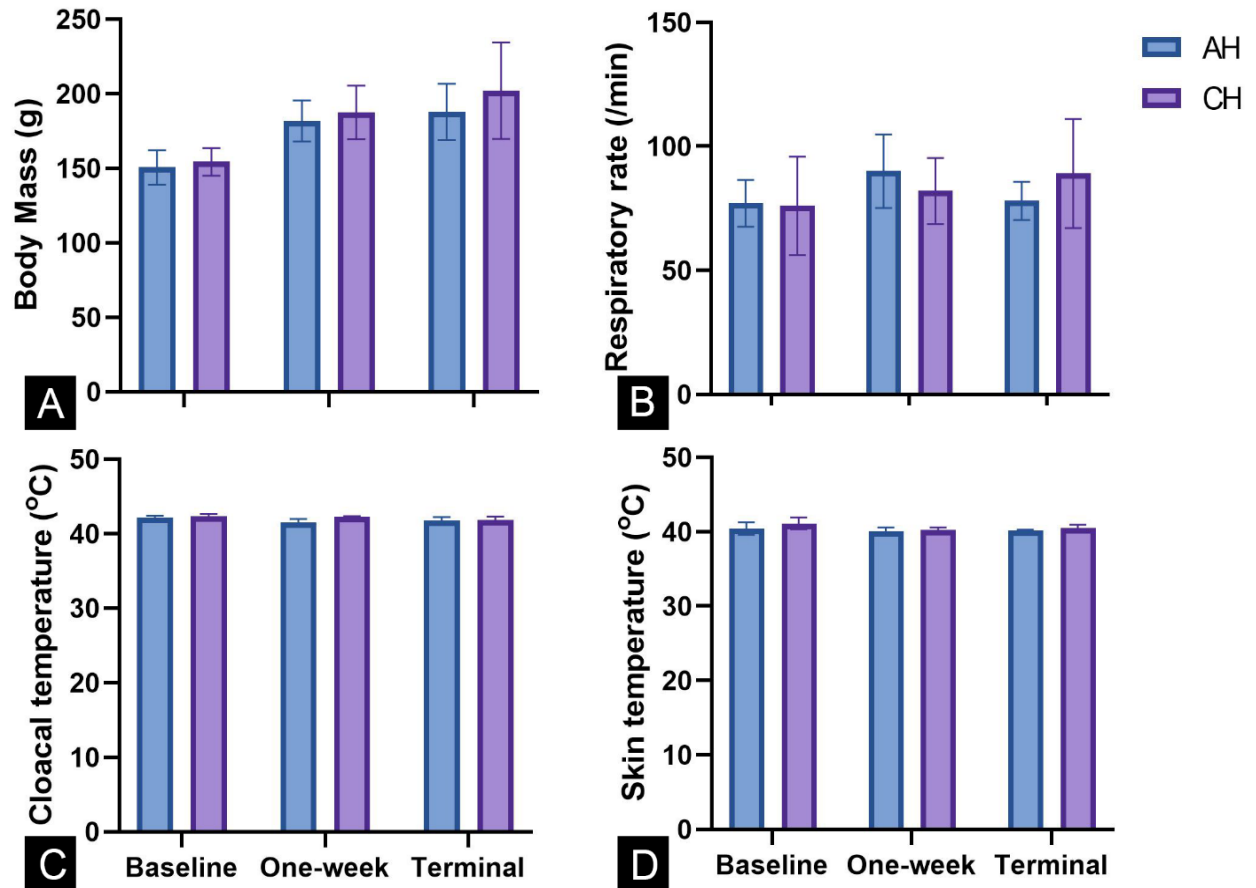


Figure 1: Summary of the daily body weight, respiratory rate, skin temperature and cloacal temperature measured from the Japanese quails ($n = 4$, for each group) during the pilot study. Baseline measurements were taken one day after arrival at the WRAF. Mean bars with standard deviation error bars.

AH – Acute heat stress, CH – Chronic heat stress.



Figure 2: Photograph of the thermometers used for the pilot study. A is the infrared thermometer used for the skin temperature measured under the wing. B is the digital thermometer used to measure cloacal temperature. For cloacal measurement, the silver-coloured tip was completely inserted into the cloaca.

Conclusion

The pilot study illustrates that no mortality occurred throughout the testing period at the said temperatures and the expected coping mechanism that birds employ to cool was also observed. As such, the proposed 38°C temperature stress regimen will be incorporated into the experimental paradigm and the method for measuring body temperature will be done using a digital thermometer inserted into the cloaca of the birds.

7.4 Appendix 4 — Registered scoping review protocol

THE IMPACT OF HEAT STRESS ON THE MORPHOLOGY OF THE JAPANESE QUAIL (COTURNIX JAPONICA) ORGANS: A SCOPING REVIEW PROTOCOL

Objectives

This review will identify available literature in databases and grey sources for studies on the effects of heat stress on the morphology of organs in the Japanese quail.

Question

Does heat stress have a negative impact on the morphology of organs in the Japanese quail?

Introduction

The climate changes occurring because of ozone layer depletion due to human activities are causing significant phenomenal fluctuations around the globe (IPCC, 2021). Extreme heat conditions are witnessed in several parts of the world, especially in sub-Saharan Africa (Yusuf et al., 2017). Increased ambient temperature affects all life forms, especially in poultry farms where many birds are kept in a small space (Nyoni et al., 2019). In addition, small birds like the Japanese quail are likely to face massive mortality, as predicted in a model study (McKechnie and Wolf, 2010). Excessive evaporative water loss due to high temperatures is expected to cause those mortalities. In addition, wild birds were noticed to lose body weight over two decades, mainly attributed to heat stress (Prokosch et al., 2019). Thus, heat stress is a global problem that will affect Japanese quail, which is a reliable alternative complement to provide protein food security in Africa (Mnisi et al., 2021, Olorunfemi et al., 2016). The research community also applied the quail as a model for various biomedical and poultry research (Baer et al., 2015, Huss et al., 2008). For example, Japanese quail were used to study senescence because of the neuroplasticity of its hypothalamus (Baer et al., 2015).

However, when exposed to heat stress birds reduce feed intake to minimize metabolic heat production hence glucose will be sourced by breakdown of fats and muscle protein. This happens because heat stress increased cellular energy demand multiple fold. The consequence will be body weight loss and propagation of oxidative stress. Furthermore, there is increased respiratory rate to combat heat stress by evaporative water loss through the respiratory surfaces. While there is relief from the heat, carbon-dioxide is lost rapidly from the blood leading to respiratory alkalosis, which affects blood ion balance including loss of calcium. Consequently, egg production will be affected because calcium is needed for quality egg production. The consequence of heat stress does not stop at this in the Japanese quail, because oxidative stress will develop and cause cellular and tissue damage leading to severe morbidity and even mortality. Few literatures can be found on the impact of heat stress on the Japanese quail organs but there are several on the biochemical and hormonal derangements caused by heat. For example, Mehaisen et al reported on the negative impact of heat stress on the gut of the Japanese quail in addition to other parameters analysed in the study. In a follow-up study immune system impairment was also analysed. Another example is Sahin et. al. (2003) in which impact of heat stress in the tissue by propagating oxidative stress was analysed. Furthermore, these studies aimed to test a dietary additive as a strategy to mitigate the impact of heat stress on the welfare of the Japanese quail.

In this scoping review, the aim is to review available data on the impact of heat stress on the Japanese quail organs, which will help identify gaps in the research on Japanese quail and the most appropriate mitigating strategy out of the several being tested.

Methodology

This study will follow the scoping review framework of the Joanna Briggs Institute, and reporting will be based on the PRISMA-ScR guideline.

Inclusion criteria

Primary experimental studies, reviews, reports and thesis of any language and all time that report on Japanese quail of any age, exposed to acute or chronic heat stress, will be included.

Studies that report on Japanese quail eggs or specific cell lines exposed to heat stress or studies that reported on Japanese quail experiments not exposed to heat stress or are exposed to cold stress will be excluded. In addition, studies on other types of quail will be excluded. Studies that reported morphological analysis of any organ in the Japanese quail but without heat stress will be excluded.

Search strategy

This review search will include PUBMED, SCOPUS, CAB direct, and WEB of SCIENCES (including BIOSIS previews, Current Content Connect, KCI-Korean Journal Database, MEDLINE, SciELO citation index) databases. Grey literature will be searched in BIORXIV (including MEDRXIV), ProQuest and google scholar.

Key terms identified are “Japanese quail”, “Heat stress”, and “Morphology”. The search query was developed using the Colandr web app (Cheng et al., 2018) as follows:

("Japanese quail" OR "Coturnix japonica" OR "Coturnix coturnix japonica") AND ("Heat stress" OR "Thermal stress" OR "Heat exposure" OR "Heat exposed" OR "Thermal exposure" OR "Thermal exposed") AND ("Morphology" OR "Histology" OR "morphometry" OR "morphometric" OR "morphological" OR "histological").

In a pilot search for studies, using the key terms, the researchers pooled 829 sources from Biorxiv (51), CAB direct (34), ProQuest (first 200 results selected), PubMed (108), and Web of Science (280). Following the removal of 271 duplicates, 28 (out of 558 unique studies) were identified to report morphological analysis of organs in Japanese quail exposed to heat stress. The selection process followed title and abstract screening using the Rayyan web application (Ouzzani et al., 2016).

Evidence selection

All the search results will be exported as .ris files from the databases and pooled in endnote library, then exported as endnote export, i.e. .txt, as explained in the Rayyan web application guide. The file will be imported into the Rayyan web application (Ouzzani et al., 2016), an

intuitive online tool for managing systematic reviews. Once imported, automatic de-duplication will be conducted any duplicate entry will be resolved. Two independent researchers involved in the study will select studies to be included, first based on the title and abstract of the sources and then secondly based on the full-text perusal of the sources. At this point, only included full-text sources will be assessed for data extraction.

Data extraction

Data charting for this review will be done using a unique data form piloted before the extraction (see appendix 1). There will be two data extraction stages, including the source's main characteristics, such as the country where the studies were conducted, the study's primary objective, the number of animals used for the study, and the study design. Details on the study design will include the temperature level, the duration (acute or chronic), and the style (cyclical or continuous) of exposure to the heat. The second stage will extract any data reported on any organ, whether morphological (descriptive) or morphometric (quantitative), at the gross or microscopic level.

Analysis of the evidence

Analysis of data from this study will be purely descriptive of both the morphological and morphometric data. No meta-analysis of quantitative or meta-synthesis of qualitative data will be conducted.

Presentation of the results

Results of this review will be presented using a table unless otherwise indicated by the nature of the data extracted.

Dissemination

The study results will be published in peer-review journals and presented at conferences.