Growth and development responses of *Gallus gallus domesticus* embryos to *in ovo* bromide exposure

by

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Declaration

I, Heike Luise Lucht declare that the thesis, which I hereby submit for the degree PhD Animal Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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List of Publications

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List of Abbreviations

Br^-	Bromide
CAM	Chorioallantoic membrane
Cl^-	Chloride
COC	Constituent of concern
CR	Crown-rump length
ED	Embryonic day
EDC	Endocrine disrupting chemical/s
F^-	Fluoride
FFU	Fitness-for-use
GH	Growth hormone
GIT	Gastrointestinal tract
GW	Groundwater
HCl	Hydrochloric acid
HD	Hatch day
HPGH	Hypothalamic-pituitary-growth hormone axis
HPT	Hypothalamic-pituitary-thyroid axis
I^-	Iodide
IGF	Insulin-like growth factor
K^+	Potassium
KBr	Potassium bromide
MB	Methyl bromide
MTL	Maximum tolerable level
Na^+	Sodium
NaBr	Sodium bromide
NaCl	Sodium chloride
NIS	Sodium/iodide symporter
NOAEL	No observed adverse effect level
PHCC	Potentially hazardous chemical constituent
RH	Relative humidity
SA WQG	South African Water Quality Guidelines
SP	Sampling point
T	Treatment
T_3	Triiodothyronine
T_4	Thyroxine
TDC	Thyroid disrupting chemical/s
TDS	Total dissolved solids
TG	Thyroglobulin
TH	Thyroid hormone
TR	Thyroid receptor
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
TWQR	Target water quality range
UP	University of Pretoria

WQ	Water quality
WQC	Water quality constituents
WQG	Water quality guidelines
WRC	Water Research Commission

Summary

Collated data showed that the concentration range of bromide (Br^-) in groundwater (GW) was between 0 – 132mg/L in selected provinces in South Africa where livestock production occurs. The no observed adverse effects level (NOAEL) of 0.01 mg/L was proposed and validated using the chicken embryo model.

The objective was to investigate the effect of a range of 0 – 1 mg/L Br^- on the pre-hatch growth and development of the chicken embryo to demonstrate the risk that Br^- exposure may pose to livestock and humans residing in the regions where livestock production depends on GW as main drinking water source.

The chicken embryo model was used to investigate the effects of Br^- on the growth and development of the heart, liver and brain because the thyroid hormone (TH) receptors are similar to that in mammals and the effects on TH are independent of maternal TH fluctuations. A volume of 200 µL NaBr at concentrations of 0, 0.01, 0.05, 0.5 and 1 mg/L was administered by *in ovo* injection and the wet mass of the whole embryo and the heart, liver and brain were measured on embryonic day (ED) 14 and 20, and on hatch day (HD) using a sensitive balance. The relative organ mass for each organ was calculated as individual organ mass over whole embryo mass and expressed as a percentage. Crown-rump (CR) and shank length were measured on ED 14, 20 and HD using callipers. These were not found to be reliable measures of pre-hatch growth.

Data were analysed using the GLM and REML procedures of SAS®. There was a significant treatment effect of Br^- on the relative heart mass, with the relative mass decreasing with increasing concentrations of Br^- ($P \leq 0.1$). It appeared that Br^- may have interfered with the pre-hatch growth of the heart.

The null hypothesis that different concentrations of Br^- do not have an effect on pre-hatch growth and development was rejected. The implication of this conclusion is that Br^- concentrations present in GW in excess of 0.01 mg/L could potentially pose a risk to livestock and human populations that are chronically exposed to the element in their drinking water.

CHAPTER 1

Introduction

Successful poultry production relies on a multifactorial approach. These factors include management of nutrition, genetics and general husbandry of the flock. Feed costs constitute the majority of the running costs of any production unit, but without unlimited free access to water, production will not be optimal. When an animal does not drink sufficiently to satisfy its needs, feed intake decreases and production is undermined.

Traditionally attempts to understand mineral deficiency and toxicity in animals have been approached from the feed perspective, and water was largely ignored for its contribution to pharmaco- and toxicokinetic of elements ingested from the environment.

In alignment with the five freedoms, animals must have free access to drinking water that is clean and safe for consumption. Currently a risk assessment is based on the elemental composition of water quality constituents (WQC) in a given water source against a set of predetermined water quality parameters that are set as guidelines.

The water quality guidelines (WQG) were developed for WQC that were deemed a possible health risk to livestock and humans, and extensive testing determined the safest minimum allowable concentrations for selected elements of interest.

Evolving technology widened the detection capability of testing facilities creating a need for regular revision of the WQG to include elements that were previously not considered problematic. The generic WQG are intended as a starting point for risk assessment of any water source, and it is prudent to consider that generic, concentration-based guidelines will not be sufficient for site-specific risk assessments due to a large multifactorial variation in WQC concentrations in different water sources.

Groundwater (GW) is the sole water source in many rural communities and includes all underground water (WWF, 2016). Poultry production can occur in the same rural areas and in this rural setting, it presented an interesting question about how much this dependence on a GW source affects the hatchability, chick quality and post-hatch performance of broilers produced under such conditions.

In addition to heavy metals and known toxic minerals, halogen anions or halides such as fluoride (F^-) and chloride (Cl^-) were identified as problematic elements in many GW samples collected from various sites in different provinces of South Africa after problems with livestock health and productivity were reported. As the scope of testing expanded to include more elements it emerged that bromide (Br^-) was present at concentrations well above all proposed and generally accepted safety levels in many of the water samples. Upon further investigation, it emerged that the disruptive effects of this halide on livestock physiology had been grossly underestimated.

The greatest natural reservoir for Br^- is seawater, which contains 65 mg/L Br^- and dietary sources of Br^- include salt made from brine with a high Br^- content (NRC, 2005).

Hypochloraemia and iodide (I^-) deficiency can be used as diagnostic tools to identify Br^- toxicity. The replacement of Cl^- in the body will influence Br^- toxicity (NRC, 2005) and the quantity of Br^- ingested will determine the degree of Cl^- replacement as there is an apparent threshold concentration above which Br^- is retained in the body (Casey *et al.*, 2017). It is yet

unclear how Br^- is distributed and where accumulation occurs. However, this was beyond the scope of this study.

The implication of Br^- as an endocrine disrupting chemical (EDC) along the hypothalamus-pituitary-thyroid (HPT) axis is explored in a review of literature, as well as how this effect can be measured in pre- and post-hatch growth.

The chicken embryo model is well recognised as a sensitive test for measuring the effect of potentially toxic substances on the whole body and on organ development. Further research is required to investigate the degree of Br^- transfer from water into the hen's plasma and subsequent transfer into the egg, which may have implications for the table egg industry and broiler chick production.

The purpose of this thesis was to explore the importance of water in poultry production and the risks that naturally occurring Br^- in GW pose to poultry production. The mechanism of action of thyroid hormones (TH) in growth was discussed. The study further explored the implications that hypothyroidism would have on chicken embryo growth, and how EDC such as Br^- can interfere with TH function. The study was expanded to validate the no observed adverse effect level (NOAEL) of 0.01 mg/L Br^- stated in the WQG and to investigate the response of the growth of the whole embryo and the heart, liver and brain to a range of Br^- concentrations administered as NaBr by *in ovo* injection into the albumen.

Aims and objectives

The aims of the study were numerous. Firstly, the aim was to validate the estimated minimum safe level or NOAEL of Br^- using the chicken embryo model. Secondly, it was to present a review of literature dealing with the importance of water in poultry production, the prevalence and pharmacokinetics of halides and the possible effect on TH function. Thirdly, the aim was to investigate the response of the growth of the heart, liver and brain of chicken embryos to a range of Br^- concentrations during the pre-hatch growth period.

The objectives of this study were to demonstrate the possible risk of Br^- exposure to poultry and highlight the risk of exposure to livestock production in areas where there are high concentrations of Br^- present in the groundwater.

Hypothesis

The H_0 was that a Br^- concentration range of 0.01 - 1 mg/L had no adverse effects on pre-hatch growth and development in Ross-308 broiler chickens. The H_A was that the growth and development of chicken embryos respond to *in ovo* exposure to Br^- during the pre-hatch growth period.

CHAPTER 2

Review of Literature

Introduction to review

The purpose of the review of literature was to provide an overview and background information to show what previous research had reported on in terms of water quality, thyroid disruption and the effects on embryonic growth and organ development. The background information covered four focus points: (1) the importance of body water and GW in poultry production, (2) a description of halides and the role of these in normal physiological function in the body, (3) a description of TH synthesis and the mechanism of Br^- as an endocrine disruptor and (4) the role of sentinel species and animal models in managing risk.

A total of 72 articles were selected based on the relevance to the topic to the noted focus points. Google Scholar and Science Direct databases were searched using "bromide", "thyroid hormones", "endocrine disruption", "chicken embryos" and "poultry production" among the key words. The chosen literature included historical and current research to provide an overview of baseline knowledge and what has been reported since. Historical research was not ignored since the principles of physiology were described. More recent research built on the principles reported in historical literature. Numerous Water Research Commission (WRC) reports provided historical water quality data, which was useful in identifying the water-borne chemicals that could disrupt the animal's physiology. A series of trials was designed using the chicken embryo model to address the role of Br^- in thyroid disruption that was absent from existing literature on water-borne chemicals. Thyroid function was not measured directly, but by deduction.

The importance of water in poultry production

Body water

Water in its pure chemical form (H_2O) has the important function in the body as the transport medium for all dissolved nutrients. It exists in the blood, muscles and organs, extra- and intracellular fluids. The water molecule is a polar inorganic compound with a diameter of approximately 2.75 angstroms or 0.275 nanometres. Water as a liquid can undergo various measures of dissociation into hydronium and hydroxide ions ($2\ H_2O \rightleftharpoons H_3O^+ + OH^-$). These properties of water permit two important processes in the body: The size of the molecule allows passage freely over all membranes and into all body spaces, and the dissociation permits bonding with other molecules to form composite molecules. The fluidity and density of liquid water at body temperatures (poultry body temperature is 41 – 42.2 °C) permit physical forces to move it around within the body. The strong cohesive properties permit capillary action. Water is a solvent due to its high dielectric constant. Substances that dissolve in water are hydrophilic as opposed to hydrophobic. Hydrophilic substances increase the osmotic pressure, which in turn facilitates the movement of water and the distribution of the solute. Water has a high latent heat capacity (4.1814 J/g.K at 25 °C) second to ammonia. The vaporisation rate is high and increases exponentially with increasing temperature at normal atmospheric pressure. Increased atmospheric pressure and increased density as in a solution diminishes the exponent

rate of increasing vaporisation. These properties of water determine the pharmacokinetic properties of water and solutions that underpin its vital role in living organisms.

The water requirement of an animal depends on numerous environmental factors such as temperature and relative humidity (RH), feed composition and the animal's physiological stage of production.

The percentage body water content of chicks is high during the first 7 days of the post-hatch growth phase decreasing proportionately as protein content followed by fat increase, and remains a major component of the broiler carcass throughout the growth period (Caldas *et al*., 2019). A 42-day old broiler will drink approximately 300 mL of water a day (NRC, 1994). Latshaw and Bishop (2001) stated that the broiler body consists of 72% water, whereas Chapman and Black (1967) stated that there is a difference in total body water as a percentage of body mass between sexes of 64.1 ± 2.9% for males and 62.0 ± 4.2% for females. Body water turnover differs between sexes, with females having a lower average half-life of the body water of 3.61 ± 0.33 days regardless of egg production rate compared with the average body water half-life of 7.3 ± 3.2 days in males (Chapman & Black, 1966). To maintain normal body water composition in broilers, drinking water must be available *ad libitum* (Caldas *et al.*, 2019). Chapman and Black (1967) reported an approximate body water pool turnover of 20% per day in females, translating into 125 mL/day/Kg body mass and 10% per day in males translating into 60 mL/day/Kg body mass respectively. For this reason, it is important to recognise water as a major medium of supplying elements to the growing chick, which may have synergistic or antagonistic actions within the body (Du Toit & Casey, 2010).

Du Toit and Casey (2010) showed that broilers treated with Br^- in drinking water had depressed water and feed intakes compared to the control group, and it emerged that I^- had an ameliorative effect on water intake when added to drinking water containing Br^-.

Ground water

Since water is essential for life and is the main component of body composition followed by protein (Caldas *et al*., 2019), it is important to seriously consider GW as a contributor of nutrients and anti-nutritional factors when formulating diets or choosing broiler production sites.

The water supply for livestock watering and domestic use is mostly GW, and its use is expected to increase for human consumption (DWA, 2013) especially in the regions of the country that lack perennial water sources (FAO, 2016). Water quality becomes especially important not only in terms of palatability but also from a water safety perspective.

Water was not traditionally considered as a source of microelements in livestock diets, and was viewed only as a necessity to promote feed intake and digestion. With increasing research, it emerged that the WQC in a GW source may have the ability to cause chemical pathology conditions within the animal body either in a synergistic or an antagonistic way.

A WQC is considered a constituent of concern (COC) or potentially hazardous chemical constituent (PHCC) when the concentration in a water sample exceeds the safe level reported in the South African Water Quality Guidelines (SA WQG) (Casey & Meyer, 2006).

Previous research showed that the WQC of interest in GW was F^-, for its widespread adverse effects on livestock production. It was shown that 0.1 mL of inorganic F^- administered by *in ovo* injection into the yolk was toxic to chicken embryos, which resulted in 50% fewer

live embryos and 2.5 times more stunted growth of embryos compared with the untreated control group (Khanum *et al.*, 2019). It emerged from further testing that other problematic elements were also present in GW. The growth rate in embryonic day (ED) 14 chicken embryos was depressed by F^- in the egg and this was evident by the smaller crown-rump (CR) length (Khanum *et al.*, 2019). Due to its propensity to cause severe toxic effects research identified a NOAEL value for F^-, which is reported in the SA WQG (Casey & Meyer, 1996).

Inorganic Br^- was shown to unfavourably influence broiler production parameters even after short-term exposure to a maximum concentration of 3 mg/L Br^- in drinking water (Du Toit & Casey, 2010).

In South African rural communities, GW is the most widely used drinking water source (Ncube & Schutte, 2005) and it is limited because large porous aquifers occur in limited areas (FAO, 2016). The composition of GW depends on the geological composition of the rock surrounding the aquifer, as well as geothermal conditions underground. Geo- and topographical characteristics surrounding aquifers enhance the processes of releasing ions, and this is true for the presence of naturally occurring Br^- (Magazinovic *et al.*, 2004). The concentration of WQC in GW depends on the availability and solubility of elements, the geochemical environment i.e. pH, and ion exchange processes (Marandi & Shand, 2018). The GW in aquifers changes composition and density from the top down within the aquifer, and the physical and chemical properties of the water can change when the water flows naturally between aquifers. The salinity of recharge water and the depth of the borehole can influence the WQC composition of GW (Nissenbaum & Magaritz, 1991). Aquifer depth will also influence the effect of evaporation on WQC (Marandi & Shand, 2018).

In routine GW analyses, known COC, total dissolved solids (TDS) and turbidity are tested. TDS refers to the sum of inorganic substances in solution and high TDS can affect palatability (Mpenyana-Monyatsi & Momba, 2012). Turbidity is the measure of clay and soil particles together with organic substances, and may be an indicator of microbiological contamination (DWAF, 1996 as cited by Mpenyana-Monyatsi & Momba, 2012). Ncube and Schutte (2005) reported high concentrations of F^- in GW in Limpopo, Northern Cape, North West and KwaZulu-Natal Provinces, which corresponded with the high Br^- concentrations reported for those provinces (Casey & Meyer, 2006).

Marandi and Shand (2018) noted that GW chemistry interpretation should begin with an assessment of the chemistry and origin of the source water, because recharge source would define the initial conditions of the underground water system. This is because the rate of dissolution of elements from the rock surrounding the aquifer will be influenced by water pH and the pump rate of the borehole, and in coastal areas salt intrusion may also be a contributing factor. Ncube and Schutte (2005) reported that at the time of their study, only a few boreholes had been tested for PHCC, and it was generally accepted that despite the high F^- content in the water sample, and independent of the concentrations of other WQC, the water was considered to be safe for consumption.

There is an overwhelming body of research available on Br^- as a by-product of wastewater treatment or desalinization of seawater. Much is written about organic Br^- of agricultural origin as a pollutant of water bodies and boreholes. Very little information exists on naturally occurring inorganic Br^- in GW and its effects on the physiology of livestock that are chronically exposed to low concentrations present in the drinking water. In contrast, there

is a wealth of information about dental fluorosis and the link to high F^- concentrations in drinking water, making F^- one of the common halides monitored in GW along with Cl^- (Ncube & Schutte, 2005).

Water has largely been ignored as a source of essential and non-essential microelements. As noted in previous studies, GW analysis included Cl^- and F^- (Ncube & Schutte, 2005; Mpenyana-Monyatsi & Momba, 2012) and when the concentration of F^- present in the GW exceeds the WQG limits for drinking water it can be considered to be supplementary or even toxic (Ncube & Schutte, 2005). It is possible that a WQC in excess of the NOAEL may be supplementary to livestock in a situation where the element in question has essential functions within the body and is lacking from the feed or environment. It is also possible that the element exceeding the NOAEL may become toxic to livestock when there is a cumulative effect and the body is unable to eliminate the excess through various excretion routes. For this reason it is dangerous to assume that WQC concentrations exceeding the NOAEL can be considered supplementary without considering the type of element and its pharmacokinetic properties within the body. In a similar way, low concentrations of inorganic Br^- in drinking water can become toxic in the case of long-term exposure.

In light of these findings and with improving technology, it becomes more important to broaden the scope of elements included in water analysis to include inorganic Br^- as a potential COC or PHCC for adequate risk assessment at specific sites.

The role of halides in the body

Halogens are a group of non-metallic elements forming strong acidic compounds in the presence of hydrogen, and halides are the anionic forms of these elements.

Although F^- was traditionally considered as the main problematic halide in GW, the halides discussed in more detail for the purpose of this review are I^-, Cl^- and Br^-, and how they may play a role in the electrolyte balance within the body.

Iodide

Prevalence

Rooke *et al.* (2010) stated that in humans more than half of I^- is taken in from animal products. In South Africa, I^- is ubiquitous in most regions and animals usually obtain sufficient quantities from the environment and in natural feed. In mammals, the I^- requirement increases in pregnancy to supply both maternal and foetal TH needs (Waugh, 2019). This may also be true in avian breeding females for sufficient TH to be deposited in the yolk to support the developing chick before the embryonic thyroid gland becomes functional. Röttger *et al.* (2012) reported that after in-feed I^- supplementation, the highest I^- concentration occurred in the thyroid gland, and medium concentrations occurred in the liver and blood serum of laying hens. The distribution of I^- would be similar in hatched growing broilers (Du Toit & Casey, 2010; 2012). In mammals, maternal I^- is an essential micronutrient and important in early embryonic development especially for foetal neurodevelopment (Velasco *et al.*, 2018). This would be true for avian species.

Pharmacokinetics

I^- is absorbed from the gastrointestinal tract (GIT) and transported to the thyrocytes via the bloodstream. (Carvalho & Dupuy, 2017). Body I^- levels are regulated by uptake into the thyroid gland and excretion of excess I^- in urine (Rooke *et al.*, 2010). The competitive nature of Br^- may mean that Br^- is preferentially absorbed from the GIT and travels to the thyrocytes to displace the I^- necessary for TH synthesis. In layers, there is a high carry-over effect of I^- from feed into eggs (Rooke *et al.*, 2010; Röttger *et al.*, 2012). Broilers exposed to I^- in drinking water showed an accumulation of I^- in the thyroid gland, liver and kidneys (Du Toit & Casey, 2010; 2012), thus carry-over effect could occur in eggs from broiler breeders exposed to I^- in drinking water. Yalcin *et al.* (2004 as cited by Rooke *et al.*, 2010) reported a linear relationship between I^- intake and I^- content in the egg, showing a significant response to I^- supplementation (Rooke *et al.*, 2010). If I^- in the feed moves easily into the egg, and I^- and Br^- have similar pharmacokinetic properties then it is possible that Br^- in drinking water may also move easily from the hen's plasma into the egg.

The translocation of I^- into the follicular lumen of thyrocytes is known as I^- efflux, and can be mediated by the Cl^- channel, using a Cl^-/I^- transporter (Carvalho & Dupuy, 2017). It is possible that Br^- could be transported into the follicular lumen by the same mechanism, since it competes with Cl^- and is preferentially transported. Apparent I^- deficiency in the presence of excess competing halides can prevent the normal I^- recycling in the halide reservoir of the body (Waugh, 2019).

The Na^+/K^+-ATPase pump on the membranes of thyrocytes actively generate a transmembrane Na^+ gradient, which drives I^- transport (Carvalho & Dupuy, 2017). Excess F^- has been shown to inhibit Na^+-K^+-ATPase pump functioning, and thus sodium/iodide symporter (NIS) function, by elevating thyroid stimulating hormone (TSH) and thyroglobulin (TG) to cause hypothyroidism, contributing to I^- depletion in the body reservoir (Waugh, 2019). The NIS mediates I^- uptake across cell membranes in the thyroid gland, salivary glands, gastric mucosa and the placenta (Waugh, 2019). The same mechanism may function to transport Br^- into thyrocytes where it competes with I^- preferentially. Transport of iodothyronines (T_4 and T_3) into cells of different species relies mostly on Na^+- and energy dependent transport, and cellular influx of TH is independent of intracellular metabolic capacity (Hennemann *et al.*, 2001).

Studies have shown that supplementation of I^- to laying hens increased the I^- concentration in the blood serum, thyroid gland, liver and whole egg, and that the concentration in the yolk is 93% compared with 7% in the albumen (Röttger *et al.*, 2012). This stands to reason since the liver is a storage organ for I^- (Röttger *et al.*, 2012) and the yolk components deposited into the egg originate from the liver (Trampel *et al.*, 2003).

In ovo I^- supplementation was shown to increase IGF-I and IGF-II gene expression (Goel *et al.*, 2016). Somatotropin expression was higher for I^- supplemented embryos on ED 20 and hatch day (HD), which decreased in the first week post-hatch (Goel *et al.*, 2016).

Chloride

Prevalence

Cl^- is one of the principal electrolytes in the body responsible for regulating fluid distribution between intravascular and cellular spaces in the body (Kataoka, 2017). It makes

up 70% of the total anion content in the body, which makes it the most important extracellular anion (Berend *et al.,* 2012). Sufficient quantities of Cl^- are usually available to livestock from premixes added to feed. Excess serum Cl^- can occur when Br^- is preferentially transported across the cell membrane into the intracellular fluid.

Phamacokinetics

Absorption of Cl^- across the cell membrane is coupled to Na^+ transport in an electrically neutral way (Frizzell *et al.,* 1979). Cl^- is absorbed from the GIT and secreted as hydrochloric acid (HCl) in gastric juice (Berend *et al.*, 2012). For water to move into the intestine, Cl^- is necessary to create an osmotic gradient (Berend *et al.,* 2012). The increased vascular volume resulting from serum Cl^- accumulation could increase the burden on the cardiovascular system (Kataoka, 2017).

Active Cl^- absorption stops when Na^+ is absent, and the inverse is true (Frizzell *et al*, 1979). Different tissues of the GIT of various species demonstrate Na^+-coupled Cl^- transport into cells, such as the ileum in humans and rabbits, the colon in rats and the rumen in ruminants (Frizzell *et al.*, 1979).

Cl^- transport occurs by three mechanisms including Na^+-coupled mechanisms, potassium (K^+) channels and the Na^+-K^+-ATPase pump (Berend *et al.*, 2012). Since Br^- moves across membranes via the same mechanism as Cl^-, the translocation of Br^- across the rumen epithelium may give Br^- the opportunity to interfere with physiological processes that require Cl^- in ruminants. The same may be true in monogastric livestock such as poultry.

Excess Cl^- is excreted in the urine by the kidneys, and Cl^- channels are present along the entire nephron in mammals, interacting with Na^+ to maintain fluid balance (Berend *et al.*, 2012). The quantity of Cl^- excreted depends on intake from diet and water, and body requirements (Berend *et al.*, 2012).

There are eight Cl^- channels in the heart (Duan, 2009) and these are distributed unevenly across different regions of the heart (Mulvaney *et al.*, 2000). At rest, opening of the Cl^- channels causes an efflux of Cl^- from the cell, which generates an inward current (Mulvany *et al.*, 2000). Cardiac Cl^- can generate both inward and outward currents, which can have an effect on the pacemaker activity of the heart (Duan, 2009). These channels are necessary for the correct function of the heart muscle and different channels have different affinity to halides, such as I^- (Mulvany *et al.*, 2000). This may be a potential site for Br^- to interfere with heart muscle function by competing with both I^- and Cl^-.

Hypochloraemia is a condition where excessive serum Cl^- is lost from the body as a result of renal or gastric loss, or as a result of dilution by excessive fluid accumulation as seen in congestive heart failure (Berend *et al*., 2012). Preferential reabsorption of Br^- in the kidney may result in Br^- toxicity that can lead to hypochloraemia and interference with normal fluid balance maintenance.

Bromide

Prevalence

Both inorganic and organic Br^- pose a potential health risk to livestock and humans alike. There is a wealth of information about the negative effects of organic Br^- on human and animal health, specifically the water disinfection by-products trihalomethanes and haloacetic acids

(Sawade *et al.*, 2016) and the fumigant methyl Br^- (CH_3Br, MB) used in agriculture. It is known that during water treatment with Cl^-, aqueous Br^- present in the water will substitute into organic compounds more readily than Cl^- in the presence of organic matter (Sawade *et al.*, 2016). When water is treated with Cl^- it can lead to the formation of toxic disinfection by-products such as the trihalomethanes and various species of organic brominated compounds when Br^- is present in the untreated water (Sawade *et al.*, 2016).

Not much is known about the effects of inorganic Br^- on human and animal health, beyond laboratory animal studies to verify the safety of Br^--containing drugs or human cases of exposure in an industrial setting.

In nature, inorganic bromine exists as part of many mineral complexes and in the dissociated anionic form, Br^- forms salts with cations like Na^+ and K^+ that dissolve easily in water (WHO, 2009). Although it had not been FDA approved the inorganic halide salt, potassium bromide (KBr) is used as an anti-epileptic drug in dogs (Baird-Heinz *et al.*, 2012) and a calmative in horses (Raidal & Edwards, 2008).

In GW, inorganic Br^- has been found to be naturally occurring due to the geology prevalent in the aquifers of localities across South Africa. Br^- from inorganic and organic sources is a ubiquitous anion found in trace quantities in soil and crops used for animal feed. Deficiency has not yet been reported, but toxicity has been suspected in areas where the concentrations of Br^- in the environment have been high enough to cause physiological disturbances in livestock. The normal Br^- concentration in urine in humans is <16 mg/L and in serum is <5 mg/L (Budnik *et al.*, 2012).

Anthropogenic organic Br^- poses a considerable health risk to livestock and humans alike, the most notable of the methyl halides being MB, which is a colourless and odourless volatile halogenated hydrocarbon traditionally used as a fumigant in green houses and for dry foodstuff (Wilson *et al.*, 1998; Suwanlaong & Phanthumchinda, 2008; Budnik *et al.*, 2012). MB dissociates into Br^-, which has disinfectant properties, and its residual effect can cause it to become toxic to animals in environments where Br^- is routinely used as a disinfectant.

When MB is used as a fumigant against parasites, insects, weeds and fungi in soil (Suwanlaong & Phanthumchinda, 2008), it has the ability to enter the animal body through contamination of the feed. Due to its toxicity, MB was to be phased out completely as a pesticide by 2015 (Budnik *et al.*, 2012). The seepage of dissociated Br^- through the soil into the GW can contaminate drinking and irrigation water sources.

Pharmacokinetics

The half-life of Br^- differs between species and depends on the Br^- source (Raidal & Edwards, 2008). The serum half-life of Br^- in humans is 12-16 days and in rats is 1.5-3.5 days, which highlights the importance of considering the interspecies physiological differences in elimination kinetics when compounds are tested to determine NOAEL (Budnik *et al.*, 2012).

The most biologically active of the organic Br^- compounds is MB, which is a cumulative poison (Budnik *et al.*, 2012). The action of MB is mainly on the nervous system resulting in dizziness and convulsions in cases of severe toxicity (Suwanlaong & Phanthumchinda, 2008). The major excretion routes of MB are through the lungs, urine and faeces (Budnik *et al.*, 2012). The residual effect of MB is known to be carcinogenic to humans especially in genetically

predisposed individuals, and even chronic low exposure caused central nervous system (CNS) depression (Budnik *et al.*, 2012).

Due to its ease of movement throughout the body, inorganic Br^- isotopes have been used as a tracer molecule to determine body water distribution (Vaiseman *et al.*, 1986 and Cheek, 1953 as cited by Raidal & Edwards, 2008).

KBr and NaBr have slightly different gastric absorption patterns due to different molecular weights (Baird-Heinz *et al.*, 2012). Gastric and salivary glands excrete Br^- in a higher concentration in relation to Cl^- resulting in relatively higher Br^- present in gastric juice after Br^- intake (Gamble *et al.*, 1953). This has implications in ruminants where the possibility of Br^- recycling exists due to the mechanism of rumination as a key component of digestion.

The kidneys, nervous system and thyroid gland are some of the target organs of Br^- (Suwanlaong & Phanthumchinda, 2008). Br^- is excreted unchanged by the kidneys, competing with Cl^- for tubular resorption, and easily distributed into the cerebral spinal fluid and interstitial tissues of the brain (Baird-Heinz *et al.*, 2012).

There is competition between Br^- and I^- for the tyrosyl residue halogenation of TG, and brominated thyronines are known to mimic TH activity (Buchberger, 1988). NaBr appeared to have a greater affinity for the thyroid gland and a greater effect on the disruption of TH synthesis than KBr (Baird-Heinz *et al.*, 2012).

Clearance of Br^- depends on serum Br^- concentrations, kidney function, administration of intravenous saline (NaCl), the Cl^- content of the diet and the individual's sensitivity to toxicosis (Baird-Heinz *et al.*, 2012).

Toxicity

Organic Br^- compounds formed as disinfection by-products have been shown to be acutely cytotoxic in humans (Sawade *et al.*, 2016), which is why research on organic Br^- compounds has been prioritised in literature. However, since dissolved inorganic Br^- preferentially binds to organic compounds in the body, the formation of brominated TG is likely when the Br^-/I^- ratio is high due to either I^- deficiency or high Br^- intake (Buchberger, 1988). Sawade *et al.* (2016) found that the toxicity of treated water increased with increased Br^- concentration, where a 4-fold increase in organic Br^--related cytotoxicity was seen when the Br^- concentration of the water samples was increased from 0.06 mg/L to 0.39 mg/L.

The purpose of this study has been to focus on the potential toxicity mechanisms of inorganic Br^- in the body.

Maternal hypothyroidism induced by fluorosis may lead to biochemical disturbances in the foetus that can potentially cause irreparable damage to the physiological processes in offspring (Waug, 2019). In a similar way, Br^--induced hypothyroidism can affect foetal growth and postnatal offspring viability. Normal foetal development is dependent on correct thyroid function because TH is necessary for maintaining energy metabolism, cardiac function and bone remodelling (Boas *et al.*, 2012). This has implications in broiler and layer production where these physiological processes are the cornerstone of production efficiency.

The halides play an important role in maintaining fluid balance and regulating metabolism. Any imbalance as a result of interactions between halides can have deleterious consequences on the animals' optimal physiological functioning. An over-supply of Br^- beyond

the capacity of the body to effectively deal with the physiological disturbances will result in Br^- toxicosis.

Thyroid hormones and endocrine disruption

Development encompasses differentiation and maturation of tissues and TH is critical in the development of specific tissues in birds (Buzała *et al.*, 2015). TH is necessary for protein synthesis and cell growth (Basu & Mohapatra, 2012). TH is vital in regulating metabolism and is required for normal reproduction (Scanes & McNabb, 2003). The development and maturation of most chicken organs depends on TH (Darras *et al.*, 2011). Anderson (2001) reported that there is a window period during which TH exerts its effect on brain development. If TH is insufficient during this window period then potentially irreversible brain damage may occur. Normal brain development depends on TH (Heuer, 2007) and it was shown in humans that even small degrees of *in utero* TH disruption could result in neuropsychological deficits in children despite normal thyroid status at birth (Gilbert & Lasley, 2013).

Growth hormone (GH) secretion is under the control of thyrotropin-releasing hormone (TRH) (Buzała *et al.*, 2015) and TH regulates TRH production. TH has been shown to be responsible for cardiac growth and angiogenesis (Rutigliano & Zucchi, 2017). In the kidney, TH is required for renal development and function (Basu & Mohapatra, 2012). Decreased concentrations of TH in yolk results in smaller chicks at hatch (Buzała *et al.*, 2015).

The two major hormones T_4 and T_3, collectively referred to as TH, regulate metabolism within the body both generally or at organ level. T_3 is the functionally active form of TH and plays a pivotal role in control of metabolism within the body (Mohammadalipour *et al.*, 2017). TH is critically important in the development of the CNS and the musculoskeletal system, as well as vital to controlling thermoregulation (Scanes & McNabb, 2003). The tissue thyroid status depends on the TH secretion as well as normal TH metabolism, and availability and distribution of thyroid hormone receptors (TR) in the target tissues (Malik & Hodgson, 2002).

Thyroid hormone synthesis

Thyroid epithelial cells produce TG, which is secreted into and iodinated in the follicular lumen after which degradation of TG produces T_4 and T_3 that are released into the blood (Ikegami & Yoshimura, 2017).

The synthesis of T_4 occurs in two steps, first by iodination of tyrosines in TG followed by coupling of two iodinated TG (Waugh, 2019). I^- recycling is a crucial process in TH synthesis (Carvalho & Dupuy, 2017) but if Br^- is preferentially taken up by the thyrocytes then this process is disrupted and hypothyroidism results even in the presence of sufficient I^-. When there is insufficient T_4 uptake there is a decreased quantity of I^- released due to the unavailability of T_4 for deiodination to T_3 (Hennemann *et al.*, 2001). Normal embryonic TH production depends on I^- availability for uptake into the thyroid gland (Forhead & Fowden, 2014).

TH metabolism is regulated by three groups of enzymes that are responsible for the conversion, activation and deactivation of T_3 and T_4 (Malik & Hodgson, 2002). Conversion of T_4 to T_3 is mediated by the enzymes deiodinases type I and II. Different tissues will express different enzymes for the local regulation of TH. Deiodinase type I is primarily expressed in

the liver and kidney and has a much lower affinity for T_4 whereas deiodinase type II action is more widely expressed and is responsible for T_3 concentrations in plasma and in different tissues (Janssen *et al.*, 2007). Deiodination plays an important role in the bioactivity regulation of TH (Darras *et al*., 1999).

Increased T_4 secretion is the result of increased metabolic rate, and peripheral deiodination to T_3 occurs mainly in the liver and kidneys (Luger *et al.*, 2001). Free T_3 and T_4 are present in the plasma at a steady concentration whereas free TH concentrations in different tissues vary according to the deiodinase activity of a specific tissue (Malik & Hodgson, 2002).

When I^- is deficient or unavailable, the thyroid gland becomes unable to synthesise sufficient quantities of TH resulting in hypothyroidism (Waugh, 2019). Iodinated TG reabsorption is a key component of TH synthesis and despite I^- being a major component of TH, its biosynthesis does not depend solely on I^- specificity, but also on other pathways outside the thyrocytes (Carvalho & Dupuy, 2017).

The iodination of tyrosyl residues is catalysed by thyroid peroxidase and is the mechanism by which TH is synthesised using I^- and hydrogen peroxide (H_2O_2) in the thyroid gland (Buchberger, 1988).

Deiodination of T_4 to the biological form T_3 is tissue specific, such as in the heart muscles where T_3 functions to enhance diastolic and systolic functions to increase heart rate (Rutigliano & Zucchi, 2017). The bioavailability of TH depends on the presence of intracellular TR in the cells of target tissues (Heuer, 2007; Forhead & Fowden, 2014). Nuclear TR mediate TH action through gene transcription at the nuclear level in the target cell (Anderson, 2001; Janssen *et al.*, 2007). Where T_4 contains Br^- and not I^-, the TR in target tissues will not recognise the molecule mimicking TH, and correct biological action will be inhibited (Hennemann *et al*., 2001).

Mechanism of action

Hormones such as GH, TSH and cortisol have diurnal secretion patterns (Roelfsema *et al*., 2017). T_3 controls GH and IGF-I activities, which influence body growth (Cabello & Wrutniak, 1989).

TH is essential for developmental and metabolic processes throughout the lifecycle of an animal (Velasco *et al.*, 2018). Conversion of T_4 to T_3 occurs at tissue level, is tissue specific and different between species (Ikagami & Yoshimura, 2017). Different tissues have different sensitivity to TH and thus respond differently to physiological stimuli (van der Spek *et al.*, 2017). The liver, heart, kidney and brain are organs in which TH plays an important role (van der Spek *et al.*, 2017).

In the liver various plasma proteins are synthesized that bind TH to provide a large, rapidly exchangeable pool of circulating hormone (Malik & Hodgson, 2002).

TH specifically targets the cardiovascular system, and is necessary for the expression of myofibrillar proteins and maintenance of the normal cardiovascular haemodynamic (Janssen *et al.*, 2007; Shuvy *et al*., 2009; Delitala *et al.*, 2017). Hypothyroidism results in low cardiac output (Basu & Mohapatra, 2012). Selection pressure on modern broiler genetic lines has led to cascade effects on muscle and cardiovascular systems (Schmidt *et al.*, 2009).

The kidney requires TH for optimal renal plasma flow, vascular resistance and glomerular filtration rate (GFR) (Schairer *et al.*, 2020) and consequent urinary concentration

and dilution (Katz *et al.*, 1975). In the kidney, TH influences Na^+ reabsorption at the proximal convoluted tubules and affects the renin-angiotensin-aldosterone axis (Basu & Mohapatra, 2012). If Br^- interferes with TH action in the kidney it will eventually exert a cascade effect on the electrolyte balance in the blood. The metabolism of I^- and TH occurs outside of the thyroid gland in peripheral tissues and the kidney plays an important role in this (Katz *et al.*, 1975).

In the mammalian brain, TH regulates the migration and differentiation of neural cells, synaptogenesis, and myelination (Bernal, 2007). This may be similar in the avian brain as all vertebrate embryos develop in a similar way up to a specific point where after species-specific development occurs (Hamburger & Hamilton, 1951). The actions of TH are mediated through TR in the cell nucleus and regulation of gene expression within the cells (Nakai *et al*., 1988; Bernal, 2007). There are differences in the expression patterns of individual TR isoforms during early and late brain development respectively and the expression patterns change over time (Anderson, 2001).

The mechanism of thyroid homeostasis is complex and can be disrupted by any changes in the thyroid gland (Boas *et al.*, 2012). Serum T_3 and T_4 concentrations were shown to decrease when I^- was deficient, coupled with excess F^- (Waugh, 2019). There is a possibility that I^- deficiency can be induced even when a sufficient quantity is ingested, when competing halides such as Br^- are present in the body to interfere with sufficient uptake.

Free hormone concentration is an extracellular factor regulating cellular TH uptake and T_3 production, and in humans 80% of plasma T_3 is produced peripherally outside of the thyroid gland by target tissues (Hennemann *et al.*, 2001).

The HPT axis regulates TH production by means of a negative feedback loop (Fig. 1), and energy homeostasis, growth and development depend on TH (van der Spek *et al.*, 2017). Downregulation of the HPT axis and altered peripheral TH metabolism are driven by the reduction of circulating TH concentrations (Janssen *et al.*, 2007).

Disruption of TH synthesis by Br^- interference would potentially disrupt the negative feedback loop causing compensation mechanisms by the thyroid gland to mitigate hypothyroidism and leading to thyroid hypertrophy. Negative feedback on TSH secretion is necessary and occurs by TH action on the gene transcription of TRH and peptide synthesis (Roelfsema *et al.*, 2017). The regulatory feedback loop is disrupted when TH synthesis is compromised

Increased plasma T_3 concentration will increase metabolic rate (Mohammadalipour *et al.*, 2017) stimulating increased oxygen consumption by mitochondria (Harvey & Williams, 2002). T_3 is involved in cell proliferation and TR are expressed in tissues in varying numbers, orchestrating tissue-specific responses to T_3 (Harvey & Williams, 2002). In avian embryogenesis, the sequence of cell proliferation, differentiation and maturation of each tissue and organ is controlled by TH (Darras, 2019).

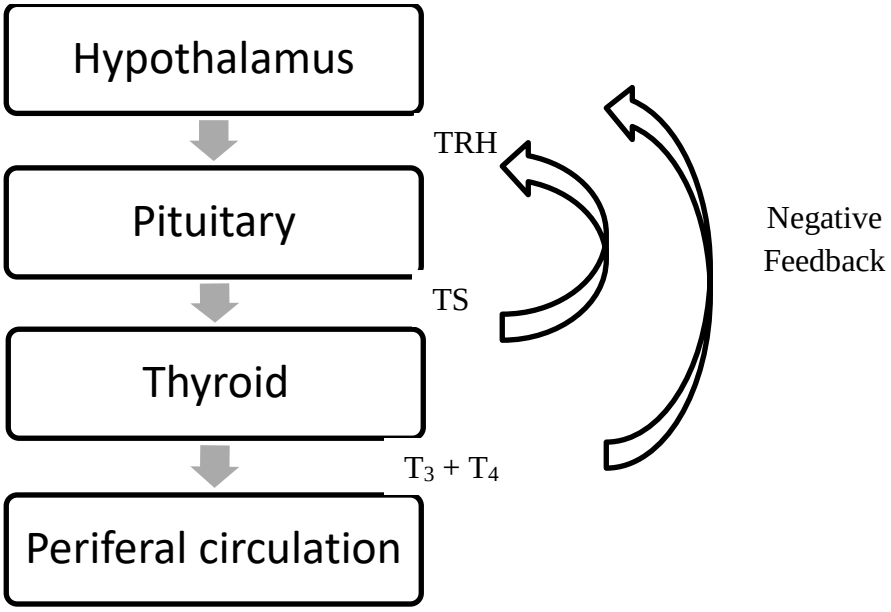

Figure 1 The hypothalamus-pituitary-thyroid axis feedback loop.

In vertebrates, the role of TH is to control growth and differentiation of nearly every organ (Darras *et al.*, 2011; Jugan *et al.*, 2010). Essential functions in vertebrate metabolism and development are controlled by TH, and TH degradation to inactive metabolites is controlled by iodothyronine deiodinase type 3, which serves as a mechanism guarding against an oversupply of TH in target organs such as the liver (Tsai *et al.*, 2002).

It is well known that TH is pivotal in regulating cardiac function (Martinez, 2016; Rutigliano & Zucchi, 2017). A higher growth rate results in faster metabolism and thus greater turnover of T_4 to T_3 in tissues, which predisposes fast growing birds to lower thermal stress tolerance when plasma TH concentration is insufficient to support higher metabolic rate (Al-Rukibat *et al.*, 2017).

Endocrine disruption and hypothyroidism

The definition of an EDC in the broad sense is any substance that can potentially interfere with the endocrine system (Jugan *et al.*, 2010). Traditionally the mechanism of EDC has been investigated within the context of how receptor binding interference impairs reproduction in mammals. However, with the evolution of technology, endocrine disrupting mechanisms are no longer limited to receptor-only actions, but expand to include the interference of protein transcription (Scanes & McNabb, 2003). The effects of EDC on thyroid signalling have been explored more recently (Jugan *et al.*, 2010). Hens exposed to goitrogens under experimental conditions decreased maternal TH deposition in the yolk, which may have irreversibly inhibited later-stage embryonic thyroid gland functioning (Darras, 2019).

Chemicals that mimic TH actions may disrupt TH function at the level of TR (Jugan *et al.*, 2010). When Br^- out-competes I^-, T_4 production is hampered in the thyroid gland and T_3 levels in the body will decrease thus disrupting the regulation of TH production in the HPT axis.

The subset of EDC that specifically disrupt thyroid function, TH synthesis and action are referred to as thyroid disrupting chemicals (TDC) (Jugan *et al.*, 2010).

Transport of TH across cell membranes and subsequent interactions with cytoplasmic proteins determines the distribution of TH within the target cells (Tata, 1999). Nutrient deficiency, high ambient temperature and goitrogenic substances can induce hypothyroidism and disruption of the TH axis can occur at any level of the cascade (Jugan *et al.*, 2010).

It has been shown in birds that toxicant-induced growth restriction can occur in the whole body or be limited to depressed growth rates of specific body parts through local endocrine and non-endocrine effects on growing tissue, or from interference with the hypothalamic-pituitary-growth hormone (HPGH) axis (Scanes & McNabb, 2003). Propylthiouracil-induced hypothyroidism in chickens resulted in a significant reduction of IGF-I in the circulation (Tsukada *et al.*, 1998). Japanese quail embryos from quail hens treated with the goitrogen ammonium perchlorate were shown to have decreased TH and body weight at ED 14 (Darras, 2019). Perchlorates competitively inhibit I^- transport into thyroid follicular cells (Jugan *et al.*, 2010) and Br^- may act in a similar way.

Increased thyroid activity in late embryogenesis increases the plasma T_4 concentration necessary to support fast growth in the latter stages of incubation (Al-Rukibat *et al.*, 2017).

Subclinical hypothyroidism is characterised by elevated TSH and a normal range of TH concentrations in circulation (Williams & Bassett, 2018). Functional hypothyroidism either expressed as decreased TH production or altered peripheral T_4 metabolism may be the result of selection for higher growth rate because thyroid function regulates metabolic rate (Decuypere *et al.*, 2000).

Regulation of TH metabolism and action is complex and there may be multiple targets for interference by EDC along the complex regulatory network (Schmutzler *et al.*, 2004). The possibility of Br^- interfering with TSH receptors may result in disruption of TH synthesis and inhibition of all thyroid mediated endocrine axes (Boas *et al.*, 2012). TDC can target any level of the HPT axis and are able to affect multiple levels simultaneously (Jugan *et al.*, 2010). Plasma TSH levels can be used as an indicator of the degree of hypothyroidism in the body (Anderson, 2001).

Knock-out mice lacking genes for the TR responsible for cardiac function had low heart rate and resultant reduced energy expenditure, whereas those lacking all types of TR genes showed depressed growth, severe hypothyroidism and even death at weaning (Harvey & Williams, 2002).

Regulation of the HPT axis can be the target of TDC, affecting TH clearance in the liver and kidneys (Jugan *et al.*, 2010). Renal physiology and function are compromised when TH dysfunction occurs (Basu & Mohapatra, 2012). If Br^- is reabsorbed preferentially in the kidney, then brominated TG may be retained and TH balance disrupted. Accumulation of Br^- in the liver (Mamabolo *et al.*, 2009) can influence TH clearance rate by the mechanism of forming brominated TG.

Action of EDC at critical stages of foetal development could lead to irreversible adverse effects unlike the possible reversible effects on mature adult hormone pathways or axes that are more resilient (Bigsby *et al.*, 1999). Long-term TDC disruption can cause hypothyroidism when compensatory mechanisms of the thyroid gland become ineffective after TDC accumulation (Boas *et al.*, 2012).

Maternal thyroid function during gestation in mammals can affect foetal neurodevelopment, but also foetal growth and development (Velasco *et al.*, 2018). In

mammals, insufficient free T_4 as a consequence of maternal hypothyroidism or hypothyroxaemia translates into insufficient T_4 availability to the foetus for normal growth and development (Velasco *et al*., 2018). Where there is interference in T_4 production by a TDC such as Br^-, the foetus may be at a disadvantage during gestation due to insufficient T_4 crossing the placenta. In birds, this may be similar in that the hen supplies all nutrients for the developing embryo by depositing yolk proteins and lipoproteins from the liver before shell formation (Husbands *et al.*, 1972). When Br^- accumulates in the liver (Mamabolo *et al.*, 2009), the embryo may be at risk for developing *in ovo* hypothyroxaemia due to the interference of T_4 production even when sufficient I^- is available. In mammals, impaired maternal TH can affect the growth and development of the foetus at critical stages of gestation (Boas *et al.*, 2012).

Hypothryoidism causes changes in remodelling of the heart muscles and vasculature (Martinez, 2016). Fast growth rate increases the metabolic rate and oxygen demands of the body, which challenges the heart to increase cardiac output to meet the additional demand (Wideman, 2000). Greater body weight of broilers was associated with lower plasma T_3 concentration and T_3/T_4 ratio compared with layer and unimproved indigenous chickens (Hassanzadeh *et al.*, 2005).

Hypothyroidism can occur at tissue level when the regulation of T_4 uptake occurs at the level of the plasma membrane in specific target tissues (Hennemann *et al.*, 2001). The most devastating effect of I^- deficiency occurs during foetal development (Waugh, 2019). In humans, F^- directly affects I^- bioavailability, and high exposure to F^- was associated with high serum TSH, which is an indicator of hypothyroidism (Waugh, 2019). The halides behave in a similar way, thus there is the possibility that Br^- toxicity can interfere with physiological processes in a similar manner as that found in fluorosis.

Hypothyroidism can occur at tissue level, such as in the liver of humans due to insufficient transport of T_4 into hepatocytes, and consequent biological disruption due to lowered T_3 production within the target tissue (Hennemann *et al.*, 2001).

Roelfsema *et al.* (2017) found that in subclinical hypothyroidism, TSH secretion increased by ten-fold and by 200-fold in severe hypothyroidism because T_4 feedback is the driving force behind TH regulation in the body. TDC have the potential to mimic T_3 action on TR, which will disrupt the negative feedback mechanisms on TRH and TSH (Jugan *et al.*, 2010).

Growth and development

Genetic factors as well as nutrition, metabolism and hormones work in concert to influence muscle growth (Caldas *et al*., 2019), which is important in broilers selected specifically for high breast meat muscle accretion. To understand where in the metabolic pathways a TDC such as Br^- may interfere with production parameters is it useful to understand the different growth stages of the broiler from egg to one-day old chick. The effects of TDC on post-hatch growth were beyond the scope of this study.

Egg production

The egg is created to protect and supply all the necessary nutrients to the developing embryo throughout incubation (Willems *et al.*, 2014). Yolk formation occurs in the ovary, and the albumen and eggshell are deposited as the yolk descends through the reproductive tract

ending in oviposition. At lay the shell, the albumen and the yolk contain all the nutrients needed by the growing embryo except oxygen. The egg component composition is influenced by hen nutrition (Vieira, 2007). Each component contributes different nutrients to the developing embryo, thus harmful elements deposited in any of these components may become available to the embryo during critical developmental stages throughout incubation.

Henriksen *et al*. (2011) found that maternal stress around the time of egg formation transferred maternal cortisol to the eggs during albumen and yolk deposition, which exposed developing embryos to cortisol during development. The implication is that the transfer of elements in maternal plasma can occur at the time of albumen and yolk deposition, exposing the embryo to these elements at critical stages of development.

The yolk comprises of 50% water, 32% lipid, 16% protein, 1% carbohydrate and 1% inorganic ions (Romanoff & Romanoff, 1949 as cited by Willems *et al*,. 2014). Trampel *et al.* (2003) showed a strong linear relationship between blood lead concentration and the lead concentration in the yolk. This showed that the harmful elements present in the hen's blood and transferred into the yolk expose the embryo to those elements throughout incubation.

The albumen is considered to be the main water source of the embryo during incubation because it contains 88.5% water, 10.5% protein and 4% inorganic ions, among which the principal electrolytes K^+, Na^+ and Cl^- (Willems *et al.*, 2014). Albumin can be iodinated and contributes to the I^- reserves in the total halide pool (Waugh, 2019). Iodinated albumin deposited during egg production could serve as an I^- reservoir for the developing chick embryo throughout the incubation period. Similarly, plasma Br^- may be bound to albumin and be a reservoir of Br^- to the embryo during incubation.

Vertebrate embryos were shown to have access to TH well before the embryonic thyroid gland was fully functional, by trans-placental transfer in mammals or by deposition in the yolk in egg laying non-mammals (Prati *et al*, 1992; De Escobar *et al*, 2004 and Walpita *et al.*, 2017 as cited by Darras *et al*., 2011). In birds, all hormone deposits are made in the yolk, thus embryonic development occurs independently of maternal physiology (De Groef *et al*., 2008).

Incubation

Embryonic growth and development in chickens takes 21 days to complete (Darras *et al.*, 2011), thus *in ovo* TDC exposure has a relatively short window in which lasting negative effects are ingrained that may affect post-hatch chick quality.

In the chicken embryo, the thyrotropic axis changes towards the end of incubation to support the growth and differentiation of the embryo in preparation for post-hatch life (De Groef *et al.*, 2008).

There are two main determinants for neonatal size across egg laying species, namely length of development and egg mass (Christensen *et al.*, 2002) because chick mass on HD is correlated with egg size (Wolanski *et al.*, 2006). The immature physiology of a young breeding hen may affect egg and chick quality, as smaller eggs may present with nutritional deficiencies in yolk nutrient content (Burnham *et al.*, 2001). Most lipophilic nutrients required by the embryo during incubation are deposited in the yolk (Goel *et al.*, 2016). The albumen serves as the reservoir for water and electrolytes necessary for embryo development.

Hens supplemented with I^- have the ability to balance high I^- intake by excretion into the albumen and yolk during egg production, and accumulated concentrations were found to be

higher in yolk than in albumen (Röttger *et al.*, 2012). The possible inability of a young bird to temper plasma concentrations of elements like older birds can, may exacerbate the risk of transferring harmful substances into the yolk. Similar mechanisms could be used to temper excess Br^- in the plasma halide pool since Br^- competes with I^- for uptake.

Eggs laid by younger hens and kept in prolonged pre-incubation storage produced embryos with lower liver mass according to strain/line, and the heart mass was reduced in embryos from eggs stored for 14 days before incubation compared to 1 day pre-incubation storage (Christensen *et al.*, 2002). Chicks hatched from older breeding flocks had shorter incubation times and thus more time out of the shell to lose body moisture at hatch, and despite this the body mass at hatch was heavier than for chicks hatched from younger breeding flocks (Peebles *et al.*, 2001). Prolonged pre-incubation storage time was shown to delay hatching by 11 hours (Christensen *et al.*, 2002). This indicated that chick quality may be compromised in eggs laid by younger flocks, and extra care should be taken to ensure chick survival by supporting young flocks to optimise their productivity through formulating the diets to balance the micronutrient content of the feed according to the WQC content of the drinking water.

Due to the shortening of the rearing period, embryonic growth has become a larger part of the growth cycle, which necessitates attention to maintaining optimal incubation conditions for best chick quality at hatch (Baghbanzadeh & Decuypere, 2008; Molenaar *et al*., 2011; Hulet *et al.*, 2007 as cited by Afsarian *et al.*, 2018). Modern broiler chicks were reported to be heavier at hatch than layer and unimproved indigenous chickens, and grew at a faster rate than the layer and indigenous chicks (Hassanzadeh *et al.*, 2005).

In work done by McIndoe (1960) it appeared that during the last week of incubation 30% of the albumen protein was assimilated into the yolk sac. During the last week of incubation, the embryo absorbs the yolk sac, which then becomes the main source of nutrients until a few days post-hatch (Burnham *et al.*, 2001).

Peebles *et al*. (2001a) reported that at ED 16 and 19 of incubation the percentage crude protein of embryos from 28-week-old breeders was higher at 63% relative humidity (RH) during incubation than at 43% RH, and intermediate at 53% RH. This highlights the importance of optimum RH during incubation to control egg dehydration rate that occurs naturally and to prevent dehydration of the growing embryo, which could lead to embryonic death or poor chick quality at hatch. An increased rate of incubational water loss lead to decreased yolk uptake in embryos (Peebles *et al.*, 2001b). Optimum RH for incubation is 40 to 70% (Lundy, 1969; Preez, 2007). Eggs will lose up to 15% of the initial egg weight under optimal incubation conditions (Ar & Rahn, 1980).

An increased wet mass during early incubation is the result of fluid absorption from the albumen (McIndoe, 1960). At ED 18, the percentage wet liver mass was found to be greater in embryos from older breeding flocks and the percentage wet embryo mass increased between ED 6 and 18 (Peebles *et al.*, 2001b).

Throughout the last week of embryonic development the T_4 levels increase, reaching a peak at hatching, and the consistently low T_3 levels throughout incubation increase sharply when the hatched chick begins breathing independently (De Groef *et al.*, 2008).

Al-Rukibat *et al.* (2017) reported that chickens subjected to thermal manipulation during incubation had significantly higher plasma T_4 concentrations and lower plasma T_3 concentrations in response to thermal challenge conditions at Day 14 post-hatch than untreated

birds. Thermal tolerance increased with thermal manipulation of embryos during incubation (Al-Rukibat *et al*., 2017), which affirmed that incubation temperature must be correct at critical stages of embryogenesis to optimally support correct development of the HPT axis during incubation.

In the chicken, dramatic changes in plasma TH occur in the hatching process (De Groef *et al.*, 2006). The changes in TH activity are important in regulating pipping and hatching in chick embryos when the transition from allantoic to pulmonary respiration occurs (Decuypere *et al*., 1991 as cited by Hassanzadeh *et al*., 2008).

Growth indicators

The CR length is an indicator of whole body growth. It is measured from the tip of the head to the root of the tail. The femur and tibia lengths are important in terms of product quality. Studies on the tibia and femur of growing birds revealed a clear difference in tibia and femur lengths between sexes, with males having longer bones than females (Applegate & Lilburn, 2002).

The tarsus or shank length is a conformation trait that is an indicator of skeletal growth, which is in proportion to body mass of the growing bird (Bhonsle *et al*., 2018). Shank length measurement is a non-invasive growth indicator in live birds. Shank length may be stunted when the developing embryo is exposed to a high concentration of cortisol (Henriksen *et al*., 2011, Mabelebele *et al.*, 2017).

At Day 14 post-hatch, shank length and body length correlated more strongly with body mass than initial hatch mass (Wolanski *et al.*, 2006), making it a suitable growth indicator. In broiler strains selected for high growth rate, Day 14 shank lengths were longer than for strains that were less heavily selected for growth rate (Wolanski *et al*., 2006). When comparing chicks of the same strain reduced shank length may be linked to lower offspring viability (Henriksen *et al.*, 2011).

EDC are elements that can include vitamins and minerals. The body does not discern between EDC and essential nutrients taken up from feed and water. The uptake of EDC can influence bone growth and development by interfering with normal bone growth processes (Mabelebele *et al.*, 2017). TH affect linear growth and skeletal development (Williams & Bassett, 2018), suggesting that any interference with correct TH expression could have similar deleterious effects on embryogenesis. Shank length can therefore be an indicator of adverse effect of chemical constituents on embryonic growth.

In human studies hypothyroidism was shown to completely arrest postnatal growth with associated bone dysplasia (Williams & Bassett, 2018). It is important to weigh eggs before incubation as this gives information on what to expect at hatching. Chicks will not thrive if eggs are too small or too large compared to what is considered normal average egg size (Mortola & Al Awam, 2010). Egg mass is highly correlated with body mass at hatching and can be used as a marker for the nutrients available to the embryo during incubation (Henriksen *et al.*, 2011). It is important to note than hatch mass includes the mass of the internalised yolk and it is not possible to separate chick mass from yolk mass in live chicks (Wolanski *et al*., 2006). During the second half of incubation, embryos from larger eggs grow at a faster rate than those from smaller eggs despite the similar size of the chorioallantoic membrane (CAM) between large and small eggs (Mortola & Al Awam, 2010). This could be the result of larger

eggs containing larger yolk compared with smaller eggs, which supply more nutrients to support the fast growing embryo.

The role of sentinel species and animal models in risk management

Sentinel species have been used in ecotoxicology to reflect the potential risk a pollutant or toxicant poses to livestock or game in a given area (Scanes & McNabb 2003). There are interspecies differences in thyroid gland development (Forhead & Fowden, 2014). For example when considering the role of TH on brain function, human and rat models focussed on the effects of TH on learning ability. Human brain development would differ from avian brain development in that chicks are independent and fully functional at hatch, whereas human brain development continues postnatally. It can however be hypothesised that TH deficit in early brain development may have negative and lasting effects in avian embryos, since all embryos follow a similar growth and development pattern in early development (Hamburger & Hamilton, 1951). Hamburger and Hamilton (1951) categorised embryo developmental stages according to the timing of the differentiation of the different tissues rather than incubation time or stage of pregnancy so as to standardise comparative embryology across species. When choosing a model animal it is important to look at the comparative physiology between species so that experimental results can be extrapolated as much as possible. Nakai *et al.* (1988) reported that there was a 78% DNA similarity between the human kidney and chicken embryo thyroid receptor type α1. This makes the chicken embryo a good model for studying embryonic thyroid function development. Another reason the chicken embryo model is favourable is because the TH processes occur independently of maternal influences (Darras *et al.*, 1999).

It is important to distinguish between model species and sentinel species, as their purposes are different and the choice of species depends on the objectives of the research requirements. This does not mean that sentinel species cannot also be used as model species (Scanes & McNabb, 2003).

Choosing an appropriate animal model to investigate toxicant effects on embryonic development and growth requires consideration of developmental maturity differences between species at hatch, or birth for mammals, before comparisons are made. Avian embryos are established models in teratology and developmental toxicology and this is useful in human pharmacology where there was a need for juvenile and perinatal animal models in pharmacological testing to understand the pharmacokinetics of paediatric drugs on pre- and perinatal children (Bjørnstad et al, 2015).

Similarly, differing developmental rates between species and individuals within species called for the development of a standardised evaluation system based on embryonic developmental stage rather than age (Hamburger & Hamilton, 1951). Timing of toxicant exposure is essential because there are known critical stages in embryo development regardless of species, and disruption in any or all of these stages can have dire consequences for embryo survivability (Scanes & McNabb, 2003). Knowledge of comparative physiology is necessary for accurate cross-species translation of results from animal models and observation of similar results in animal models of different species increases the likeliness of the results being translatable to humans (Bjørnstad et al, 2015).

It has long been recognised that the use of the chicken embryo model is a cost effective method to investigate how foetal development and growth are influenced by direct effects independent of maternal effects (Scanes & McNabb, 2003). The chicken embryo has been a long-established model for early development studies (Darras *et al*., 2011) as early as the 17th century and is thus considered to be a "classic" model organism to show that embryos are not preformed but develop body parts progressively (Wittig & Münsterberg, 2016).

There are many advantages of using the chicken embryo as animal model. The costs are low, it is easy to obtain multiple embryos at the same time, and the embryos are large enough to be accessed during all stages of development (De Groef *et al.*, 2008). The similarity between the physico-chemical properties of TH receptors in the chicken and in mammals makes it an appropriate animal model to study the effects of Br^- in humans (Bellabarba et al., 1988).

In mammalian models the actions of TH can be easily observed in postnatal and adult animals, but not accurately during the prenatal period due to interference of trans-placental movement of maternal TH to the foetus (Tata, 1999). The chicken embryo model is a good model to study prenatal TH actions, because it is a free-living organism in a contained environment and therefore excludes direct maternal effects during the embryo development period in incubation (Tata, 1999).

Classical teratology tests, or Segment 2 tests, are commonly used in industry to test EDC, and the end-points are either gross malformations and/or embryonic death (Bigsby *et al.*, 1999). In these tests the highest experimental dose is selected based on some measurable sign of toxicity, such as decreased body mass, and the lowest experimental dose is selected to be 50 times less than the highest dose (Bigsby *et al.*, 1999). The endocrinological differences between species require careful selection of animal models to examine *in vivo* hormone actions and possible mechanisms for physiological disruption by EDC (Ikagami & Yoshimura, 2017). Maternal physiological fluctuations will affect foetal physiology in both mammals and birds, but mammals are exposed to maternal hormone fluctuations at critical periods throughout gestation whereas avian embryos are only affected by maternal hormones during egg formation and develop independently as a closed system throughout the incubation period (Henriksen *et al.*, 2011).

The function of endocrine axes are similar in mammals and birds (De Groef *et al.*, 2008), making chickens a good choice of sentinel species. Broilers are also a good model animal for observing the link between selection for increased growth rate and changes in specific organs and organ systems (Schmidt *et al.*, 2009).

Maternal stress may negatively affect the physiological traits of offspring such as when unsuitable environmental factors are present at critical stages of embryo development, and result in the occurrence of disease due to inadequate capacity of the embryonic physiology to respond and adapt to the environment (Henriksen *et al.*, 2011). Timing of TH exposure is key during embryogenesis because many essential developmental genes will only respond to TH signalling during predetermined window periods (Darras, 2019). The concentration of maternal plasma TH fluctuates naturally and the level of TH deposited in the yolk varies accordingly (Darras, 2019). In the case of maternal hyperthyroidism the concentration of TH deposited in the yolk remains stable, whereas maternal hypothyroidism has dire consequences for embryonic development due to insufficient T_3 and T_4 present in the yolk, and only small

concentrations of maternal TH are present in the albumen because TH are lipophilic (Darras, 2019).

Despite complete glandular development within the embryo, the relevant endocrine axes will only become functional once all glands and target tissues become linked i.e. the target tissues must develop the receptors to be able to respond functionally to hormones acting on them (De Groef *et al.*, 2008). The embryonic thyroid gland of the chicken becomes functional by ED 10 even though TH signalling begins from ED 1 (Darras, 2019).

Precursor T_4 and active T_3 are necessary for the correct development, growth and metabolism regulation in vertebrates, and negative feedback mechanisms of T_4 and T_3 inhibit TSH release (Ikagami & Yoshimura, 2017). The mammalian foetus is continuously exposed to minimal quantities of maternal TH via the placenta (Tsai *et al*., 2002; Darras, 2019). In contrast, the developing chicken embryo is wholly reliant on TH deposited in the yolk during egg formation for the duration of the incubation period (Darras, 2019). In mammals, TH is functionally able to inhibit TSH production by a negative feedback mechanism at ED 19 (De Groef *et al*., 2008). The synthesis and secretion of TH in mammals and birds are regulated by the TSH produced in the *pars distalis* of the anterior pituitary gland (Ikagami & Yoshimura, 2017).

Correct timing of TH signalling is pivotal for normal perinatal development, and in mammals constant exposure of the foetus to maternal hormones including TH occurs *in utero* (Ikagami & Yoshimura, 2017), which puts the mammalian animal model at a disadvantage for demonstrating the effect of TH disruption on foetal growth and development.

Another reason why broilers or indigenous chickens can be used as sentinel species is because they are widely kept by populations residing in rural areas, where a multitude of elements present in the environment can have direct effects on livestock and poultry health, and potentially on human health. Chickens have a relatively short life span and problems can be identified in a timeous manner using information from tissue samples for risk assessment.

CHAPTER 3

Prevalence of bromide in groundwater in selected regions in South Africa

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Introduction

Many regions across South Africa are dependent on groundwater as the only water source for extensive and intensive livestock production, and wildlife in game farming and game reserves. In remote regions, domestic households might also be dependent on groundwater, as access to surface water or roof-harvested rainwater could be erratic, and therefore exposed to potentially hazardous elements. Initially health problems were reported in livestock in specific areas and fluorosis was identified as a major problem (Coetzee *et al*., 1997; 2000). Water samples were collected at various points from source and use-points for the purpose of compiling reports addressing the risk presented to livestock. Chemical analysis of water quality constituents (WQC) of the earliest water samples confirmed the presence of fluoride in excess of reported water quality guideline (WQG) safe levels. This information was used to determine an appropriate method of risk assessment and subsequent decision-making on fitness-for-use (FFU) of available water sources.

The initial focus of early research was to formulate and test solutions to most frequently identified palatability and toxicological water quality (WQ) problems of groundwater drawn from wells, springs and boreholes (Casey *et al*., 1998). Increased efforts to measure and assess the potential risk posed by geochemistry-related factors resulted from advances in identifying the role of inorganic constituents on the epidemiology of non-differential clinical symptoms commonly observed in livestock (Meyer *et al*., 2000).

As more information emerged, objectives of the research projects changed to include analysis of multiple elements in water sampled from various regions across South Africa. It emerged from those research projects that bromide (Br^-) was present in many of the samples collected from areas already identified as at-risk of exposure to known hazardous elements. It further emerged that Br^- was present at potentially harmful concentrations for many of the selected sites.

There were no formal international or local WQG available for Br^- because it was initially merely acknowledged as a ubiquitous micro-element of unknown essentiality and not considered problematic. Similarly, water was ignored as a nutrient. Traditionally the focal point of deficiency or toxicity research was limited to the contribution of micronutrients from premixes to nutrient composition of feed. As research progressed over time, a very different picture emerged and raised many questions. It emphasised that water should be given closer attention as a potential source of micro-elements in human and livestock nutrition (Casey and Meyer, 2001).

This paper presents collated results of data collected in selected regions in 6 provinces in South Africa and published in various reports between 2001 and 2016. The aims of the Water Research Commission (WRC) and other sponsored research were to determine FFU of water for livestock production. This was extended to include game such as ostrich production. The aim of this paper was to show the considerable range of Br^- concentrations present in groundwater in selected regions, and to draw attention to the reality that Br^- may have a much greater impact on animal production than previously considered.

Materials and Methods

Data of groundwater Br^- concentrations were collated from water sample results (Table 1). The reports from which the data were collated were not all in the same format. Sampling for each report was done according to the objectives of the individual report, which resulted in an uneven dataset. There were no GPS co-ordinates available for points sampled for earlier reports and thus compilation of a distribution map for this paper was not possible.

Table 1 WRC Reports used for data collation

Report	**Author**
WRC 857/1/01	Casey and Meyer, 2001
WRC 857/2/01	Casey, Meyer and Coetzee, 2001
032005/02/26	Meyer, 2005
WRC 1175/1/06	Casey and Meyer, 2006
WRC 2175/2/16	Korsten, Casey and Chidamba, 2016

Data were sorted according to sample source (Table 2) and locality across years and seasons to obtain information of overall Br^- concentrations present in groundwater used for livestock production and household use across selected regions. The majority of the samples were collected in areas where the human and livestock populations were dependent on groundwater as the only source of water for drinking and domestic use.

Initially potentially problematic physiologically significant trace elements were identified according to the specific requirements of each individual project prior to water sample collection. All water samples were collected for the completion of individual reports using the same method, and all samples were analysed using inductively coupled plasma atomic emission spectrometry (ICP-AES) techniques with full quantitative and semi-quantitative procedures by the Institute for Soil, Climate and Water at the Agricultural Research Council (ARC-ICSW), Pretoria. Elements present in the samples were classified as constituents of concern (COC) or potentially hazardous chemical constituents (PHCC) according to their presence at concentrations relative to local and international WQG standards. Not all elements were present in all water samples, and for the purpose of this paper, only Br^- concentrations were considered.

Where possible, water samples were collected at source from wells, springs and boreholes, from the surface of reservoirs, tanks or drinking troughs supplied by boreholes and at use-points such as from taps or water lines in poultry houses (Table 2). In WRC reports published before 2001, Br^- was not included as a COC, but as time progressed and the objectives of the projects expanded, it emerged from the various case studies that Br^- should be included in further testing. A total of 350 groundwater samples were ranked from highest to lowest Br^- concentrations across all years, sources, seasons and localities to determine the overall range of Br^- present in groundwater in a South African context. Water samples were grouped by collection source within province to determine an overall picture of how Br^- concentration may vary in different sample sources within a locality.

Results and discussion

Inconsistent sampling techniques, such as sample collection by different technicians and variations in sample collection depth, and inconsistent or seasonal use of boreholes in some areas could explain the occurrence of unavoidable sampling errors with effects on the accuracy of measurements.

It emerged from the collated data that the overall range of Br^- concentrations present in groundwater sampled across selected regions in South Africa was 0–132.68 mg/L.

Samples taken from dams, source, reservoirs and use-points included in the dataset were limited to those supplied by boreholes (Tables 2 and 3).

Table 2 Br^- concentrations (mg/L) within source across all years, all seasons, all localities

Sample	*n*	min	Max	μ	SE
Dam	2	0.79	0.84	0.81	0.04
Source	269	ND*	132.68	3.52	14.53
Reservoir	63	0.01	6.44	0.80	1.19
Use-point	16	0.01	0.38	0.17	0.12

*ND = not detected

There were distinct differences in Br^- concentrations between sources within and across regions (Tables 2 and 3). The majority of water samples collected were for selected areas in the North West and Limpopo Provinces in accordance with the specific objectives of projects undertaken to address area-specific problems reported within those provinces.

Groundwater sampled from reservoirs showed higher Br^- concentration compared with groundwater sampled at source, with the exception of the 97 water samples collected at source in Limpopo Province (Table 3). Many factors, such as depth of sampling, sampling site, water flow rate, water usage rate, and pumping frequency could influence the accuracy of the measurement of a WQC in a definitive water sample of the source, and Br^- is no exception. However, since it is not possible with current technology to verify the measured concentration, it was assumed that each sample was an accurate representative sample and the measurement an accurate estimation of the concentration of a WQC at the time of sampling.

Water stored in open reservoirs or held in drinking troughs that are not subject to high stocking rates or frequent use are expected to contain higher concentration of Br^- than water sampled at source or at use-point. This is because exposure of open troughs and reservoirs to evaporation results in a concentrating effect on Br^- within that water body, which is true for all WQC. Groundwater pumped into open reservoirs exposed to UV radiation is subject to speciation of Br^- in the presence of oxygen to form bromate (BrO_3). The rate of conversion is dependent on pH and presence of other elements. This speciation of Br^- is potentially hazardous since BrO_3 is a known carcinogen (Jain *et al*., 1996; DeAngelo *et al*., 1998; Magazinovic *et al*., 2004; Bonacquisti, 2006; Moore and Chen, 2006). Similarly, water sampled from pipes exposed to sun may differ in composition to water sampled at source because the rate of elemental interactions within water accelerates with heating (Table 2).

The current South African WQG for livestock watering does not list Br^- as either a COC or PHCC (Casey and Meyer, 1996). It is common for products of endocrine disrupting chemical (EDC) metabolism to be more toxic than the parent compound (Burger, 2005). An EDC is any

naturally occurring or synthetic chemical that interferes with the structure or function of hormone receptor complexes, either in an antagonistic or synergistic way, to alter the correct function of an endocrine response within a target organ (Bornman *et al*., 2007). The USEPA (1997) expands the definition of an EDC to include that the exogenous substance causes adverse health effects in the intact organism, its progeny or (sub)populations. EDCs commonly monitored for hazards to human and animal health are usually lipophilic organic compounds with oestrogenic properties (Bornman *et al*., 2007). Naturally occurring Br^- is a hydrophilic inorganic element identified as an EDC in rats (Loeber *et al*., 1983) and chickens (Du Toit and Casey, 2012) and is expected to have the greatest direct disrupting effect on metabolism in vulnerable livestock and human populations.

The report by Casey and Meyer (2001) lists Br^- with a maximum permissible level (MPL) of 1–3 mg/L and a crisis level of 6 mg/L, with the recommended limit set at 1 mg/L due to risk of BrO_3 formation at that concentration. A subsequent report (Casey and Meyer, 2006) introduced 0.01 mg/L as a maximum contaminant level (MCL) to align it with USEPA (2005) guidelines. Faced with conflicting reports of what constituted a safe minimum concentration against which to compare results obtained from field samples, Casey (2016) accepted a minimum level of 0.01 mg/L as a point of departure for analysis and interpretation of the test results. This was in line with the accepted default maximum residue level (MRL) of 1 mg/kg used for most food additives not yet validated in terms of Regulation (EC) No. 396/2005 (European Parliament, 2005).

Traditional WQG propose generic safety levels of elements based on concentration-based estimates, which assume a linear relationship between the concentration of an element in source and its effects in vivo. Limitations of such a generic approach are that the accepted safety limits of elements in feed and water are seldom published in the same guidelines, and limited differentiation exists between different types of livestock or game species where applicable. Further limitations are that interactions between elements in the same source are ignored and the assumption that all groundwater sources in the same area are of equal quality.

The disadvantage of a concentration-based approach is that exposure risk is disregarded as being multifactorial when it is influenced by any factor that affects water intake rate or physiological state of an individual. Intake-based guidelines that are site-specific will better estimate exposure risk of a target population, thus allow for better mitigation of adverse effects.

All elements, whether essential or nonessential, can exert toxic effects when consumed in excess through water or feed, which includes minerals occurring in feed and water at trace levels otherwise regarded as incidental contaminants with no obvious important nutritional role (NRC, 2005). PHCC have adverse effects at relatively low levels, and magnitude of exposure risk depends on exposure period duration (Plant *et al*., 1996). Low-dose, long-term exposure to PHCC will most likely manifest in subclinical responses where toxicity is expressed as secondary induced deficiencies, making toxicity symptoms difficult to identify (Meyer and Casey, 2004). Similarly, EDCs exert their effect at very low exposure levels (Bornman *et al*., 2007).

Table 3 Br^- concentrations (mg/L) in groundwater in selected regions clustered by province

North West						Western Cape					
Sample	n	min	max	μ	SE	Sample	n	Min	max	μ	SE
Source	141	0.00	2.14	0.31	0.37	Source	13	0.04	6.60	3.11	2.01
Reservoir	36	0.03	2.09	0.42	0.55	Reservoir	5	2.43	6.44	3.82	1.62
Use point	16	0.01	0.38	0.17	0.12						
Limpopo						KwaZulu-Natal					
	n	min	max	μ	SE		n	min	max	μ	SE
Dam	2	0.79	0.84	0.81	0.04	Source	5	0.01	0.23	0.12	0.09
Source	97	0.01	132.68	8.87	23.29						
Reservoir	22	0.01	2.98	0.74	0.88						
Mpumalanga						Eastern Cape					
	n	min	max	μ	SE		n	min	max	μ	SE
Source	6	0.05	0.25	0.14	0.09	Source	6	0.06	0.37	0.21	0.13

The vast range in Br^- concentrations in water sampled from Limpopo Province compared with other provinces (Table 3) draws attention to the importance of site-specific analysis of groundwater sources when determining FFU of such sources and the potential risk of vulnerable population exposure to hazardous chemical constituents. Site-specific risk assessment requires that geochemical factors on soil and plant concentrations be included in the total exposure risk estimation for a given area to enable formulation of contextual solutions (Meyer *et al*., 2000).

Some areas were chosen for sampling to determine the quality of alternative water sources in provinces that were not solely dependent on groundwater, in line with the research objectives to generate specific reports. This resulted in collection of relatively few groundwater samples from those provinces compared with areas where groundwater played a greater role (Table 3).

Many environmental health effects caused by nutritional element excess and deficiencies in South African agricultural systems have been documented, yet there are still health impacts of potentially harmful elements that are less known (Davies and Mundalamo, 2010). Heavy metals are known to be toxic due to their cumulative nature and cause increasing damage to brain, kidney and nervous system with extended exposure periods (Ezekwe *et al*., 2012). Similarly, Br^- has been shown to accumulate in liver, kidney and thyroid tissue (Du Toit and Casey, 2012; Mamabolo *et al*., 2009). Further fieldwork done by Meyer (2005) included tissue sampling and revealed evidence that Br^- had histopathological effects on thyroid and other tissues in commercial broilers reared in areas where Br^- concentrations in groundwater were high. It is known that Br^- has the ability to circulate freely and rapidly into the extracellular fluid and various tissues of the body except the central nervous system (Pavelka *et al*., 2000). This free movement throughout the body affords Br^- the opportunity to interfere with multiple biochemical processes. Although further validation is required, it appears that Br^- could be labelled an EDC, which is a concern for livestock farmers and people who might be exposed to Br^- in drinking water.

Table 4 Estimated intake of Br^- through water by humans

Persons	WQG	Br in water (mg/L)		Water Intake	Br/day by WI (mg)	
	mg/L	Max	μ	L/day*	Max	μ
Males: adults and adolescents	0.01	133	3	2.30	305	7
Children: both sexes 4–12 yr	0.01	133	3	0.55	73	2
Children: both sexes 0–3 yr	0.01	133	3	0.40	53	1
Women: pregnancy < 18 yr	0.01	133	3	2.30	305	7
Women: pregnancy 19–50 yr	0.01	133	3	2.30	305	7
Women: lactating < 18 yr	0.01	133	3	2.90	385	8
Women: lactating 19–50 yr	0.01	133	3	2.90	385	8

*Assumed for normal healthy people of moderate lifestyle at 95 % of the empirical distribution (EPA, 2004)

Identifying Br^- as a COC or PHCC in water sources of areas where no alternative water sources were available raised further questions about the best ways to define and identify vulnerable populations to determine FFU of these water sources. Risk assessment relies on the identification of vulnerable populations within an area, because water requirements will differ between groups within a population according to age and physiological state (Table 4). Vulnerable populations in livestock production include neonates, very young and actively growing animals, immunocompromised animals and pregnant and lactating females. Where multispecies water use is common, such as in game reserves with watering holes supplied by borehole water, interspecies differences in mineral tolerance must be considered in the FFU decision-making process due to the different species-specific metabolic requirements related to physiological state. Bornman *et al*. (2007) stated that EDCs can pose risks to reproductive function, immunity, thyroid function and neurodevelopment, dependent on the type of substance and its toxicodynamic and toxicokinetic mechanisms of action.

The geochemical character of groundwater depends on mineral chemistry of aquifer materials and biomediated ion exchange reactions (Ezekwe *et al*., 2012). Changes in environment such as ambient temperature, feed composition and water palatability influence water intake. Physiological differences between groups translate to differences in metabolism and assimilation rates of elements. Exposure risk depends on the per capita consumption of an element relative to body weight (Ezekwe *et al*., 2012) and this is clearly shown in Table 4. Immature and actively growing individuals are thus at greatest risk of developing toxicity symptoms from relatively lower concentrations of COC or PHCC due to a combination of limited capacity of immature organs for adequate detoxification, and greater rates of assimilation of elements by tissues with high metabolic activity (Table 4). As a result, it is common practice to assign water sources of relatively poorer FFU scores to the least vulnerable groups within a population when alternative water sources are unavailable. In some cases, where practical, water treatment can improve its elemental quality sufficiently to make it safe for use.

The use of sentinel species is a useful tool to evaluate risk to vulnerable populations over time. Meyer (2015) used indigenous chicken breeds and commercial broiler chickens produced in a specific locale as a reference point for risk assessment of groundwater containing high concentrations of Br^- for the selected area. The additional collection of multiple tissue sample types from sentinel species, together with single water samples, allowed for better

identification of chronic exposure risk to PHCC than water sampling alone, with liver samples reported to be the most appropriate tissue sample for assessment of Br^- exposure risk (Casey and Meyer, 2006). The most suitable choice of sentinel species in an area will depend on specific monitoring objectives and the practicality of tissue sample collection for testing. Future consistent sampling of the same sites over time will garner more information on the toxicity risk that Br^- in groundwater poses to populations in the area at different times of the year and in different situations. Monitoring specific groundwater sources could indicate which water usage patterns could effectively limit exposure of vulnerable populations to COC and PHCC.

Conclusion

The considerable range of concentrations of Br^- occurring in South African groundwater presented in this paper draws attention to the importance of monitoring site-specific WQ for FFU assessment for domestic and livestock use. It further highlights that Br^- may be a greater toxicity risk factor to livestock production and human health than previously considered. In order to be included in WQG, further validation is required on the physiological effects of Br^- and associated risk factors. Identification of vulnerable populations is paramount to the selection of the best solution to alleviate risks of exposure to Br^- in groundwater. Continued seasonal monitoring is recommended to identify potential risks linked to changes in WQ and to assist in the diagnosis of physiological anomalies.

CHAPTER 4

Bromide: A potential risk to livestock production in South Africa

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Introduction

Groundwater is a vital resource for animal agriculture, wildlife and the local human populations over vast areas of South Africa. A constant supply of water is necessary to support the welfare of the population and to sustain economic activities. Drawing groundwater from aquifers occurs in all regions of the country and is not confined to the drier, low rainfall areas. The climate that prevails over the sub-continent is erratic and prone to alternating periods of adequate and sub-adequate rainfall. Intermittent seasonal dry periods and drought conditions require supplementation from groundwater resources where surface water is insufficient. In addition to erratic rainfall patterns, the topography favours rapid drainage off the central plateau and escarpment.

Historically, in areas where rainfall was erratic and rivers were non-perennial, farming practices were predominantly nomadic systems in which livestock moved to areas where water was available. The ability to access deep underground water sources with industrial drilling equipment made fixed farming systems possible because of the availability of a constant water source. This seemingly simple step changed the landscape of agriculture. On the one hand, it brought relief, but on the other, it led to consequences.

With time, noticeable differences in water properties and the quality of various groundwater sources allowed categorizing according to palatability and observed adverse effects that occurred in livestock and humans. This prompted in-depth investigation of water source compositions and subsequent development of water quality guidelines (WQG), which clustered water sources with similar properties and provided designations for use according to suitability, be it for irrigation, livestock watering, washing or as potable water for human consumption.

The variations in the quality of groundwater are well known and are recognised scientifically. Dissociated ions and conjugated forms of elements are collectively referred to as water quality constituents (WQC). The WQC determine water quality and its user specificity. These are published as WQG, which serve as classifications of water quality for defined purposes (Casey *et al*., 1996; Meyer *et al*., 1997; WRC, 2010; WHO, 2011). The Ministry of Environment, British Columbia (2015), noted that the principle of risk- based assessment is to represent safe levels of substances that protect different water uses. The approach is not to develop WQG that protect water quality per se or to provide direction on policy and decisions that affect it. Technological evolution has allowed for more sensitive testing of WQC and the capacity to identify more elements of interest to establish site-specific exposure risk. Direct comparisons between WQG reveal discrepancies in recommended 'safe values', which can be ascribed to observations from case studies with few imperative validations (Meyer & Casey, 2012).

This article discusses the presence of bromide (Br^-) in South African groundwater as a silent threat to livestock production.

Bromide occurs naturally in groundwater in combination with other ions such as chloride (Cl^-) (Davis *et al.*, 2004). This is a consequence of salt intrusion and local geographical

situations in coastal regions (Agus *et al.*, 2009), while for the inland areas it is a result of geological features and historical geographic conditions (Davis *et al*., 2004).

The dissociated anionic form of the diatomic halogen bromine (Br) is Br^-, which has chemical properties similar to other halides (fluoride (F^-), iodide (I^-) and Cl^-). It forms salts, which are mostly soluble in water with the exception of water insoluble salts, which are formed with copper (Cu), mercury (Hg), silver (Ag), lead (Pb), platinum (Pt), and thallium (Th) (Jolles, 1966; NRC, 2005). Like Cl^-, Br^- will attach to mineral surfaces at low pH, and to organic solids (Davis *et al*., 2004).

In the presence of organic matter and oxygen, Br^- forms organic compounds such as methyl bromide, which is a known endocrine-disrupting chemical. For the purpose of this article, the focus is on the interaction of inorganic Br^- with I^- and Cl^-. Organic bromides are mentioned to draw attention to the probability of health risks when the many forms of Br^- enter the production chain and, by extension, the food chain, although the topic is beyond the scope of this article.

The ocean is the greatest reservoir of Br^-, with concentrations of approximately 67 mg/L (Heeb *et al.*, 2014) and trace quantities are found in most groundwater (Davis *et al.*, 2004). In the United States it was reported that Br^- concentrations were highest in the coastal areas, decreasing further inland (Davis *et al.*, 2004), which has implications for producing potable water for domestic use by desalinating sea water. In groundwater across South Africa naturally occurring Br^- was reported to be present in concentrations that ranged from 0 to 132 mg/L according to collated data of the Water Research Commission (WRC) reports (Casey & Meyer, 2001; Casey *et al.*, 2001; Meyer, 2005; Casey & Meyer, 2006; Korsten *et al*., 2016).

Water quality constituents do not occur in groundwater in isolation. Their presence in groundwater varies, depending on the geochemical properties of the bedrock surrounding the aquifers. Different aquifers can therefore have varying WQC composition, even when in close proximity.

Research on the WQC has focused on the constituents that are potentially harmful and lead to toxicity (Meyer & Casey, 2012). Water is increasingly being recognized as a nutrient and a source of micro-minerals rather than merely a medium that promotes the uptake of nutrients from feed (Meyer & Casey, 2004; Korsten *et al*., 2016). The dissociated state of WQC in solution allows them to interact and potentially have high bioavailability. Thus, groundwater of a specific composition has the potential to supplement trace elements that are deficient in feed. Conversely, WQC can react in such a way that they render essential trace elements biologically unavailable, resulting in deficiency. This is interesting because high bioavailability allows WQC access to biological systems in which they can exert a synergistic or an antagonistic effect. In practice, recommended safe levels of trace elements in water and livestock feed are seldom reported simultaneously (Meyer, 2015). The focus of this article is on the toxicity of WQC, because it is more complicated to mitigate toxic effects than to supplement deficient trace elements.

Groundwater that is pumped into open reservoirs and exposed to UV radiation is subject to speciation of Br^- in the presence of oxygen to form bromate (BrO). This form of Br^- is a known carcinogen (Jain *et al*., 1996; DeAngelo *et al*., 1998; Magazinovic *et al*., 2004; Bonacquisti, 2006; Moore & Chen, 2006) and a potential by-product of water ozonation,

which is used to produce pathogen-free potable water. Oxidative water treatment, as with ozonation, results in Br^- oxidizing to hypobromous acid (HOBr) and hypobromite (OBr^-), which are the dominant Br species in chlorinated fresh water system. Hypobromous acid is the more reactive of the two forms, and readily reacts with many inorganic compounds (Heeb *et al.*, 2014). It is essential to know the level of naturally occurring background Br^- in a groundwater source that is specific to a site, because some water purification treatments may utilize chemicals that react with Br species to form carcinogens (Davis *et al.*, 2004). This underlines the importance of a multifactorial approach to risk assessment instead of relying on a linear concentration-based model that is commonly used to establish WQG. The WQG are set at a concentration safety level for various elements, against which the actual WQC composition in a water sample can be compared to establish the fitness-for-use of that water source for livestock or domestic use.

The South African WQG for livestock watering (Casey *et al.*, 1996) do not list Br^- as a COC or potentially hazardous chemical constituent (PHCC). Similarly, there are no WQG in the British Columbia, Canada that reference Br (Ministry of Environment, British Columbia, 2015). In contrast, the Australian and New Zealand WQG for human consumption, which were updated in December 2013, set the maximum exposure level at 0.02 mg/L (National Health and Medical Research Council and the Natural Resource Management Ministerial Council, Australian Government, 2016). In the absence of established WQG for local conditions, international guidelines can be useful as a point of reference, but it would be ill advised to rely solely on these as a benchmark, because climate and production systems differ between countries. Additionally, the variations in aquifer composition necessitate a site-specific evaluation as the best strategy to determine fitness-for-use. Anthropogenic factors, such as pumping rate and seasonal use of boreholes, can influence aquifer WQC composition.

Determining fitness-for-use of a groundwater source in the livestock context is important as it directs decision making regarding which groundwater sources are most suited to a specific livestock group. Livestock in various production phases differ in resilience to poorer quality groundwater. The toxicity of WQC depends on the intake rate and exposure time, the physiological state of the animal, and the interaction between WQC in the water.

The chemical form of a WQC determines its potential bioavailability in the animal after it has been ingested. Ions regulate cell membrane permeability, as with the principal electrolytes sodium (Na^+) and Cl^- in the extracellular fluid and potassium (K^+) in the intracellular fluid and the heart (Hribar *et al.*, 2002). In the gastrointestinal tract, Br^- forms HBr in mucosal cells in the same manner as hydrochloric acid. It is readily absorbed via the passive chloride transport mechanism and transported to the liver, where it is not metabolized (Baird-Heinz *et al.*, 2012), and to various other tissues and organs via the blood to move freely to multiple sites. In white blood cells, Br^- and Cl^- are oxidized with hydrogen peroxide and react with biomolecules of pathogens as part of the mammalian host defence system (Heeb *et al.*, 2014). Plasma Br^- levels vary linearly with their concentration in the diet (NRC, 2005).

The distribution of Br^- throughout the body is similar to that of Cl^-, which relates to Br^- being competitive with Cl^- (Pavelka *et al.*, 2000b). The half-life of whole-body Br^- at normal

dietary Cl^- levels is 10 to 12 days in humans, 3 to 8 days in rats and 15 days in dogs. Although many authors have stated that the half-life depends on the Cl^- level in the body, and that depressed Cl^- levels dramatically increase the half-life of Br^- (Pavelka *et al*., 2000b; Frances *et al*., 2003; Babicky *et al*., 2005; NRC, 2005), Pavelka *et al*. (2005) showed that the biological half-life of Br^- is dependent on the intake of Na^+ rather than Cl^-. It is also dependent on the physiological state of the animal: rat dams have the lowest Br^- half-life, just 44 hours, compared with their non-weaned offspring at 269 hours (Babicky *et al*., 2005). The short half-life of Br^- in the lactating female is probably because Br is excreted in the milk, and in the urine and faeces. Half-life also depends on the organ in which Br^- accumulates. According to Pavelka *et al.* (2000a), the liver has the longest half-life of Br^- at 235 hours, with the thyroid gland and brain having the shortest half-life at 94 hours. Whole-body half-life was 198 hours, which was longer than that of most of the tissues that were studied (Pavelka *et al*., 2000a). These authors reported significant correlation between values of steady state concentration of Br^- and the biological half-life in tissues, the liver being an exception.

There is competition between Br^- and Cl^- in all organs – with the exception of the thyroid – where Br^- competes with I^-. In the thyroid, Br^- is preferentially taken up to replace I^-, thus disrupting the production of thyroid hormones T_3 and T_4 over longer periods of exposure in broilers (Du Toit & Casey, 2012). Compensatory action by the thyroid may induce hypothyroidism and Br^- toxicity, which are expressed as secondary I^- deficiency. High exposure to Br^- is associated with damaged thyrocytes (Meyer, 2015), and lower feed and water intakes, adversely affecting production (Du Toit & Casey, 2010).

The body does not distinguish between Br^- and Cl^-, and excess Br^- replaces Cl^- in the total halide pool owing to its pharmacokinetic properties. The blood-brain barrier is permeable to Br^-, in which Br^- uptake exerts a sedative effect in the central nervous system. Similarly, the placental barrier is permeable to Br^- and trans-placental movement exposes the foetus to Br^- during critical stages of development. The toxicity effects in the foetus are dependent on the quantity and exposure time of Br^- that is ingested by the dam, resulting in impaired growth, negative impact on differential development, and the possibility of congenital goitre and hypothyroidism as a result of secondary I^- deficiency. The excretion of Br^- into the milk further compromises the viability of mammalian neonates. In female rats, a high concentration of Br^- reduced I^- accumulation in the mammary glands, increased renal excretion, and the pups exhibited thyroid abnormalities and reduced weight gain (Pavelka *et al*., 2002; Pavelka, 2004).

The primary excretion route is renal at approximately 5% of the ingested rate per 24 hours (Pavelka *et al.*, 2000b), while excretion also occurs via faeces, saliva and sweat. Preliminary results showed that despite high Br^- ingestion, the urinary excretion rate in Merino sheep is constant (Casey *et al*., 2017). The phenomenon of Br^- being competitive with Cl^- and probably infiltrating the Cl^-space (Pavelka *et al*., 2000a), the relatively low urinary excretion rate (5%) found in rats (Pavelka *et al*., 2000b) and the recycling of body water in ruminants, may exacerbate the likelihood of Br^- accumulation in the body from groundwater that contains low concentrations that are ingested over extended periods. This has implications for the safety of animal products that enter the food chain if Br^- that is retained in the tissues, milk and eggs is at concentrations above the NOAEL set in the South African WQG. In the body,

Br^- is preferentially absorbed when competing with Cl^-, which is evident in the kidney, where reabsorbed Br^- triggers preferential excretion of Cl^-.

Decreased fertility in male and female animals are expressions of endocrine-disrupting effects. In male rats, observed disturbances of endocrine function of the thyroid, testes and adrenal glands resulted in hypothyroidism and reduced spermatogenesis following exposure to high concentrations of Br^- (Loeber *et al*., 1983).

Risk assessment because of WQC is complex and requires observations of a number of parameters. A proposed model that is based on the intakes, physiology, and environmental factors illustrates the parameters that could be included in an assessment (Casey *et al*., 1998a,b; Meyer, 1998; Meyer & Casey, 2012):

∫(risk) = X1[Animal (or person) type] * X2[Animal (or person)'s physiological status] * X3[environmental demands] * X4[water (PHCC) intake and turnover rate] * X5[level of PHCC] * X6[physiological effect of PHCC] * …. Xn * e

This is essentially a metric system to estimate more accurately the probability of a WQC becoming a COC or PHCC. Text box 1 illustrates a practical approach to risk assessment.

Textbox 1 Guide to water quality constituents risk assessment

Sample water at source and at point of use.
Use correct preservation methods of water samples Use reliable analytical techniques.
Focus analyses on water quality constituents that have a bearing on livestock (e.g., Fluorine, Selenium) and watering facilities (e.g., Iron).
Review each of the water quality constituents in the analysis.

- Concentration (mg /L).
- Potential physiological effect of the water quality constituents.
- Possible alleviatory factors such as total dissolved solids and competitive water quality constituents Note the source of the sample

Note the water-user group (sheep: lactating, broiler chickens: one week old, horses: endurance) Note the site-specific factors that can influence water intake:

- Dry rations
- High physiological demand for a high water-intake as with young animals, lactation, high physical activity
- High altitude

Note the expected or given exposure time Note the general management practices
Repeat the sample and analysis for wet and dry seasons

(Meyer & Casey, 2012)

Casey and Meyer's (2001) report lists Br^- as having a target water quality range of 0–3 mg/L. A subsequent report (Casey & Meyer, 2006) introduced 0.01 mg/L as a WQG value to align it with similar limits. Although no conclusive evidence has supported the value, 0.01 mg/L was adopted as an acceptable norm and applied as a WQG value (Korsten *et al*., 2016). This relates to 0.01 mg/L as the default level that is recommended by Regulation (EC)

No 396/2005 on many residues in which the maximum residue level (MRL) has not been validated (European Parliament, 2005).

Subsequently, Lucht *et al*. (2018), having tested concentrations of Br^- that was injected into fertilized chicken *(Gallus gallus domesticus)* eggs, reported that Br^- is toxic to embryos at 1 mg/L and lethal at concentrations that are greater than 1 mg/L. Embryo survival was significantly negatively correlated (R^2 = - 0.92) with increasing Br^- concentrations. Concentrations that were greater than 0.01 mg/L showed potentially severe effects on developing embryos. The NOAEL target water quality range in developing chicken embryos was ≤0.01 mg/L.

This was the first conclusive validation of COC, PHCC and NOAEL for Br^- of 0.01 mg/L that appeared in literature. The chicken embryo, after direct injection of the substance to be tested into the albumen of the fertilised egg, is a highly sensitive assay for toxic effects of substances on a whole organism level over a short period, enabling identification of a stage at which embryonic death or other effects might occur (Korhonen *et al*., 1982). It also enables testing of direct effects on growing embryos independently of maternal influences that are present in mammalian animal models. The method has been used for this reason to study toxicological effects of various substances on target organ development and on the whole organism, especially in current nano-medical research (Sawosz *et al*., 2014). Although the chicken embryo model is highly sensitive, and could be regarded as a sentinel species test, the same conclusion may not apply to other types of livestock, which would require validation through controlled experiments.

The use of sentinel species for monitoring PHCC in the environment is not a new concept and is implemented mostly to monitor organic EDC in aquatic systems, as the aquatic animal models are highly sensitive to pollution. Areas were flagged as high risk when Br^- concentrations were greater than 0.01 mg/L. Site-specific observational recording of pathological conditions of livestock, which measure blood parameters and the analysis of harvested organs, have contributed significantly to associating the presence of WQC concentrations with these parameters. The effect of naturally occurring Br^- in groundwater, and that resulting from contamination of anthropogenic origin such as disinfectants and pesticides, in these areas was tested using broiler chickens and free-roaming village chickens in the area as sentinel species (Meyer, 2015). Tissue and blood sampling of these birds indicated that birds that were exposed to elevated Br^- concentrations in drinking water presented with thyroid anomalies and dysfunction (Meyer, 2015).

Vulnerable populations include neonates, females in gestation and lactation, individuals in an active growth phase, and immunocompromised individuals. Each production phase presents specific physiological demands. Water intake is a key factor to consider when identifying vulnerable individuals. Actively growing weaned animals and lactating females have higher water intake to meet the physiological demands of muscle and milk production, respectively. The risks of toxicity for these groups are simple to identify because high intake of water with high concentrations of PHCC expose them to acute toxicity symptoms.

Low concentrations of WQC, in this case Br^-, that are ingested over a longer period are the reason that it is considered a silent threat. When risk assessment is done in terms of metabolic body mass ($BW^{0.75}$) it is clear that foetuses, neonates and young animals are at greater risk of toxicity than older, larger animals because they are physically smaller and

in a state of active growth and tissue accretion. Mammalian neonates have an additional factor that elevates the risk of toxicity, which is the excretion of Br^- into the milk on which they are dependent until weaning.

Similarly, in the human context, vulnerable populations could mirror those found in livestock, with the exception of including older adults owing to the comparatively longer lifespan of humans. An additional factor that contributes to Br^- toxicity risk in humans is the compounding effect of Br^- in the drinking water that enters the food chain, where livestock ingest Br^- through drinking water and transfer it into tissues, milk and eggs. People that reside in affected areas use the same groundwater source as their livestock for drinking and cooking meat and organs, in addition to the consumption of milk and eggs. The compounding effect of natural Br^- paired with chronic exposure puts humans at risk of adverse toxicity effects on health.

Conclusion

Across South Africa, there are areas in which high concentrations of Br^- are naturally present in groundwater. The prevalence of high-risk groundwater overlaps with areas where groundwater is the main water source for livestock watering and human consumption. Although concentrations of Br^- in the groundwater may be low according to South African WQG for livestock watering, chronic exposure increases the risk of toxicity. Livestock may remain asymptomatic, since the production cycle is far shorter than the natural lifespan of the animals. However, risk to a vulnerable human population of concern because their longer lifespan results in a longer period of exposure. Regular seasonal sampling of groundwater is necessary to mitigate risk in areas that report problems in livestock production and human health. When site-specific WQC composition of groundwater has been established, fitness-for-use is determined from comparisons between Br^- and other element concentrations, and the NOAEL values in the South African WQG. This permits the allocation of poorer quality groundwater to more resilient livestock groups, reserving safer groundwater for use by vulnerable populations. When suitable alternative water sources are unavailable, the recommendation is to treat groundwater that is intended for human use, and, where necessary, for livestock watering.

CHAPTER 5

Survival and development of embryos of *Gallus gallus domesticus* treated with inorganic bromide

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Introduction

Inorganic bromide (Br^-) occurs naturally as a water quality constituent (WQC) in groundwater sources across South Africa with concentrations ranging up to 18.4 mg/L (Casey and Meyer, 2001; 2006; Meyer, 2015; Casey, 2016). The inorganic WQC in water sources have distinct influences on the quality of water according to their absolute and relative concentrations for the specific purpose of use and whether the exposure to the WQC is acute or chronic.

Water quality guidelines (WQG) are intended to assist user groups to gauge the suitability of the water according to the chemical constituents and physical properties. The WQG for inorganic WQC designate concentrations in generic terms as no observed adverse effect levels (NOAEL), as constituents of concern (COC) where concentrations are approaching the WQG-values and as potentially hazardous chemical constituents (PHCC) where the WQC concentration exceeds the WQG-values. These designations assume chronic exposure. The WHO (2011) Guidelines for Drinking Water notes however that not all water sources will contain WQC with WQG-values and that WQC without WQG-values, or that are not addressed in the WQG, may be of legitimate local concern. Inorganic WQC occurring naturally in groundwater are due to geological characteristics (Meyer *et al*., 2000). These might include the chemical nature of the rock and soil deposits; historic sediments in aquifers; current geothermal, surface agricultural and mining activities; and migration of water between aquifers. As a result, the concentrations of WQC in groundwater are largely site-specific.

The current South African Water Quality Guideline for livestock watering does not list Br^- as either a COC or a PHCC (Casey and Meyer, 1996). The report by Casey and Meyer (2001) lists Br^- as having a Target Water Quality Range (TWQR) of 0 - 3 mg/L. A subsequent report (Casey and Meyer, 2006) introduced 0.01 mg/L as a WQG value to align it with similar limits. Although no conclusive evidence supported the value, 0.01 mg/L was adopted as an acceptable norm and applied as a WQG value (Casey, 2016). This relates to 0.01 mg/kg as the default level recommended by Regulation (EC) No 396/2005 on many residues where the maximum residue level (MRL) has not been validated.

Bromine as a non-conjugated anion (Br^-) has been shown to have negative effects in mammals and poultry. Br^- exerted dramatic changes on the endocrine status of rats at high doses resulting in increased thyroid mass and depressed thyroxin (T_4) production, which caused decreased growth hormone (GH) production and increased insulin production leading to growth retardation (Loeber *et al*., 1983). Acute 31-day exposure to Br^- as sodium bromide (NaBr) via feed to hatchling chicks resulted in negative effects noted by Bosshardt *et al.* (1956) and Doberenz *et al.* (1965) as stated in NRC (2005). Administered to broilers as NaBr at 1 and 3 mg/L in drinking water respectively, over a 42-day growth period, Br^- significantly decreased water and feed intakes (du Toit and Casey, 2010). Accumulation of Br^- occurred in the thyroid gland, liver and kidneys and although there was limited damage to thyroid and kidneys, explicit liver damage was reported in birds receiving higher Br^- concentrations in their drinking water

(du Toit and Casey, 2012). The liver is a site of increasing concentrations of Br^- accumulation with increasing treatment levels of 0.005 and 0.1 mg/L (Mamabolo *et al*., 2009); and 0, 1 and 3 mg/L (du Toit and Casey, 2012). Yolk lipoprotein precursors originate in the liver, and it has been shown that drugs that deposit in the yolk are actively accumulated during the time the yolk undergoes rapid growth (Goetting *et al*., 2011). Albumen, which is deposited 2 - 3 hours following yolk maturation, is a possible accumulation site for drug residues (Goetting *et al*., 2011). The route for Br^- to be present in the albumen and yolk is most likely via the liver. The strong negative correlation between embryo and albumen mass indicates albumen is a nutrient source during the critical stages of growth and development in incubation (Akil and Zakaria, 2015). The presence of Br^- in albumen and yolk would expose embryos to the risk of adverse effects.

The question is to what extent could embryos be sensitive to Br^-? The hypothesis of this research was that should embryos be sensitive to Br^-, a differential response occurs in the survival and development of chicken embryos following direct exposure to varying concentrations of Br^-.

Materials and Methods

The animal model chosen to investigate the potential toxic effects of Br^- was the chicken (*Gallus gallus domesticus*) embryo. It has the advantages of testing the toxic effects of a substance on a whole organism level over a short period and enabling identification of a stage at which embryonic death or other effects might occur (Korhonen *et al*., 1982). It also enables testing of direct effects on the growing embryo independent of maternal influences present in mammalian animal models. The method has been used for this reason to study toxicological effects of various substances on target organ development and on the whole organism, especially in current nano-medical research (Sawosz *et al*., 2014).

This investigation of Br^- toxicity was done in three phases. For all experiments, fertilized Ross-308 broiler eggs were disinfected prior to treatment by wiping down shells with potassium permanganate ($KMnO_4$) solution and exposing eggs to UV light for 50 seconds. Following disinfection, the shell of each egg assigned to a treatment other than 0 mg Br^-/L (Control), was swabbed with ethanol and a small hole made in the shell using a sterile 18 G needle one third the distance from the narrow end of the egg. Each egg was injected into the albumen with 200 µL NaBr solution, the concentration of which was dependent on the assigned treatment group, using a sterile 23 G x ½ inch needle for each egg. Excess albumen was wiped off the shell using cotton wool and the hole sealed using medical tape.

The eggs for all treatment groups were placed in the ALMD-1N3-7 incubator (FHU Walenski, Gostyn, UK) according to the method used by Sawosz *et al*. (2014), under the following standard conditions: day 1 - 17 at 37.7 °C and humidity 60%; day 18 - 20 at 37 °C and humidity 70%.

Phase 1: Five fertilized Ross-308 eggs were injected with 10 mg Br^-/L ($Tr_{10mg/L}$). On day five of incubation, one egg was chosen at random and broken open into a clean, non-sterile petri dish for evaluation of angiogenesis with the naked eye. The embryo anatomy and the network of blood vessels were further evaluated using a Model SZX2-ILLB binocular stereomicroscope (Olympus Corporation, Japan), while attached to the yolk. The embryo was excised using a ring of filter paper and placed on a clean glass slide for a clearer field of view

of its anatomy. The microscope images were recorded. On day 10 of incubation, the remaining four eggs were broken open into individual clean, non-sterile petri dishes and evaluated with the naked eye to determine whether Br^- had an effect on embryo survivability. No microscope images were taken because no live embryos were recorded.

Phase 2: 45 fertilized Ross-308 eggs were randomly assigned to three treatments (15 eggs per treatment group): $Tr_{0mg/L}$ (Control) = 0 mg Br^-/L, $Tr_{5mg/L}$ = 5 mg Br^-/L and $Tr_{1mg/L}$ = 1 mg Br^-/L. After five days of incubation, samples were randomly selected from each treatment group to be examined for embryo survival as follows: Tr_{0mg} one egg, Tr_{1mg} two eggs and Tr_{5mg} five eggs. The eggs were broken open, their contents carefully poured into individual clean, non-sterile petri dishes and examined with the naked eye for signs of angiogenesis and embryo survival. No microscope images were taken at this stage. The following samples were taken on day 18 of incubation: $Tr_{0mg/L}$ 14 eggs, $Tr_{1mg/L}$ 13 eggs and $Tr_{5mg/L}$ 10 eggs, which were broken open as described and the results recorded.

Following the results of Phases 1 and 2, Phase 3 was designed such that 148 fertilized Ross-308 eggs were randomly assigned to five treatment groups: $Tr_{0mg/L}$ = 0 mg Br^-/L, $Tr_{0.01mg/L}$ = 0.01 mg Br^-/L, $Tr_{0.05mg/L}$ = 0.05 mg Br^-/L, $Tr_{0.5mg/L}$ = 0.5 mg Br^-/L and $Tr_{1mg/L}$ = 1 mg Br^-/L.

On day 20 of incubation, eggs were randomly removed from the incubator and broken open until a minimum of six live embryos were collected per treatment group. The distribution of the total number of eggs removed (n = 59) differed between treatments according to the incidence of non-viable eggs occurring within a treatment group. After breaking the shell, the embryos were removed and placed on a clean, non-sterile petri dish. The number of live embryos and the macroscopic evaluation were determined according to the procedure of Hamburger and Hamilton (1951). The embryos were allocated as live embryos when they showed signs of being alive *in ovo* at breakout and had died immediately post breakout. Any embryos showing signs of embryonic death before the shells were broken were recorded as dead embryos. Six live embryos per treatment were weighed and dissected to harvest the brain, heart and liver. The collected organs were individually weighed and the relative organ mass (g) was calculated against the mass of the whole embryo (g) and expressed as a percentage of the total embryo mass. The remaining eggs (n = 89) were placed in hatching boxes in the incubator to hatch. The relative embryo survival was calculated as a percentage of live vs. dead embryos for each treatment.

Statistical analysis was done for Phase 3 only. The FREQ procedure was used to determine the chi-square goodness-of-fit test for percentages of live or dead embryos within and between treatments (P <0.05) (Statistical Analyses System®). The GLM procedure was applied to test for differences in relative organ mass between treatments by means of the F-test (P <0.05).

The project was done at the Department of Animal Nutrition and Biotechnology, Warsaw University of Life Sciences (SGGW), Poland and conformed to the requirements of EU Directive 2010/63/EU for experimental animals.

Results

Table 5 shows the results of Phases 1 and 2. The embryo from Phase 1 ($Tr_{10mg/L}$) evaluated on day five of incubation appeared to be viable to the naked eye, which was confirmed with subsequent microscopy. Of the remaining eggs (n = 4) evaluated on day 10 of

incubation, one egg was unfertilized and the remaining three showed various stages of embryonic death.

In Phase 2, randomly selected eggs from each treatment evaluated with the naked eye showed that embryos exposed to 1 mg Br^-/L presented with varying degrees of impediment of angiogenesis observed on a macro scale when compared with angiogenesis of the control group. Embryo mortality was observed for all eggs treated with 5 mg Br^-/L.

On day 18 of incubation, the eggs evaluated from $Tr_{0mg/L}$ showed one non-fertilized egg, and all other embryos were alive with normal development. All remaining eggs (n = 10) sampled from $Tr_{5mg/L}$ showed embryo mortality and various stages of proteolysis. Two live and eleven embryo deaths were observed for $Tr_{1mg/L}$. The live embryos showed normal development on a macroscopic scale. It was deduced from these results that Br^- is lethal to embryos at concentrations >1 mg/L and toxic at 1 mg/L.

Table 5 Embryo survival between treatments (10, 5, 1, 0 mg/L Br) on selected days of incubation

Treatments		Eggs	Sampling at days of incubation								
			5			10			18		
Phase	(mg/L Br)	N	NF	L	D	NF	L	D	NF	L	D
1	10	5		1		1		3			
2	0	15		1					3	11	
2	1	15		2						2	11
2	5	15	1	4							10

NF = Not fertilized; L = Live embryo; D = Dead embryo

The results of Phase 3 in Tables 6and 7 where the chi-square determination was applied for live or dead embryos within and between treatments show a decline in the relative number of live embryos with increasing concentrations of Br^- (P <0.05).

Table 8 shows the linear relationship between Br^- concentrations and chicken embryo survival. A high negative correlation (R^2 = -0.92) occurred between increasing concentrations of Br^- and the percentage of embryo survival, and a high positive correlation (R^2 = 0.82) occurred between increasing concentrations of Br^- and the percentage of embryo mortality.

Table 9 shows the differences in mean relative mass for the heart, liver and brain respectively between treatment groups. Relative organ mass (g), expressed as a percentage of the whole embryo mass (g), was compared between treatments within each organ type, but no comparison was made between the relative masses of different organs within treatment.

Table 6 Relative embryo survival (%) within treatments

Treatment	Eggs	% of embryos alive / dead	
mg/L Br	N	Alive	Dead
0	28	85.7[a]	14.3[b]
0.01	30	86.7[a]	13.3[b]
0.05	30	60.0[a]	40.0[b]
0.5	30	43.3[a]	56.7[b]
1	30	40.0[a]	60.0[b]

[a,b] Row means with different superscripts differ significantly at P <0.05; F-test

Table 7 Relative embryo survival (%) between treatments

Treatment	Eggs	% of embryos	
mg/L Br	N	Alive	Dead
0	28	25.8[a]	7.3[b]
0.01	30	28.0[a]	7.3[b]
0.05	30	19.3[a]	21.8[b]
0.5	30	14.0[a]	30.9[b]
1	30	12.9[a]	32.7[b]

[a,b] Row means with different superscripts differ significantly at P <0.05; F-test

Table 8 Linear relationships between bromide (Br^-) treatments (0 mg/L, 0.01 mg/L, 0.05 mg/L, 0.5 mg/L and 1 mg/L Br) and chicken embryo survival (%)

	R^2	Covariance	r^2	Rt MS	Rt MSE	P >0.05
% Embryo mortality	0.68	88.43	0.4574	63.73485	12.7470	**
% Hatched	-0.92	65.32	0.8467	92.0071	18.4014	**
% Total live embryos	-0.92	44.16	0.8486	141.0762	28.2152	**
% Total mortality	0.82	73.62	0.6798	70.6786	14.1357	**

Table 9 Percentage mean (± SE) of relative organ mass by treatment with correlation (R^2) and slope relationships between treatments and percentage mean relative organ mass.

Treatment	Relative organ mass (%)		
mg/L Br	Heart	Liver	Brain
0	0.8232[a] ± 0.07	2.1687[ab] ± 0.12	2.3658[a] ± 0.15
0.01	0.8588[ab] ± 0.07	2.0048[a] ± 0.12	2.5268[a] ± 0.15
0.05	0.7921[a] ± 0.07	1.8526[a] ± 0.12	2.7115[ab] ± 0.15
0.5	1.0305[b] ± 0.07	2.5169[b] ± 0.12	2.6346[a] ± 0.15
1	1.0212[b] ± 0.07	2.1177[a] ± 0.12	3.1456[b] ± 0.15
R^2	0.88	0.38	0.88
Slope	3.40	0.67	1.31

[a,b] Column means with different superscripts differ significantly at P <0.05; F-test

Discussion

The treatments that ranged from 0 - 1 mg Br^-/L simulated the levels of exposure to Br^- measured in groundwater throughout South Africa. Although levels as high as 18.4 mg/L have been recorded in the field, the maximum treatment concentration of 1 mg/L was set following the results of the Phases (1) and (2) (Table 5). This maximum treatment concentration was 100 times greater than the 0.01 mg/L taken to be the maximum value for a no observed adverse effect level (NOAEL) or a critical TWQR-value. The concentrations of 0, 0.05 and 0.5 mg/L on either side of 0.01 mg/L were intended to further test the sensitivity to the recommended WQG level of 0.01 mg/L. The treatments were by injection into the albumen in order to have controlled treatments. In addition, the treatment method was intended to mimic, under controlled conditions, the exposure to and deposition of Br^- in the albumen and the yolk, though direct injection into the yolk was not done. Both albumen and yolk could be exposed to Br^- present in the blood plasma of hens exposed to concentrations of Br^- in drinking water. This is possible as shown by the negative effects ingested Br^- had on the endocrine system and organs of developing broiler chickens. The concentration of Br^- in the hen's plasma is a function of the level in the drinking water, the ingestion rate via the quantity of water the hen drinks and the absorption of Br^- from the digestive tract into the blood plasma as reported for growing broiler chickens (Mamabolo *et al*., 2009; du Toit and Casey, 2010; 2012). Since the albumen is a possible accumulation site for circulating drug residues (Goetting *et al*., 2011), ingested Br^- could transfer a similar concentration to the albumen in a manner that mimics trans-placental movement of elements from dam to foetus in mammalian species.

TWQR-values are given as a range, for example fluoride (F) which has a recommended TWQR of 0 - 2 mg/L for no adverse effects, 2 - 4 mg/L yielding mild adverse effects and levels >4 mg/L causing debilitating adverse effects in livestock. Meyer and Casey (2012) listed particular circumstances that contribute to WQC becoming COC or PHCC. These include chronic intakes at concentrations below WQG-values if the constituents accumulate in the body, or the clearance rates are relatively low leading to an exponential accumulation until a toxicity threshold is crossed. A PHCC situation evolves under short-term or acute intakes at concentrations exceeding WQG-values and when animals are in hypersensitive physiological stages that require high water intake, such as during the early growth period, during lactation in mammals when there is an increased demand for water, or during a period of adverse temperature-humidity index. Du Toit and Casey (2012) found Br^- to be potentially toxic and an endocrine disrupting chemical (EDC) by mechanism of depressing the levels of thyroid hormones in broiler chickens.

The high correlation coefficients shown in Table 4 indicate that chicken embryos are highly sensitive to increasing concentrations of Br^- injected into the albumen. The concentrations 0.5 mg/L and 1 mg/L Br^- had the most detrimental effect on embryo survival (Tables 6 and7). This raises the possibility of Br^- influencing the differential development of organs and/or exerting teratogenic effects during embryo development.

The development of the three organs monitored is presented as relative organ mass against total embryo mass (Table 9). The relative organ mass was introduced to equalise mass differences that might have occurred between embryos. An increasing relative mass due to increasing treatment concentrations would indicate a differential response and hypertrophic

growth. The heart showed a sequential relative organ mass response to the increasing concentrations of Br^-. The mean relative mass of the heart was significantly greater for embryos treated with 0.5 mg/L and 1 mg/L Br^- respectively, compared with those from $Tr_{0mg/L}$ and $Tr_{0.05mg/L}$ with a correlation coefficient of $R^2 = 0.88$ and the slope or linear rate of relative increase 3.4.

The mean relative liver mass in $Tr_{0.5mg/L}$ was significantly greater against $Tr_{0.01mg/L}$ and $Tr_{0.05\ mg/L}$, but non-significantly different from $Tr_{0mg/L}$. The correlation coefficient ($R^2 = 0.38$) and the low incremental rate (<1.00) indicate the possibility of hypertrophic growth with increasing Br- concentrations, but this is inconclusive.

Increasing concentrations influenced the differential increase in relative brain mass significantly with the $R^2 = 0.88$ and the slope or linear rate of increase = 1.31. The relative mean mass of the brain was significantly greater in embryos treated in $Tr_{1\ mg/L}$ compared with those in $Tr_{0mg/L}$, $Tr_{0.01mg/L}$ and $Tr_{0.5mg/L}$ groups.

These results show differential responses in relative mass gain with increasing concentrations of Br^- and that the heart had the greatest response and possible hypertrophic growth compared with the brain and liver.

It appears from the results that 0.01 mg/L Br^- is sufficiently stringent and concurs with the conclusion of du Toit and Casey (2012). In reality the wide range and excessively high Br^- concentrations reported to occur naturally in groundwater indicate that many animals and humans are chronically exposed to Br^- concentrations exceeding 0.01 mg/L level.

Conclusion

The results show a differential response of embryo survivability and development of the heart, liver and brain to different Br^- concentrations. The concentration of 0.01 mg/L Br^- emerged as the watershed value between concentrations that have limited or no negative effects. Concentrations >0.01 mg/L Br^- have potentially severe effects on developing chicken embryos, concurring with previous research on broiler chickens. In the absence of similar evidence from other livestock species, the recommendation is to accept 0 - 0.01 mg/L Br^- as the TWQR-value.

CHAPTER 6

The response of relative organ mass to different Br^- concentrations

Introduction

Broiler performance is dependent on embryonic development (Tona *et al.*, 2010). Hamburger and Hamilton (1951) quantified embryonic growth according to stage of development in order to compare development equally between different embryos, including interspecies comparisons. The days of incubation were denoted as embryonic day (ED) one to 21 and hatch day (HD) to distinguish pre- and post-hatch development.

In order to interfere with embryogenesis of the developing chicken embryo, potentially harmful WQC would need to enter the egg when the yolk and albumen are deposited, but before the shell is formed, because the embryo has limited nutrient availability once confined to its *in ovo* environment (Khanum *et al.*, 2019). During the first half of incubation the water in the albumen moves into the yolk causing the albumen to reduce in volume (Willems *et al.*, 2014). The implication is that all ions present in the albumen could be transferred into the yolk by either active or passive transport mechanisms, to be utilised by the developing embryo.

In mammals and non-mammals, TH were shown to be involved in both early prenatal or pre-hatch development and postnatal or post-hatch development (Darras *et al.*, 2011).

From the first day of incubation (ED 1), T_3 was shown to have a role in chicken embryo growth (Darras *et al.*, 2011). T_3 nuclear binding sites were shown to be present in early embryonic liver and brain development in chicken embryos (Bellabarba & Lehoux, 1981; Ballabarba *et al.*, 1983 and Haidar & Sakar, 1984 as cited by Darras *et al.*, 2011).

The membranes surrounding the embryo appear to be the same between the *in ovo* membranes of birds and the mammalian placental membranes (Henriksen *et al*., 2011).

In the chicken, embryonic development is completed within 21 days and by the ED 7 the embryonic thyroid gland is functional (De Groef *et al.*, 2008). There appears to be localised control of T_3 concentrations preventing an oversupply of T_3, which could be detrimental to normal embryogenesis (Tsai *et al.*, 2002). It is known that TH is necessary for early embryonic development in mammalian and non-mammalian vertebrates, and that maternal TH is the sole source of TH available to the early embryo (Darras, 2019). Maternal TH present in the yolk supports early embryonic growth and development until the embryonic thyroid gland becomes functional around mid-incubation (Darras, 2019).

A rapid decrease in albumen can be detected between ED 12 and ED 16 as the embryo utilizes the proteins for growth when they are swallowed from the amniotic fluid or stored in the yolk for utilization in the early post-hatch phase (Tona *et al.*, 2010).

Embryonic organ development depends on metabolic function, which is a corollary of thyroid function (Afsarian *et al.*, 2018). Any factor that compromises organ development during the embryonic phase will compromise the viability of the growing bird in post-hatch life (Druyan *et al.*, 2007).

The heart, liver and brain are crucial organs for the optimal functioning of the body. The heart and liver are considered to be supply organs (Christensen *et al.*, 2002). The heart is the first supply organ to develop within the first few days of incubation to supply blood to all developing organs and tissues. The liver follows negative allometric growth in respect to increasing body mass with increasing age, and has a slower growth rate relative to the overall

growth of the embryo (Schmidt *et al*., 2009). The liver is an important organ for newly hatched chicks and develops rapidly after hatching (Bhanja *et al.*, 2009). The brain orchestrates all hormonal functions including TH regulation. Br^- has been shown to have a neurological effect as it moves freely into the cerebrospinal fluid and interstitial tissues of the brain (Baird-Heinz *et al.*, 2012). There is a strong possibility that Br^--induced hypothyroidism can impair embryonic organ development, thus increasing the risk of poor chick quality and depressed post-hatch growth.

The question was whether the growth of the chicken embryo liver, heart and brain is sensitive to Br^- treatment because an embryo is the most sensitive organism of all populations. The aim of this study was to investigate the responses of the heart, liver and brain of the chicken embryo to different treatment concentrations of Br^- during incubation.

The null hypothesis was that a differential response occurs in the mass of the heart, liver and brain following direct exposure to varying concentrations of Br^-.

Materials and Methods

The experiment was divided into 5 Runs and the trials were designed to investigate the possible responses of chicken embryos to different Br^- concentrations from ED 0 to HD.

The trials were approved by the Animal Ethics Committee of the University of Pretoria before commencing (Certificate EC038-17).

356 fertilised Ross-308 hatching eggs were divided into 5 incubation runs due to logistics, which were carried out at the hatchery at the University of Pretoria Experimental Farm, Pretoria.

Run 1 was carried out to validate the incubator efficacy before commencing the *in ovo* injection trials. 36 fertilized Ross-308 eggs were sourced from National Chicks in Pretoria, Gauteng. The age of the source flock was unknown.

Eggs (n=80) for Runs 2 and 3 were sourced from National Chicks in Pretoria, Gauteng on two separate occasions, where 40 eggs were used for each Run. For Runs 4 to 5, 120 eggs were sourced on two separate occasions (n=240) from a National Chicks egg production facility in the Kwa-Zulu Natal Midlands due to a local egg shortage resulting from the quarantine measures implemented following an avian influenza outbreak in Gauteng Province. The age of the source flocks was unknown.

For each Run, the eggs were collected and stored on a flat surface at room temperature for up to five days before treatment and setting. In each Run, eggs were randomly assigned to one of five treatments according to Br^- concentrations:

$T_{0mg/L}$ (Control) = 0 mg/L
$T_{0.01mg/L}$ = 0.01 mg/L
$T_{0.05mg/L}$ = 0.05 mg/L
$T_{0.5mg/L}$ = 0.5 mg/L
$T_{1mg/L}$ = 1 mg/L

The lowest dose of 0.01 mg/L Br^- was chosen to be in line with previous research (Lucht *et al*., 2018) that validated the dose as an acceptable NOAEL for Br^-. The treatment concentrations were chosen to be in line with conventional dose response trials where the highest dose was between 50 and 100 times that of the lowest dose.

For Run 1, eggs were set in the incubator under standard conditions after the storage period. Standard incubation conditions were: ED 0 through ED 17 at temperature 37.7 °C and RH 60%; ED 18 through ED 20 at temperature 37°C and RH 70%. Eggs were turned automatically at regular intervals from ED 0 through ED 18, after which the eggs were kept in an upright position until ED 20. The incubator temperature and RH were logged using a logger (DWL-20, Hairius Instrument Co. Ltd) placed on the floor of the incubator and set to record data at 3-hour intervals for the duration of incubation.

For Run 1 only, each egg (n=40) was first disinfected using 0.8% $KMnO_4$ solution followed by disinfection of the injection site with 91% isopropyl alcohol. The use of $KMnO_4$ was discontinued for subsequent Runs and only 91% isopropyl alcohol was used as a surface disinfectant for all remaining eggs to rule out the interference of K^+ with Br^- uptake.

For Runs 2 to 5, eggs were marked with a unique identification using pencil and weighed individually. After each egg was weighed, the shell was disinfected with 91% isopropyl alcohol before a small hole was made in it using a sterile 18G x 1½ inch needle, one-third the distance from the narrow end of the egg.

After the hole was made in the shell, 200 µL NaBr of each treatment was injected into the albumen at a 45˚ angle using a sterile 25G x $^5/_8$ inch needle for each egg. Excess albumen was wiped from the shell with clean cotton wool and the hole sealed with medical tape (Micropore™, 3M). All eggs were distributed randomly throughout the egg tray and incubated.

Treated eggs were incubated for 20 days at standard conditions and turned automatically from ED 0 to ED 18. The incubator temperature was automatically controlled, but RH was controlled manually by placing pans of water on the incubator floor and refilled as needed.

For Run 1, one egg was opened on ED 10 to determine whether embryo growth had occurred by observing the presence of eyes, limb buds and CAM, and embryo viability. On ED 16, 10 eggs were broken open to examine the progression of embryo development. On ED 20, the remaining 24 eggs were broken open and examined for signs of internal pipping.

For Runs 2 to 5, break out analysis was done on ED 14 and ED 20 respectively. For all Runs only viable embryos were used for collection of data and any embryo deaths were noted to calculate hatchability. Viable embryos showed signs of life at break out but did not survive after the shell and membranes were broken, and were thus not alive when weights and measurements were collected. Embryo and organ masses and growth indicator measurements collected during each stage were pooled for all Runs within sampling point (SP).

For Runs 2 and 3, four eggs per treatment group (n=20) were randomly selected on ED 14 and ED 20 respectively for breakout analysis. For Runs 4 to 5, 12 eggs per treatment (n=60) were randomly selected from each treatment group for breakout analysis on ED 14 and ED 20 respectively. The crown-rump (CR) length and shank length were measured with callipers in millimetres. Whole embryo mass (grams) was measured before the collection of heart, liver and brain.

For Runs 2 to 5 the yolk sac and membranes were removed and the embryo dried off using paper towels before measurements and masses were taken. For Runs 2 and 3, after the yolk sac was removed and the embryo weighed, the heart, liver and brain were removed and placed in a petri dish containing 0.72% NaCl solution (Salex™) to prevent tissue damage before weighing. For Runs 4 and 5, phosphate buffered saline (PBS, Merck Millipore Oncogene) solution was prepared using one tablet of PBS dissolved in 1 L deionised water to

yield a solution of 140 mM NaCl, 10mM phosphate buffer and 3 mM KCl at a pH of 7.4. Collected organs were placed in a petri dish containing PBS to prevent tissue damage before weighing. The wet masses of the yolk-free whole embryo and the heart, liver and brain were measured in grams using a sensitive balance.

For Runs 2 to 5 the relative masses of embryos and organs on ED 14 and ED 20 were calculated as organ mass over whole embryo mass for respective SP.

Statistical analysis was done using the GLM procedure of Statistical Analysis Software® (SAS 2019) to determine the interactions between treatments and the relative body and organ masses on each of the SP. Embryos with less than 50% relative body mass at breakout on ED 20 were excluded from the data set. Significance was set at ($P \leq 0.1$) and non-significance (NS) is noted. Means are least square means (LSM) and standards errors (SE) are noted.

Results

The inside temperature of the incubator remained stable throughout the incubation period but the RH fluctuated due to the necessity of manual management of the water levels in the pans (Fig. 2).

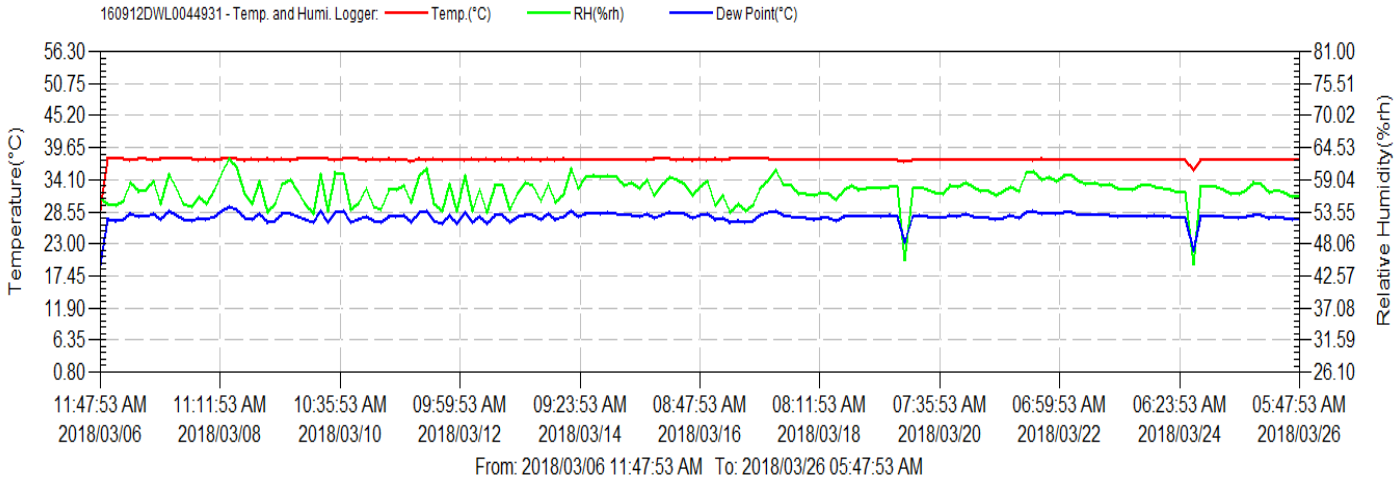

Figure 2 Temperature (Temp), relative humidity (RH) and dew point inside incubator over 20 days incubation period

The data for ED 14 and ED 20 were analysed separately using a one-way ANOVA (SAS®, 2019). Within ED, the data showed that the treatment differences of relative organ mass for heart, liver and brain on ED 14 were NS ($P \leq 0.1$) (Table 10). On ED 20, the difference of relative heart mass between treatments was significant ($P \leq 0.1$), but there were NS differences between liver and brain masses between treatments ($P \leq 0.05$) (Table 11).

Table 10 The difference between relative organ mass (g, LSM±SE) between treatments (mg/L) on embryonic day 14

Treatment	n	Heart	N	Liver	n	Brain
0	29	1.25±0.03	29	2.41±0.08	29	4.36±0.10
0.01	6	1.29±0.07	6	2.40±0.18	6	4.41±0.23
0.05	16	1.25±0.04	16	2.40±0.11	16	4.50±0.14
0.5	15	1.31±0.04	15	2.54±0.11	15	4.40±0.14
1	14	1.31±0.05	14	2.53±0.12	14	4.50±0.15

On ED 14, the brain had the largest mass relative to the whole embryo, followed by the liver and heart respectively. This followed the expected normal progression of differential development of the embryo. There were only six viable embryos available for $T_{0.01mg/L}$, which accounted for the low n reported in Table 10.

Table 11 The difference of relative organ mass least square means (g, LSM±SE) between treatments (mg/L) on embryonic day 20

Treatment	n	Heart	n	Liver	n	Brain
0	19	0.75±0.04 [a]	19	1.84±0.09	19	2.63±0.14
0.01	9	0.74±0.06 [a]	9	1.93±0.14	9	2.49±0.21
0.05	8	0.90±0.06 [b]	8	1.93±0.15	8	2.95±0.22
0.5	12	0.80±0.05 [ab]	12	1.75±0.12	12	2.50±0.18
1	11	0.76±0.05 [a]	11	1.94±0.12	11	2.63±0.19

[ab] Column means are significantly different ($P \leq 0.1$)

On ED 20 (Table 11), the relative organ masses followed the same order of magnitude as at ED 14, but it was shown that the differences between the respective relative organ masses were less than on ED 14 (Table 10). These results were expected because the whole embryo grows to maturation during incubation and the organs occupy a relatively smaller space within the body on ED 20 compared to ED 14, when organ development is favoured over whole embryo growth.

There were only nine viable embryos available for $T_{0.01mg/L}$ and eight viable embryos available for $T_{0.05mg/L}$ which accounted for the low n values reported for these treatments reported in Table 2. It is unclear whether the low hatchability was due to treatment effect.

At a treatment concentration of 0.05 mg/L Br^- the relative organ masses were not as expected with increased dose response, and presented as outliers (Table 11).

Congenital defects were noted only in the context of determining hatchability and were not found to be related to Br^- dose.

Discussion

The incubator temperature and RH remained constant and within the range of standard incubation conditions throughout incubation (Fig 2) and thus growth anomalies were noted not to be in response to incubation conditions.

The concentration range of 0 - 1 mg/L Br^- was chosen to satisfy the recommendation of Bigsby *et al.* (1999) of an upper level dose 50 times that of the low dose and to test the response of relative organ mass to a potentially toxic concentration validated in previous research. NaBr was chosen as the Br^- source since according to Baird-Heinz *et al.* (2012) it appeared to have a greater affinity for the thyroid gland and a greater effect on the disruption of TH synthesis than KBr.

The growth of the heart, liver and brain followed normal allometric growth in relation to whole embryo mass between ED 14 and 20, showing decreased relative organ mass with increasing age. This was expected because with increased whole embryo mass over time, the relative organ mass will decrease proportionately. When the relative masses of the supply organs are less than those for the control group chick quality may be compromised.

It emerged from the data that the greatest dose response of relative organ mass to Br^- treatment was apparent for the heart but not for the liver or brain.

The liver controls TH transport and metabolism by extracting up to 10% of plasma T_4 and synthesizing plasma proteins to bind the lipophilic TH, which provides a pool of TH in which T_3 and T_4 exchange can occur (Malik & Hodgson, 2002). This metabolism control measure in the liver may temper the effects of Br^- interference with TH synthesis for a time, and only in severe hypothyroidism will liver disease occur. Prolonged Br^- exposure may lead to hypothyroidism severe enough to cause liver disease. This should be further investigated and was beyond the scope of this study.

The significant difference in relative heart mass on ED 20 may be indicative of a treatment effect, and the greater relative heart mass for $T_{0.05mg/L}$ compared to all other treatments may indicate a threshold value at which Br^- has an effect on whole body growth. The larger relative heart rate with increasing Br^- concentration may indicate that Br^- interfered with TH action on whole body growth rather than influencing the growth of the heart. The results were in agreement with previous research done on the effect of Br^- on relative organ mass. It was expected that Br^- would have an interference effect on TH action but it appears from this data that on ED 20 this was not the case. From the data it can be seen that there is an apparent effect of Br^- on the growth of organs in the embryo, thus it is possible that Br^- interference with TH function may occur at earlier stages of embryonic development, though results of this study were inconclusive.

Conclusion

The results indicated that Br^- may have an effect on the growth of the whole embryo and that of the heart during incubation, which may influence post-hatch viability of chicks. There was an apparent effect of TH function at different stages of development, which affected different aspects of development. Since the heart was the organ that responded to different concentrations of Br^- at later stages of development further investigation of the response of the relative heart mass to Br^- concentrations over the period of incubation until HD is recommended.

CHAPTER 7

The response of relative heart mass to different Br^- concentrations

Introduction

Modern broiler selection has focused on fast growth rates and increased breast muscle mass (Hassanzadeh *et al.*, 2005). Selection for greater breast meat yield may exacerbate the inverse relationship between muscle mass and the organs of the cardiopulmonary system, as glycolytic white muscle fibre development will not drive an increase in heart mass because the oxygen requirement is lower compared to red muscle fibres (Decuypere *et al.*, 2000). This results in the disproportionate development of internal organs in relation to skeletal muscle accretion, and an insufficient ability of the cardiopulmonary system to support the high metabolic demands of fast muscle growth (Hassanzadeh *et al.*, 2008; Mohammadalipour *et al.*, 2017). It is necessary for incubation conditions to be optimal to support optimal embryo development during the pre-hatch phase.

There is a possibility of an inverse relationship between selection of greater muscle yield and producing birds with a relatively smaller cardiovascular system (Decuypere *et al.*, 2000). Organ development during the pre-hatch phase will become important in the post-hatch phase, when the supply organs must support rapid growth of the broiler. During the development of the embryo, normal allometric growth of organs occurs, resulting in organs to be proportionately larger or smaller than the whole body growth depending on the developmental stage.

The heart is one of the major supply organs (Christensen *et al.*, 2002) and is the first organ to develop to supply blood to all other developing tissues throughout embryonic development. Selected changes in proportional growth produce birds with small cardiopulmonary systems relative to whole body mass (Hassanzadeh *et al.*, 2005).

Insufficient heart development in the early phases will predispose the fast growing bird to developing circulatory problems during the post-hatch growth phase (Druyan *et al.*, 2007) because the development and growth of the supply organ eventually fail to keep up with the rapid muscle accretion characteristic of modern broiler strains (Buzała *et al.*, 2015).

Many physiological functions depend on TH regulation, making normal thyroid function essential for maintaining homeostasis (Boas *et al.*, 2012). *In ovo* T_4 treatment resulted in decreased relative heart mass (Afsarian *et al.*, 2018). TH respond to higher metabolic rate and promote cardiac output by action on the heart muscles (Rutigliano & Zucchi, 2017). Shuvy *et al.* (2009) hypothesised that in humans, hypothyroidism could impair cardiac muscle function. The various Cl^- channels distributed across the heart demonstrate different selectivity sequences, such as in the rabbit ventricle where the Ca^+-activated Cl^- current has the sequence $I^->Br^->Cl^-$ (Sorota, 1999). It is possible that Br^- may interfere with TH synthesis and action, which may impair heart growth. Furthermore the possibility exists that Br^- may interfere with the Cl^- channels in the heart, resulting in impaired heart function.

The heart is the major supply organ to the developing embryo and the question was whether the growth of the chicken embryo heart is sensitive to Br^- treatment.

The aim of this study was to investigate the response of the relative heart mass to different concentrations of Br^- during the pre-hatch period.

The hypothesis was that a differential response occurs in the mass of the embryonic heart following direct exposure to varying concentrations of Br^-.

Materials and Methods

A total of 900 fertilised Ross-308 eggs were sourced from Festive flocks aged 45 weeks. The number of eggs chosen was based on a 60% hatch rate and the flock age was considered to be that of birds in peak production. Broken and severely cracked eggs were discarded from the trial before eggs were injected. The trial was conducted at the Astral Festive Research Farm in Doornkloof, Pretoria. Ethical approval was obtained before the commencement of the trial (Certificate EC038-17).

Eggs were randomly assigned to one of four treatments according to Br^- concentrations:

$T_{0mg/L}$ (Control) = 0 mg/L

$T_{0.01mg/L}$ = 0.01 mg/L

$T_{0.5mg/L}$ = 0.5 mg/L

$T_{1mg/L}$ = 1 mg/L

Once assigned to a treatment group the eggs were weighed collectively within the treatment group at the start of the trial and marked with pencil to identify the treatment groups. Average egg mass per treatment group was calculated. All eggs, except those in the control group ($T_{0mg/L}$), were injected using a sterile 27G x ½ inch needle for each egg, with 200 µL Br^- as NaBr according to the experimental procedure described by Sawosz *et al*. (2014).

Each treatment contained 225 eggs, divided into 10 replicates per treatment. All eggs were incubated at standard conditions and automatically turned at a 45˚ angle every 15 minutes. 44 viable eggs from each treatment were set into each tray, so that the incubator was filled at half the capacity per tray to allow for better airflow during incubation. The trays were divided evenly across two incubators.

Eggs were pre-warmed at 30 ºC and 55% RH for 7 hours after which the temperature was set at 37.7 ºC and 60% RH until ED 18. Temperature and RH inside one incubator was logged using a temperature and humidity logger (DWL-20, Hairius Instrument Co. Ltd) set at a 3-hour logging interval and placed on the floor of the incubator for the duration of incubation. The room temperature was logged daily using a standard thermometer and recorded in the morning and afternoon.

On ED 18 all the eggs were candled, and unfertilised eggs (UF) and eggs containing non-viable embryos were removed. Non-viable embryos were identified by the arrested growth compared to viable embryos, which had grown to the full size and filled the entire egg. After candling and selection, 880 eggs were selected and placed into hatching trays and placed in the incubator at 37 ˚C and 70% RH. A hatch window of 26 hours was allowed starting on ED 20.

On HD, 10 chicks per treatment were randomly chosen for euthanasia by cervical dislocation. Whole body mass and heart mass were measured in grams using a sensitive balance. The CR and shank lengths were measured in millimetres using callipers. The relative heart mass was calculated as heart mass divided by whole body mass. The remainder of the hatched chicks were humanely euthanized by cervical dislocation.

On HD, breakout analysis was done on unhatched eggs to determine the age at which embryos had died. The number of pipped eggs was noted. Any anatomical anomalies were noted, but these appeared not to be linked to treatment concentrations.

Statistical analysis was done using SAS® software (2019). A one-way ANOVA was used to determine the interactions between treatments and the relative body and heart masses. The interaction between treatment (T) and SP was analysed using REML (SAS®, 2019). The CR and shank lengths were analysed using a mixed model with the REML procedure (SAS®, 2019). Significance was set at $P \leq 0.05$ and $P \leq 0.1$ and non-significance (NS) is noted. Means are least square means (LSM) and standards errors (SE) are noted.

Results

The incubator temperature and RH remained stable for the duration of the incubation period only decreasing sharply when the eggs were removed from the incubator for transfer into hatching baskets (Fig. 3). Room temperature was lower than incubator temperature and fluctuated over the incubation period but the incubator temperature remained stable regardless (Fig. 4).

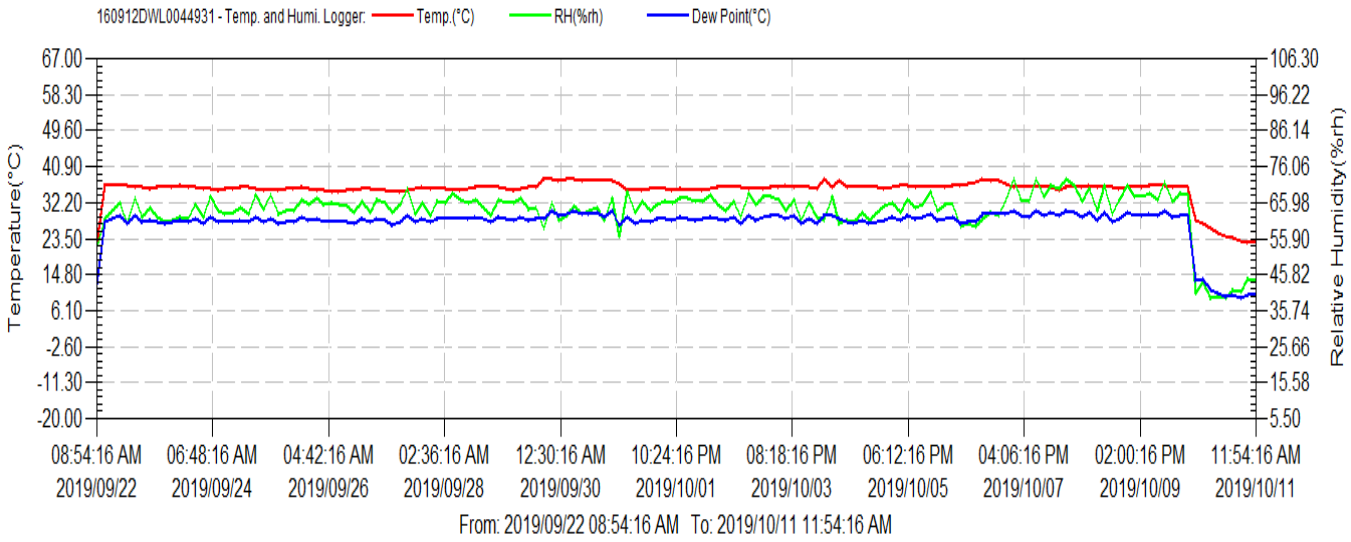

Figure 3 The temperature (Temp), relative humidity (RH) and dew point inside the incubator throughout the incubation period.

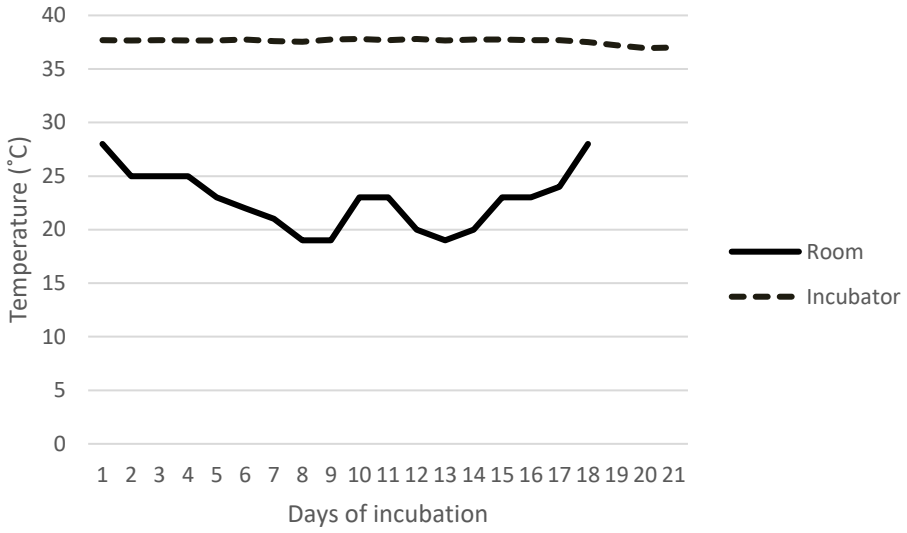

Figure 4 Incubator and room temperature during incubation period.

The average egg mass at the start of the trial and the average chick mass on HD between treatments was approximately the same at 63 g. For $T_{0.01mg/L}$ and $T_{0.5mg/L}$ the chicks were smaller than expected compared to the Control group on HD.

A one-way ANOVA using the GLM procedure of SAS® (2019) was performed to compare the pooled data of relative heart mass. It emerged that there was a highly significant difference (P≤0.05) between relative heart masses between treatments only on HD (Table 12). There were significant differences between relative heart masses on ED 14, ED 20 and HD within treatment (P≤0.1) (Table 13). When the REML procedure of SAS® (2019) was used to analyse TxSP, the differences were NS (P≤0.1).

Table 12 The difference of relative heart mass least square means (g, LSM±SE) between treatments (mg/L) on embryonic day 14, 20 and hatch day

Treatment	n	Embryo Day 14	n	Embryo Day 20	n	Hatch Day
0	29	1.25±0.03	19	0.75±0.04	10	0.88±0.03 [a]
0.01	5	1.23±0.07	9	0.74±0.05	10	0.72±0.03 [b] ***
0.5	14	1.30±0.04	12	0.80±0.05	10	0.68±0.03 [b] ***
1	14	1.31±0.04	11	0.76±0.05	10	0.68±0.03 [b] ***

[ab] Column means are significantly different ($P \leq 0.05$)

*** highly significant for treatment within day

Table 13 The difference of relative heart mass (g, LSM± SE) between treatments and between sampling days

Treatment	N	Day 14	n	Day 20	n	Hatch Day
0	10	0.13 ± 0.05 [a]	10	0.73 ± 0.05 [b]	10	0.88 ± 0.05 [c]
0.01	5	1.23 ± 0.07 [a]	5	0.70 ± 0.07 [b]	5	0.71 ± 0.07 [b]
0.5	11	1.29 ± 0.05 [a]	11	0.82 ± 0.05 [b]	11	0.68 ± 0.05 [c]
1	10	1.29 ± 0.05 [a]	10	0.77 ± 0.05 [b]	10	0.68 ± 0.05 [b]

[ab] Row means are significantly different ($P \leq 0.1$)

The CR lengths (mm, LSM±SE) increased with increasing age and were significantly different within T between SP (P≤0.1) (Table 14). On ED 14 there were significant differences in CR length between treatments, with CR length of $T_{0.5mg/L}$ being significantly smaller than that of the control (P≤0.1) (Table 14). There were NS differences between treatments on ED 20 or HD at P≤0.1 (Table 14).

Table 14 Crown-rump length (mm, LSE±SE) at embryonic day (ED) 14, ED 20 and hatch day (HD)

Treatment	N	ED 14	n	ED 20	n	HD
0		53.21±1.09 [a1]		72.05±1.25 [2]		93.15±1.85 [3]
0.01		49.35±2.39 [ab1]		73.22±1.95 [2]		89.78±1.95 [3]
0.5		48.93±1.51 [b1]		73.75±1.69 [2]		93.05±1.85 [3]
1		52.71±1.56 [a1]		72.27±1.76 [2]		89.85±1.85 [3]

[ab] Column means with different superscripts differ significantly at P<0.1

[1,2] Row means with different superscripts differ significantly at P <0.1

The shank length (mm, LSM±SE) increased with increasing age and the difference in LSM within T was significantly different between SP (P≤0.1) (Table 15). On ED 14, the shank length (LSM±SE) of $T_{0.01mg/L}$ was significantly smaller than that the Control and $T_{1mg/L}$ groups respectively (P≤0.1). All other LSM were NS different from one another (Table 15). On ED 20, the shank length (LSM±SE) of $T_{1mg/L}$ was significantly smaller than that for all other treatment groups (P≤0.1) and on HD, $T_{0.01mg/L}$ shank length (mm, LSM±SE) was significantly larger than that for all other treatment groups (Table 15).

Table 15 Shank length (mm, LSE±SE) at embryonic day (ED) 14, ED 20 and hatch day (HD)

Treatment	N	ED 14	n	ED 20	n	HD
0		10.56±0.27 [a1]		16.39±0.31 [a2]		28.95±0.46 [a3]
0.01		9.33±0.59 [b1]		16.67±0.48 [a2]		30.78±0.48 [b3]
0.5		10.07±0.37 [ab1]		16.67±0.42 [a2]		29.40±0.46 [a3]
1		10.79±0.39 [a1]		15.36±0.43 [b2]		28.80±0.46 [a3]

[ab] Column means with different superscripts differ significantly at P<0.1

[1,2] Row means with different superscripts differ significantly at P <0.1

Discussion

The egg mass was considered to be an indicator of the expected chick mass on HD. Larger eggs contain greater quantities of nutrients than smaller eggs, and have physically more space for the embryo to grow to a larger size. For $T_{0.01mg/L}$ and $T_{0.5mg/L}$ the chicks were smaller than expected compared to the Control group on HD. This may indicate that at these concentrations Br^- interfered with TH function with regard to whole body growth, since the incubator conditions were optimal. It also appeared that $T_{0.01mg/L}$ and $T_{0.5mg/L}$ may have been threshold concentrations at which a response occurred. In the absence of serum concentration data, it was unknown whether this was the case. Further investigation is required.

The growth of the heart, as measured by calculating relative heart mass, followed the expected negative progression with increasing age. The relative heart mass increased with increasing Br^- concentration on ED 14 and 20, which was in agreement with previous research on the response of relative organ mass to increasing Br^- concentrations. This may be indicative of stunted whole embryo growth during the early stages of development, causing the heart growth to appear greater than that of the control. On HD, relative heart mass decreased with increasing Br^- concentration, which had the possibility of negatively influencing chick quality.

The heart is a principal target of TH. It is known that an increase in circulating TH will result in a higher haemodynamic load and cardiac hypertrophy (Janssen *et al.*, 2007). This is in contrast to findings where embryos of eggs treated with Br^- showed greater relative heart mass compared to the control group. This may be attributed to a treatment effect on whole body mass where decreased whole body growth may make the organs appear relatively larger than under control conditions. A link between a cell-level hypothyroid state in the heart and the development of heart failure was found (Janssen *et al.*, 2007). This may have implications for broilers in the growth phase that are chronically exposed to Br^- in the water, which may result in interference with TH production and put additional strain on the heart to deliver adequate blood supply to the muscles. Janssen *et al.* (2007) found that inactivation of TH was linked to heart failure. Br^- may interfere with the action of TH on heart muscle growth.

The smaller relative heart mass with increasing Br^- concentration on HD compared to ED 14 and 20 respectively could be indicative of Br^- interference in TH function on heart growth at later stages of embryo maturity. However, this needs further investigation into the post-hatch growth period and was beyond the scope of this study.

The significant difference in relative heart mass within T between SP was as expected due to allometric growth that occurred with increasing age. According to Forhead and Fowden (2014), in mammals the foetal TH have major implications on heart function *in utero*. Similarly, in the chicken embryo TH have an effect on *in ovo* cardiac growth, development and function.

The stable incubator temperature ensured that the embryos were kept at the correct conditions for optimal growth and development during critical stages of development, and differences in relative heart mass may be attributed to the treatment effect.

CR length and shank length were considered to be reasonable indicators of growth in the chicken. The CR length increased with increasing age within treatment group for all treatment groups. This followed the normal allometric growth of the body that occurs as the embryo develops and was an indicator of optimal incubation conditions. On ED 14 the CR was significantly smaller for $T_{0.5mg/L}$ than that of the Control, and this may have been the response

to Br^- interference with TH function during the earlier stages of incubation, which stunted whole body growth. There was no effect on CR length on ED 20 or HD, indicating that the effect of Br^- on whole body growth mainly occurs in the earlier stages of embryogenesis.

The shank length increased with increasing age, which followed the normal growth pattern of a chicken embryo. On ED 14, the significantly smaller shank length of $T_{0.01mg/L}$ compared with that of the Control group was expected as the expectation was that Br^- could interfere with TH function in bone growth at low concentrations. What was unexpected is that the $T_{0.01mg/L}$ shank length was significantly smaller than that of the $T_{1mg/L}$ group, so that no clear dose response was found on ED 14. However, on ED 20, the shank length of $T_{1mg/L}$ was significantly smaller than that for all other treatment groups ($P \leq 0.1$), which could mean that Br^- at the highest concentration interfered with bone growth at the later stage of embryo development. On HD, $T_{0.01mg/L}$ shank length was significantly longer than that for all other treatment groups, which was unexpected. The lack of a clear pattern of CR and shank length indicates that these are not reliable growth indicators for pre-hatch growth.

Conclusion

The results showed that there was an apparent stage-specific effect of Br^- on CR and shank lengths, where different concentrations elicited different responses in growth at different embryonic ages. The response of relative heart mass to different Br^- concentrations at different stages of embryo development through to hatch drew attention to the fact that even at low Br^- concentrations there may be an effect on embryogenesis. This effect may affect chick quality and day-old chick production efficiency. Further research is required to test the effect of *in ovo* Br^- on the serum Br^- concentrations in day-old chicks and growing broilers.

CHAPTER 8

Discussion

Water is essential for life and a source of essential and non-essential elements. The importance of water to poultry production was highlighted and the role of GW in maintaining the physiological processes of the body during production was discussed briefly.

It was argued that water is the vehicle for elements including the halides that form part of the group of principal electrolytes. The role of halides in the normal physiological functioning of the body was presented.

It is well known that TH are critical in developmental, and metabolic effects in embryos of all species (Forhead & Fowden, 2014). Buzała *et al*. (2015) stated that TH are critical in the development in specific tissues in birds. It drew attention to the roles of I^- in TH synthesis and Cl^- as a principal electrolyte in maintaining the fluid balance within the body. The mechanisms by which Br^- could interfere with Cl^- uptake in the kidney, with TH synthesis in the thyroid gland and TH action along the HPT axis were considered and were in line with findings in literature (Waugh, 2019; Berend *et al.*, 2012; Luger *et al.*, 2001). The HPT-axis activity and TH production, the extra-thyroid conversion of T_4 to T_3 and the uptake of the TH into target tissues determine TSH and TH bioavailability (Forhead & Fowden, 2014). This drew attention to the immense potential of Br^- to interfere with multiple physiological pathways simultaneously throughout the body. In previous research Br^- intake was shown to depress water and feed intakes in treated broilers compared to the control group (Du Toit & Casey, 2010; 2012).

The importance of site-specific GW quality monitoring was highlighted by the finding that the overall naturally occurring range of Br^- concentrations in the GW sampled across selected provinces in South Africa was 0–132.68 mg/L. In terms of Br^- exposure risk, the FFU decision-making for a water source requires comparison of the Br^- content of the water to the NOAEL value stated in the South African WQG (Casey & Meyer, 2006). This is in line with the recommendation of Marandi and Shand (2018) that GW chemistry interpretation must begin with an assessment of WQC of the source water. Although the SA WQG has not been revised since its inception, the value of 0.01 mg/L was used as the safe lower limit for Br^-.

Concentrations of ≥5 mg/L Br^- were shown to be lethal to chicken embryos. The concentration range of 0 – 1 mg/L was lower than the 3 mg/L Du Toit and Casey (2010, 2012) used for growing broilers and satisfied the recommendation by Bigsby *et al.* (1999) recommended of an upper level dose 50 times that of the low dose. The Br^- highest treatment concentration used in this study was chosen to be 100 times that of the lowest dose.

It emerged from the meta-analysis of collated water quality data from various WRC reports that there was no formal NOAEL for Br^-. A value of 0.01 mg/L was arbitrarily chosen to be in line with the accepted practice for elements that have not yet been validated to obtain a default lowest maximum tolerable level (MTL) of 0.01 mg/kg (Regulation (EC) No. 396/2005 European Parliament, 2005). This value was also accepted to be the lowest value that the majority of laboratories were able to test without advanced equipment. The advances of technology meant that more sensitive testing could occur. The concentration of 0.01 mg/L had been validated as an acceptable NOAEL for Br^- by using the chicken embryo model. In literature is was found that there was a 4-fold increase in organic Br^--related cytotoxicity when

the Br^- concentration of the water samples was increased from 0.06 mg/L to 0.39 mg/L (Sawade *et al.*, 2016), which confirms that even low concentrations of dissolved inorganic Br^- can pose a significant health risk to livestock and humans when speciation occurs either in the GW source or within the body after GW intake.

There was a gap in the research on animal models pertaining to the acceptable NOAEL for Br^-. A study of literature revealed information on the effect of Br^- in rats (Loeber *et al.*, 1983; Pavelka *et al.*, 2002; Pavelka *et al.*, 2005), dogs (Baird-Heinz *et al.*, 2012) and horses (Raidal & Edwards, 2008).

In rat experiments Br^- was administered by adding NaBr to the feed, whereas in dogs and horses Br^- was administered as KBr orally or by injection.

This presented a gap in the literature of the effect of Br^- administered through the drinking water on the physiological function of an animal. One experiment used sheep and administered NaBr through drinking water to test whether excretion was equal to intake (Casey *et al.*, 2017). Pavelka *et al*. (2002) reported the effects of I^- transfer to rat pups *in utero* and later through the milk. Khanum *et al.* (2019) demonstrated that F^- administered into the yolk by in ovo injection decreased embryo viability. There was no research available on the effects of *in ovo* Br^- on chick embryo development.

Research was done using the chicken embryo model to validate that 0.01 mg/L Br^- was an acceptable NOAEL, since Br^- concentrations above this level were toxic and even lethal to the embryos. The embryo of any species is the most vulnerable of all production groups followed in order of magnitude by neonates > young growing animals > pregnant females > adult males. For this reason, the chicken embryo model was a good measure of Br^- toxicity at low concentrations. Previous research showed that Br^- accumulated in the organs and muscles of broilers (Du Toit & Casey 2010, 2012). This raised the question about the possibility of toxicity at low concentrations in GW with chronic exposure due to the cumulative effects of Br^-.

The risk assessment to determine the suitability of a water source for livestock production should be constructed to account for both interspecies and intra-species differences in tolerance for both essential and non-essential elements. Intra-species differences in tolerance must be considered according to the physiological state of the animal group. The best quality water should be offered to the least resilient groups where possible and the poorer quality water to the most resilient groups. It is also important to note that palatability is not always a measure of safety, as elements such as Br^-, unlike Cl^-, are odourless and tasteless. Water with poor palatability could be FFU for the more resilient groups based on the WQC profile of the water source (Casey *et al.*, 1998a; 1998b; Casey & Meyer, 2001; Casey & Meyer, 2006).

The use of sentinel species is valuable in determining the chemical status quo in a given environment. The chicken is a good sentinel species to monitor the response of a population to naturally occurring Br^- in GW used for drinking. The reason is that most households in rural areas where livestock production occurs and depends on GW keep chickens for domestic use (Meyer, 2015).

It was stated in the current study that the chicken embryo model was a suitable animal model for investigating the effect of EDC, specifically Br^-, on whole body growth and the growth of major supply organs. The chicken embryo model was a practical and low cost animal model to use to investigate the response of the most sensitive organism to a potentially harmful

element since all vertebrate embryos share similar critical growth stages and interspecies comparisons can easily be made with confidence.

Correct growth and development of the whole body and the supply organs depend on correct TH synthesis and function. Forhead and Fowden (2014) stated that embryonic thyroid gland function was necessary for embryonic mass accretion and specific cell type differentiation. Interference with these processes affects how the whole body and organs grow and function. If the supply organs are unable to function optimally, they will be unable to support the rapid growth and rate of muscle accretion that is characteristic of modern broiler strains. This will compromise chick quality and post-hatch growth performance.

Research showed that the transfer of hormones, such as cortisol, into the egg is possible (Henriksen *et al*., 2011). Halides have the ability to cross the trans-placental barrier freely and the body cannot discern the individual halides in the body halide pool. This allows Br^- to cross freely when it competes with Cl^- where it may then pose a significant risk to the foetus at critical stages of development. Similarly, the transfer of Br^- from the hen's plasma into the egg exposes the embryo to Br^- throughout incubation, which can disrupt normal growth and development in either early, mid or late stages of incubation.

All studies testing the response of chicken embryos to Br^- used NaBr as the Br^- source. The reason for this was that NaBr has a smaller molecular weight and thus has a slightly higher bioavailability compared with KBr (Baird-Heinz *et al.*, 2012). NaBr had a greater affinity to the thyroid gland and greater TH synthesis disrupting properties compared to KBr. Additionally, NaBr was chosen because reviewed literature showed that it did not disrupt the K^+ balance in the interstitial fluid as KBr did (Baird-Heinz *et al.*, 2012). There were clear interspecies differences in Br^- clearance rate reported by numerous authors (Pavelka *et al.*, 2005; Raidal & Edwards, 2008; Budnik *et al.*, 2012), and this drew attention to the fact that these species differences must be considered when conducting a site-specific risk assessment. *In ovo* injection into the albumen was chosen because in a scenario where the hen is exposed to Br^- in the drinking water, the plasma Br^- may be bound to albumin and become a reservoir of Br^- to the embryo during incubation much the same as with I^-.

For the purpose of the study, the breed of chicken used was the Ross-308 broiler strain. Although not reported it was found across all studies that the hatchability of the eggs was lower for the treatment groups and this did not appear to be an inherent property of the Ross-308 flocks from which the eggs were sourced and thus could be treatment related. Further investigation is required to test this theory.

The strain was chosen as the animal model for its rapid growth rate and wide availability, with many commercial populations kept under strict biosecurity conditions, ruling out any pathological causes of growth responses to Br^- treatment. The genetic composition and housing conditions were considered to be uniform throughout the country's broiler units using Ross-308 birds. Individual differences in the response of an animal to acute low concentrations of Br^- depending on its physiological state should be considered. The younger animals are more susceptible to toxicity symptoms, which present at lower Br^- concentrations. This does not discount the possibility of toxicosis presenting in older animals after chronic exposure to low concentrations of Br^-.

The rapid growth rate of Ross-308 birds in all growth stages translated into an innate high metabolic rate, which is controlled by the HPT axis. A rapidly growing embryo is a good

reference of maximum selection pressure on thyroid function and metabolism. The role of TH in normal embryonic growth and development was reviewed, and non-invasive measurement techniques were used to track the progress of growth. Khanum *et al.* (2019) showed that CR and shank lengths were good indicators of embryonic growth, and could be used to measure growth in live birds in the post-hatch growth period. In the current study, CR length was not found to be a reliable indicator of the response of embryo growth to Br^- treatment concentrations in later the stages of development. Shank length was shown not to be a reliable indicator of growth response to Br^- concentrations, as there was an apparent growth promoting effect of the highest Br^- concentration, which contradicted expectations and could not be explained.

A disturbance in TH synthesis and utilization at critical stages of development could have detrimental effects on whole embryo growth as well as the growth and development of the key supply organs. The sensitivity of the chicken embryo to minor changes in the *in ovo* environment made it the best animal model to test the response of whole body and organ growth to low concentrations of Br^- during the pre-hatch period. Bellabarba *et al.* (1988) found that the physico-chemical properties of TH receptors in the chicken were similar to that of mammals. This made the chicken embryo an appropriate choice of animal model to study the effects of Br^- treatment on embryogenesis, which could potentially be extrapolated to estimate the effect of Br^- on embryogenesis in other livestock species and even humans.

Further evidence emerged that low concentrations of Br^- negatively affected chick hatchability when administered into the albumen of eggs by *in ovo* injection when the eggs were incubated at standard conditions. Standard incubator conditions ensured that the hatchability could be linked to the Br^- treatment concentrations and not due to poor incubation conditions. It appeared that Br^- interfered with whole embryo growth up to ED 20 thus affecting the chick viability at hatch. TH is necessary for the late stage embryo to prepare for the transition to respiratory breathing and Br^- interference with the normal processes would cause failure to hatch and embryo death. This would greatly depress hatching egg production and have a cascade effect on the rest of the broiler production stages. Embryo death in the early and mid-incubation periods were thought to be signs of Br^- toxicosis where Br^- interfered with normal development processed that left the embryo body unable to support further growth.

The decreased survivability and hatchability that were shown to occur with increasing Br^- concentrations had implications for broiler egg production. If hens are chronically exposed to low concentrations of Br^- in the drinking water over the production period, and the Br^- is transferred into the eggs, there is a risk of decreasing the flock's performance by decreasing hatchability. Further research is necessary to determine whether (1) there is a transfer of Br^- into the eggs after exposing hens to Br^- during the laying period, (2) there is a latent effect of transferred Br^- in later broiler growth and (3) whether continued post-hatch exposure to Br^- would have any effect on the whole body mass and relative heart mass during the post-hatch growth period.

The heart is one of the main supply organs, as it develops first to support the growth and development of all other organs and tissues throughout pre- and post-hatch life. The evidence that increasing Br^- concentrations had an effect on relative heart mass on HD showed that Br^- apparently interferes with heart growth in the latter stages of embryo development. This has the potential to compromise chick quality and post-hatch growth, if the heart is not large enough

to support the rapid muscle accretion that is characteristic of modern broilers (Buzała *et al.*, 2015). Furthermore, there is a possibility that Br^- can interfere with not only heart growth but also its function, by competing with Cl^- and ultimately interfering with the correct function of the Cl^- channels that are necessary for the correct pacemaker function in the heart (Mulvany *et al.*, 2000; Duan, 2009).

Although further research is required, there is the potential for birds to develop cardiac failure in the later stages of post-hatch production when a compromised heart cannot support the rate of muscle accretion. The decreasing relative heart mass in response to increasing Br^- treatment may point to some disruption in TH activity during embryonic growth and development because as noted in literature, TH have been shown to be responsible for cardiac growth and function (Schmidt et al., 2009; Martinez, 2016; Delitala *et al.*, 2017; Shuvy *et al.*, 2017). The relative heart mass increased with increasing Br^- concentration and with increasing age, which was contrary to normal allometric growth where the organs become relatively smaller as the whole embryo grows. There was a strong possibility that the Br^- from the albumen was absorbed by the growing embryo, where it was free in the body to interfere with TH synthesis and action. This interference would have stunted the growth and development of the whole embryo thus making the heart appear relatively larger on ED 20 than would be found in the Control group. In the current study, the chicks from the Br^- treatment groups were observed to be smaller and appeared weaker than those of the Control group at hatch.

In the current study, the response of relative heart mass to increasing Br^-concentrations revealed the possibility of a threshold concentration at which whole body growth is severely stunted. It appeared that the range lies somewhere between 0.05 and 0.5 mg/L Br^-.

There was a response of the relative heart mass to concentrations of <1 mg/L Br^- in later stages of embryo development ED 20 and HD, whereas the liver and brain did not show a response to treatments at the same stages of incubation. Further research showed that there was no significant effect of Br^- treatment on the relative masses of the liver or brain on ED 14 and ED 20 respectively. The relative masses of the liver and brain were not further studied beyond ED 20 as there were no significant differences between treatment concentrations on ED 14 or ED 20.

The liver is known to be a crucial supply organ in the first few days after hatch (Bhanja *et al.*, 2009), providing valuable nutrients to the chick before there is access to feed. The liver has specific functions in TH transport and metabolism (Malik & Hodgson, 2002) and it appeared that during pre-hatch development it was unaffected by Br^- interference in TH production possibly due to its regulatory function of the free TH pool within the body.

The neurological effects of Br^- have been well documented in horses and dogs (Raidal & Edwards, 2008; Baird-Heinz *et al.*, 2012) but not in chickens, and it would be prudent to do further research of the post-hatch phase to observe whether the known neurological effects occur with exposure to Br^- from a GW source used for drinking. In the current study there were no neurological effects observed at hatch.

In previous research it was shown that TH is transferred across the placenta in mammals and is deposited in the yolk during egg formation in birds. Br^- was also shown to accumulate in the liver from which yolk is formed. This opens the possibility of maternal transfer of Br^- into the egg from hens exposed to Br^- in their drinking water. The consequences of this are that embryonic growth and development may be compromised by way of Br^- acting as a TDC

during incubation and affecting chick quality at hatch, which may have a cascade effect on post-hatch growth.

CHAPTER 9

Conclusion

When comparing the growth of the heart, liver and brain between treatments it was found that there was an effect of Br^- concentrations on the growth of the heart, where the heart was relatively smaller with increasing Br^- concentrations during the latter stages of embryo development and on HD. This trend was confirmed by further testing focusing exclusively on heart mass on HD. This presented the possibility of residual effects on the post-hatch broiler production phase as heart function may be compromised due to an inability of a smaller than normal heart to supply sufficient blood for high rates of muscle accretion. There were no apparent effects of Br^- treatments on the pre-hatch growth of the liver and brain.

In the current study CR length and shank length were not found to be reliable measures of pre-hatch growth.

Therefore the H_0 that a Br^- concentration range of 0.01 - 1 mg/L had no adverse effects on pre- hatch growth and development in Ross-308 broiler chickens was rejected.

The study therefore confirms that Br^- occurring in GW at concentration >1 mg/L may pose restrictions on the normal development of broiler embryos. It is therefore important that the standards given in the WQG require reconsideration.

CHAPTER 10

Critical review

A careful review of numerous WRC reports showed the WQC profile for numerous elements including the principle electrolytes. What was noteworthy was that the concentrations of Br^- many times greater than the chosen default NOAEL of 0.01 mg/L present in numerous water samples. The areas from which these samples were collected were populated by communities sharing water resources with their livestock and poultry.

It was discovered that the NOAEL for Br^- was chosen as a random default safe value. There was no formal NOAEL reported, which made decision-making about the quality of GW too vague. This opened the door for conducting research to validate the NOAEL for Br^-.

The decision was taken to use the chicken embryo model to validate the appropriate NOAEL for Br^- as this was the most sensitive and practical model to use. It was necessary for the author to travel to Warsaw, Poland to learn the *in ovo* injection technique. This technique would become the cornerstone of the research methodology of the study to determine the response of chicken embryos to different Br^- concentrations to (1) validate the NOAEL of 0.01 mg/L and (2) test which concentrations of Br^- could be toxic or lethal to chicken embryos. The value of intellectual capital became apparent as there was a team of people assisting in the data collection process in the research conducted in Poland. This made for fast and efficient data collection in an environment that was suited to this purpose. The validation of 0.01 mg/L as a formal NOAEL meant that there is now a concrete value against which concentrations present in water samples can be compared to aid in decision-making of FFU. This also means that the WQG need to be revised to include this NOAEL value as the formal safety level of Br^-.

The study was not without its limitations. The trials conducted at the University of Pretoria (UP) to further investigate the effect of different Br^- concentration on chicken embryo growth and development had some limitations. The author had to work alone, which limited the number of eggs that could be handled at one time thus complicating the logistics. Further limitations were the lack of a UV sterilization chamber and the state of the incubators that were available, which may have compromised hatchability results due to less than ideal pre-treatment shell sterilization and incubation conditions. The incubator limitations included the need to manually control RH, which may have caused an unfavourable temperature drop every time the water pans had to be refilled, although in nature it is not uncommon for the hen to leave the nest for up to 20 minutes to eat without detrimental effects on the embryos.

There was a challenge with needing to transport ED 21 eggs to the UP Hatfield Campus, which is a separate site to the UP Experimental Farm, for breakout analysis and sample collection in the laboratory on site. In an ideal situation the hatchery facilities on the UP Experimental Farm should have contained well maintained and reliable incubators and a clean space to do breakout analysis and sample collection with weighing equipment on site. It is unclear whether the transit time between the UP Experimental Farm hatchery and the laboratory on the UP Hatfield Campus had an effect on embryo viability. It is also unknown whether the cold ambient temperature in the laboratory negatively affected embryo viability before breakout analysis was completed. The absence of a team of people when breakout

analysis was done meant a long lag time from the first to the last egg broken open. This may have compromised embryo viability.

Further studies conducted at the Astral Festive facilities in Pretoria allowed for a greater number of eggs to be processed as there was a small team working on the project. One limitation was that the majority of the team members had no experience with the *in ovo* injection technique resulting in the possibility of human error in administering the Br^- treatments. The incubators of the facility were in good working order, automatically controlling temperature and RH, and the incubation conditions were optimal for chicken embryo growth and development. The author is confident that this gave reliable hatchability results where incubation conditions were concerned. It was unclear whether chick hatchability was solely related to Br^- treatment as there was a possibility that the inherent hatchability of the flock may have played a large role. Further research with repeated measures is required to test for this.

Despite the difficulties encountered along the way, the author is confident that the results and deductions are sound and make a worthy contribution to recognising Br^- as a potential health risk to humans and livestock. It further contributes to the understanding that Br^- may be toxic to humans and livestock even at chronic exposure to low concentrations. Validation of the NOAEL for Br^- allows for the WQG for Br^- to be updated with confidence.

The author is of the opinion that a fully functional laboratory space complete with a reliable incubator and measurement equipment in the UP Experimental Farm hatchery would lend itself to better hatchability testing. It would be favourable to have a team available for processing eggs in a larger volume when *in ovo* injection experiments are done in future.

Throughout the duration of the current study there was tremendous personal growth as a researcher for the author. Planning was essential and liaising with third parties was imperative, and these skills were invaluable to the completion of the current study. Regretfully time constraints created the challenge of being unable to continue with further stages of investigation into the effects Br^- may have during the egg production and post-hatch periods respectively.

There are many factors that contribute to the success of broiler production. Although feed costs are the main expense of broiler production and it is essential that feeding and watering lines consistently operate optimally there is something to be said about intellectual capital. This is the employment of staff that has the necessary appropriate training for the tasks they are responsible for in a production unit. This may include maintaining the feed and water lines, ensuring the constant supply of feed and water to the birds in their houses and the ability to identify any health problems that may arise and make decisions on whether veterinary care is required or if it something that can be dealt with in-house. The staff must be trained in administration of the requisite vaccines at the correct times and to regularly collect water samples of the drinking water source for WQC analysis to maintain flock health. These are just a few examples of the essential services necessary on a poultry unit. The same is true for researchers and support staff when conducting outcome specific research trials.

It is the author's opinion that further research is required to test the carry-over effect of *in ovo* injection of Br^- on growing broiler performance. Furthermore there is a need to test the carry-over effect of hens treated with different concentrations of Br^- administered in drinking water into the eggs produced. Further investigation of how eggs produced under such conditions would influence chick quality at hatch, and the subsequent effect on broiler growth is necessary. The addition of blood values would greatly strengthen the understanding of how

Br^- acts as a TDC in the actively producing laying hen and the growing broiler. The current study did not go beyond hatch and thus testing blood chemistry was not considered meaningful or necessary for the purpose of the investigation.

In the author's opinion there is great value in the validation of the NOAEL of Br^- as this had previously not been done. The emergence of 1 mg/L Br^- being lethal to chicken embryos highlighted the risk to developing chicken embryos, and further research is needed to determine whether the same is true for the mammalian foetus. The implications of the findings are that livestock and human populations chronically exposed to elevated Br^- concentrations in the drinking water are at risk for developing health problems. The populations most at risk are animal and human foetuses, very young animals and children, and in humans the elderly and immunocompromised.

There has been much research on the neurological effects of Br^- in humans. Despite the various limitations experienced during the duration of the experimental process, the results obtained point to some important patterns in the embryonic growth and development of the heart, which has not been previously reported. This may be a factor in birds prone to ascites in the post-hatch growth period. Further research is required to validate this hypothesis.

CHAPTER 11

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