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Emerging flame retardants and their effects on growth and development in Japanese quails (Coturnix japonica)

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Environmental Toxicology and Chemistry
Submission date: May 2016
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Abstract

Tris(1,3-dichloro-2-propyl) phosphate (TDICPP) and Dechlorane Plus (DP) are (re)emerging flame retardants widely detected in the environment and biota. These compounds have shown a potential to bioaccumulate in organisms, and DP has shown to biomagnify through the food chain. Maternal transfer of both the contaminants in birds has been demonstrated through reported levels in eggs. Information about their potential toxicity in wildlife, especially in avian species, is very limited. The aim of this study was to investigate possible effects and interaction effects of both flame retardants on development and growth in avian species, using the domestic Japanese quail (*Coturnix japonica*) as a model organism. Eggs of Japanese quail were injected with low, medium and high doses (10, 100 and 1000 ng/g egg, respectively) of either TDICPP, DP, or a 1:1 mixture of both. Biometrical measurements of body weight, head length, and tarsus length were taken regularly from newly hatched chicks for a total of 14 days. Neither the compounds nor the mixture had an effect on hatching success, size of the newly hatched chicks, overall growth rate, or body condition (at 12 days old). These results suggest that *in ovo* exposure to TDCIPP and DP resulted in no observable effects on development or growth in two weeks old Japanese quails. Because TDICPP and DP are ubiquitous in the environment and biota, and are frequently and increasingly used in industry, potential toxicological effects of these flame retardants need to be further investigated.

Sammendrag

Tris(1,3-dikloro-2-propyl)fosfat (TDCIPP) og Dechlorane Plus (DP) er flammehemmere som til stadighet har blitt detektert i store deler av miljøet og i biota. Begge forbindelsene har potensialet til å akkumulere i organismer, og DP kan biomagnifiseres opp gjennom næringskjeden. Informasjon om disse flammehemmernes potensielle toksisitet, særlig hos fuglearter, er veldig begrenset. Maternal overføring av begge kontaminantene i fugler har blitt demonstrert ved at det er blitt rapportert nivåer i egg. Hensikten med dette studiet var å undersøke mulige effekter og interaksjonseffekter av TDCIPP og DP på utvikling og vekst hos fugler. Dette ved å bruke egg fra modellarten Japansk vaktel (*Coturnix japonica*) som ble injisert med lav, middels og høy dose (10, 100 og 1000 ng/g egg) av enten TDCIPP, DP eller en 1:1 blanding av begge forbindelsene. Biometriske målinger av kroppsvekt, hodelengde og tarsuslengde ble tatt regelmessig fra kyllingene var nyutklekkede til de var 14 dager gamle. Hverken forbindelsene hver for seg eller kombinasjonen av dem hadde en effekt på klekkesuksess, størrelsen på nylig klekkede kyllinger, generell vekstrate eller kroppskondisjon (12 dager gamle). Disse resultatene indikerer at *in ovo* eksponering av TDCIPP og DP resulterte i ikke-observerbare effekter på utvikling og vekst hos to uker gamle Japanske vaktler. Ettersom konsentrasjoner av TDCIPP og DP er utbredt i miljøet og det er registrert hyppig og økende forbruk av dem i industri, bør potensielle toksikologiske effekter av disse flammehemmerne undersøkes videre.

Abbreviation

ACN	Acetonitrile
AIC	Akaike Information Criterion
ANOVA	Analysis of variance
AS	Acid silica
BCI	Body condition index
BCCP	Blood clinical-chemical parameters
BFR	Brominated flame retardant
BDCIPP	Bis(1,3-dichloro-2-propyl) phosphate
BMF	Biomagnification factor
bw	Body weight
C-I	Control injected
C-NI	Control non-injected
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DP	Dechlorane Plus
EDC	Endocrine disrupting chemical
ELISA	Enzyme-linked immunoassay
FR	Flame retardant
FR3	Free triiodothyronine
GC	Gas chromatography
GH	Growth hormones
GR	Growth rate
HBCD	Hexabromocyclodecane
HD	High dose
IS	Internal standard
LC	Liquid chromatography
LD	Low dose
LOQ	Limit of quantification
lw	Lipid weight

MIX	Mixture
MD	Medium dose
MS	Mass spectrometry
n	Number of observations
NTNU	Norwegian University of Science and Technology
OLS	Ordinary least squares
OPFR	Organophosphate flame retardant
p	Significance level
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
POPs	Persisten organic pollutants
RS	Recovery standard
SE	Standard error
SIM	Selected ion-monitoring
SPE	Solid phase extraction
T3	3,5,3′-triiodothyronine/Triiodothyronine
T4	Thyroxine
TBBPA	Tetrabromobisphenol A
TDCIPP	Tris(1,3-dichloro-2-propyl)phosphate
TDCPP	Tris(1,3-dichloropropyl)phosphate
TH	Thyroid hormones
TMB	tetramethylbenzidine
TRH	Thyrotropin-releasing hormone
TT3	Total triiodothyronine
ww	Wet weight

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1. INTRODUCTION

1.1 Flame retardants

Flame retardants (FRs) are chemicals that are able to inhibit ignition or resist the spread of fire, and for this reason they are used in a variety of consumer products that must meet fire safety standards. Examples of such consumer products are carpets, computers, clothing, electrical equipment, televisions and other household appliances. FRs are either used as additive or reactive ingredients in polymers and other materials used in these products (Wenning and Martello, 2014). Additive means that the FRs are not chemically bound to the polymers, and the compound is therefore more likely to be released into the environment during the product's lifetime than reactive and bonded FRs, which are chemically bound to the matrix (van der Veen and de Boer, 2012). FRs include more than 175 different types of chemicals. They are generally divided into four classes: halogenated organic (usually brominated or chlorinated), phosphorus-containing, nitrogen-containing and inorganic chemicals (Wenning and Martello, 2014). FRs may leak into the environment during their production, incorporation into consumer products, during the product's usage and lifetime, and when disposed as waste (Wenning and Martello, 2014). Certain commercial brominated flame retardants (BFRs), such as polybrominated diphenyl ethers (PBDEs), have been subjected to strict regulation due to concerns about their ability to persist in the environment and in tissues of animals and humans, for being prone to bioaccumulation, and for having possible toxic effects (Covaci et al., 2011). Such restrictions have led to the development of novel, alternative FRs. Van der Veen and de Boer (2012) give an overview of the environmental levels of the organophosphate flame retardants (OPFRs), used as alternatives to BFRs. Both humans and wildlife are exposed to these emerging FRs, and measurable levels have been evidenced in sediments and soil (Qui et al., 2007), indoor air and dust (Abdallah et al., 2008; Qi et al., 2014) and, among others, in different species of birds (Karlsson et al., 2006; Venier et al., 2010). More information about the occurrence in the environment, accumulation and the toxicological effects of these emerging FRs in wildlife is lacking and urgently needed (van der Veen and de Boer, 2012).

In this thesis, the possible developmental effects of two emerging FRs: tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) and Dechlorane Plus (DP) will be investigated. Figure 1 shows the molecular structure of the two compounds.

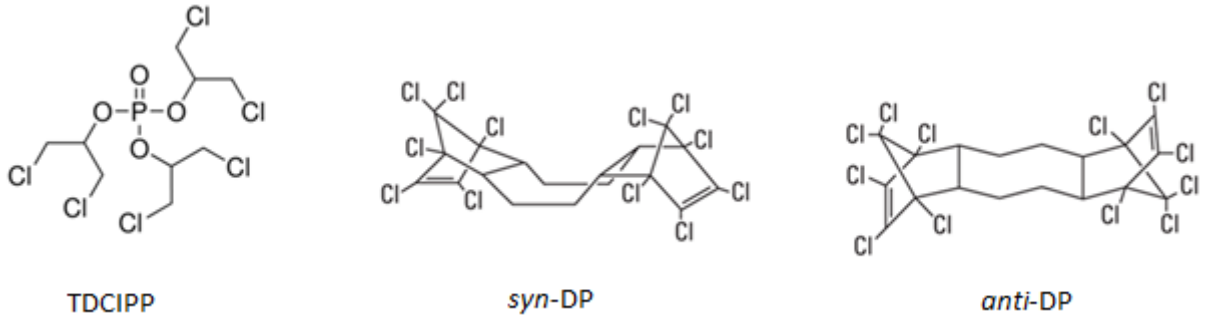

Figure 1. Illustrations of the molecular structures of Tris(1,3-dichloro-2-propyl)phosphate (TDCIPP), and *syn*- and *anti*-isomers of Dechlorane Plus (DP).

1.1.1 Tris(1,3-dichloro-2-propyl)phosphate (TDCIPP)

TDCIPP is a chlorinated OPFR used as an additive FR in the manufacturing of polymers, latexes, foams, resins and infant products. OPFRs are often used as replacements for banned or restricted BFRs (van der Veen and de Boer, 2012). Several studies have detected TDCIPP in surface water, indoor air, sediments, sludge samples, and biota, indicating a possible widespread environmental distribution of TDCIPP contamination (reviewed by van der Veen & de Boer 2012). TDCIPP is lipophilic and resistant to degradation in the environment, and thereby prone to bioaccumulation in organisms (WHO 1997). Studies have demonstrated short half-lives in organism by showing that TDCIPP is rapidly metabolized in rodents (Lynn et al., 1981; Nomeir et al., 1981), chicken embryos (Farhat et al., 2013), fish (WHO 1997), and in humans (Van den Eede et al., 2013). When TDICPP is biotransformed in biological systems, the major metabolite, bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), is readily excreted from the organism (Lynn et al., 1981; Nomeir et al., 1981). Despite a low bioconcentration factor (BCF range 3-107) (WHO 1997) and rapid metabolism, TDCIPP has been detected in wildlife. Measurable levels have been evidenced in tissues of fish (Campone et al. 2010; Green et al. 2008), and in eggs, blood and tissues of birds (Chen et al. 2012d; Leonards et al. 2011).

TDCIPP was classified as a carcinogenic compound by the World Health Organization in 1997 (WHO 1997), and again by the California Environmental Protection Agency in 2011 (OEHHA, 2011). Disturbance of development has been demonstrated in zebrafish embryos (*Danio rerio*), where increased developmental abnormalities in fish exposed to TDCIPP were found (McGee et al., 2012). Moreover, chickens exposed orally to TDCIPP exhibited weakness in legs and wings (Ulsamera et al., 1980). Other studies have suggested that TDCIPP has the potential of being a disruptor of thyroid hormone regulation in chicken and zebrafish embryos (Farhat et al. 2013; Li et al. 2013), and also of sex hormone levels in zebrafish (Liu et al. 2013). Farhat et al. (2013) also observed negative effects on growth where the beak and bill size and the body mass of the embryo were reduced. Still, more research on the potential toxic effects in biota, including effects on growth and development, is urgently needed (van der Veen & de Boer 2012). There has been some confusion in the literature regarding the use of abbreviation for tris(1,3-dichloro-2-propyl)phosphate. Different studies have been using the abbreviations TDCP, TDCPP, and TDCIPP for this compound, however the correct one is TDCIPP (Van den Eede et al., 2013).

1.1.2 Dechlorane Plus (DP)

DP is a highly chlorinated additive FR used as a replacement for toxic Mirex. It comprises two major isomers: *syn*- and *anti*-DP. The compound is unregulated and was first introduced onto the market in the 1960s. It has since then been widely applied in products such as electrical wires, cable coatings, plastic, roofing materials, and hard connectors in computers and televisions (Weil and Levchik, 2004). Studies have demonstrated that DP is a global pollutant susceptible to long-range atmospheric transport, that it is persistent and has the potential to bioaccumulate and biomagnify (Feo et al., 2012; Sverko et al., 2011). DP is ubiquitous in the environment (Li et al., 2013a; Sverko et al., 2011) and has been globally detected in ambient air (Moller et al., 2010), dust, surface soils (Yu et al., 2010), sediments (Fang et al., 2014), wildlife (review: Feo et al. 2012), and human beings (Ren et al., 2009). Levels of DP have been detected in serum samples from young nestling bald eagles (Venier et al., 2010), indicating that the compound has the ability to accumulate in birds at high trophic levels. Several studies have examined the toxicological effects of DP in vertebrates. This includes rodents (Li et al. 2013; Wu et al. 2012), birds (Crump et al., 2011), and fish (Kang et al., 2016; Liang et al., 2014). Results from former studies on effects have suggested induced hepatic oxidative damage and perturbations of metabolism and signal transduction in mice (Wu et al., 2012).

Further, a potential to disrupt both thyroid and sex hormones by altering regulatory pathways in the brain of zebrafish has been described (Kang et al., 2016). Because of concerns about the presence of manufacturing plants in the North American Great Lakes basin and in China, a majority of studies on DP in biota has been conducted in these areas (Muñoz-Arnanz et al., 2012).

1.2 Avian toxicology of flame retardants

The legacy BFRs, such as PBDEs, hexabromocyclodecane (HBCD), and tetrabromobisphenol A (TBBPA) has been extensively studied. Various toxic effects in laboratory animals and wildlife have been observed for some BFRs, in particular for PBDEs. The observed effects include carcinogenicity, neurotoxicity, teratogenicity and endocrine disruption (Legler and Brouwer, 2003). A three-generation *in ovo* exposure in zebra finch (*Taeniopygia guttata*) suggested that a low exposure dose of BDE99 may reduce clutch size, and have a possible negative effect on reproduction (Winter et al., 2013). However, passerine birds (including zebra finch) appear to be less sensitive to PBDEs than mammals or other bird species (Eng et al., 2013). American kestrels (*Falco sparverius*) exposured to PBDEs resulted in thinner eggshells, reduced fertility, decrease in egg weight, and unsuccessful reproduction (Fernie et al., 2009). In adult male rats, BDE99 has shown to interfere with hormonal response (Alonso et al., 2010).

Emerging FRs have similar physicochemical properties as banned/legacy FRs, despite being different chemical molecules, and therefore potentially have a similar fate in the environment (Ezechiáš et al., 2014). Data on toxicological and molecular responses are scarce, both for DP (Feo et al., 2012; Sverko et al., 2011) and TDCPP (Covaci et al., 2011; European Union, 2008), especially regarding avian species. More information about the occurrence in the environment, the ability to persist and accumulate, and the prospective toxic effects of these two emerging FRs in wildlife are urgently needed (European Union, 2008; Sverko et al., 2011; van der Veen and de Boer, 2012). Because of their high trophic level in both the marine and the terrestrial food chain, raptors are especially vulnerable to potentially high levels of pollutants, including FRs, due to biomagnification of contaminants through the food chain (O'Sullivan and Megson, 2014). More research on avian exposure and potential toxicological effects, especially in species at high trophic levels, is therefore particularly important.

1.3 The use of different bird species in ecotoxicology

The use of raptor species in research is not common practice. The most central reason for this is that raptors are protected in most countries, but also ethical and practical considerations play a role (Jaspers, 2015). Raptors of smaller size, like American kestrels, are bred in captivity and used in research (Jaspers, 2015). Results from studies on captive populations of raptors are important in order to properly understand toxicological observations in wild raptor populations. However, performing studies on captive raptors is not always possible, or the most convenient. In these cases, model species can be utilized and data can be extrapolated to wild raptor populations. Both songbirds and poultry have frequently been used as model species in ecotoxicological avian research. Examples of songbirds that have been used are European starlings (*Sturnus vulgaris*) and zebra finches (Jaspers, 2015). Zebra finches are small in size, which makes them convenient for use in laboratory experiments, although at the same time their size limits the amount of sample that can be obtained from each individual (Jaspers, 2015). Starlings have been used both in laboratory studies and in field studies, by using artificial nest boxes. Also poultry species are used in ecotoxicological research, such as mallard ducks (*Anas platyrhynchos*), northern bobwhite quail (*Colinus virginianus*), and especially the Japanese quail (*Coturnix japonica*) has been a popular species to use (Jaspers, 2015). Nevertheless, important species differences in biology and development exist, which should be considered when trying to extrapolate findings from ecotoxicological studies to other species.

1.3.1 Precocial and altricial development in birds

Birds are characterized as precocial or altricial species based on different developmental patterns. Galliforms, ducks, and ground-dwelling species are precocial, while many raptors, songbirds, and other passerine bird species are altricial, (Ottinger et al., 2014). Newly hatched precocial birds are at a relatively advanced developmental stage. This includes functionally and mature eyes, an almost instant capability for locomotion, and the ability to handle cooling temperatures by initiating thermoregulative processes. This makes them able to practice independent foraging, and thus being relative independent from parental care. On the contrary, the altricial birds are hatched at an earlier developmental stage, forcing them to be nest-bound and dependent of parental care and feeding. They are hatched with closed eyes, unable of coordinated locomotion, and with immature thermoregulatory responses to cooling (Ehrlich et al., 1988; McNabb, 2007). Due to varying degree of development at hatching, some species are semi-precocial or semi-altricial (Ehrlich et al., 1988; O'Connor, 1984). Also growth rate differs

between species, and their characteristic growth rates are related to body weight as an adult, food availability and their developmental pattern (Ricklefs, 1979).

1.3.2 Maternal transfer of contaminants

During embryonic development, environmental contaminants from the mother can affect embryos through maternal transfer into placenta and eggs. Critical developmental processes occur during early life stages, embryos are therefore more sensitive to adverse effects from chemicals (Zheng et al., 2014a). Maternal transfer of environmental contaminants has been evidenced in fish (Nyholm et al. 2008), mammals (Alonso et al., 2015) and in avian species (Bryan et al., 2003; Jarman et al., 1993; Verreault et al., 2006). In birds, organic contaminants get deposited into eggs together with maternal lipids and proteins during egg synthesis, which is believed to be controlled by a combination of biological factors and the physicochemical properties of the contaminants (Verreault et al., 2006). These biological factors include maternal fat reserves and body condition, clutch size, egg mass, and yolk content. Physicochemical properties include the contaminant's molecular structure, lipophilicity, degree of halogenation, rate and degree of metabolism, and affinity to macromolecules (Verreault et al., 2006). Altricial and semi-precocial species deposit smaller quantities of lipids into their eggs than precocial species, and therefore they have a lower egg:maternal tissue contaminant ratio (Drouillard and Norström, 2001).

1.3.3 The NewRaptor project

In the fall of 2014 NewRaptor, a 4-year project co-founded by the Norwegian research Council and Norwegian University of Science and Technology, was established. The project is a collaboration between different institutes and universities in Norway, Denmark, Spain and Belgium. The aim of the project is to study the exposure, toxicological, and biological effects of emerging pollutants in raptors. In the current study, as a part of the New Raptor project, the Japanese quail will be used as a model organism in order to study the effects of the emerging flame retardants, DP and TDCIPP, on growth and development in avian species.

1.4 The Japanese quail (Coturnix japonica) as a model organism

The Japanese quail is a galliform species in the Phasiandae family (Vali, 2008). The quail has been frequently used in research since the 1950s and therefore there is a lot of data and literature available (Huss et al., 2008). The species is suitable as a laboratory animal due to low cost and maintenance, and a small size which does not require a lot of space for housing. It has a high production of eggs, and reaches sexual maturity after 6 weeks making it favorable for generational exposure studies (Huss et al., 2008; Vali, 2008). Japanese quails are convenient as a model organism for studying developmental biology and ecotoxicology because of an easily accessible embryo and a short incubation period of 17 days (Barrett et al., 2000). The embryo can be manipulated by removing a small part of the eggshell and injecting contaminants into the egg, giving an opportunity to study maternal transfer of contaminants. This way, it is easy to control the exposure dose, since the embryo will digest the content in the egg before hatching. In addition, the development of the embryo can be monitored by candling the egg during the incubation period. Quails have a precocial developmental pattern, which means that newly hatched chicks are at a fairly advanced developmental stage, as they are able to see, move around and feed themselves shortly after hatching (McNabb, 2007). Quails have frequently been applied in toxicological studies, investigating effects of different chemical compounds and possible endocrine disrupting chemicals (EDC). From exposure studies with EDCs (estradiol, methoxychlor, and Dichlorodiphenyldichloroethylene), Ottinger et al. (2005) found effects on sexual behavior and neuroendocrine regulation of reproduction in male quails, and reduced fertility in hens. Results from an *in ovo* exposure study suggested that nonylphenol has estrogenic effects on quail embryos (Razia et al., 2006). In another study, DP caused alterations in hepatic enzyme activity (Li et al. 2013).

1.5 Thyroid hormones in growth and development

Thyroid hormones (TH) play an important role in the development of birds (McNabb, 2007). These hormones are essential for several important physiological processes, such as growth, differentiation, metabolism, reproduction, immune system and homeostasis of vitamins and hormones, and they are therefore crucial for normal development and function in several species (McNabb, 2007; McNabb and Darras, 2015; Nøst et al., 2012). THs interact with other hormones and hormone systems, and have an effect on physiological systems in both a direct

and an indirect way (McNabb & Darras 2015). Among others, they interact with growth hormones (GH), where they regulate the production and release of GHs by the pituitary. They do this by direct inhibition of the site where GHs are synthesized, the pituitary somatotrophs, and also by acting as a negative feedback on the thyrotropin-releasing hormone (TRH) (McNabb and Darras, 2015). The THs, 3,5,3′-triiodothyronine (T3) and thyroxine (T4), are produced in the thyroid gland, and transported through the blood stream by thyroid binding proteins (Richardson et al., 1994). In birds, the major binding proteins are transthyretin (high affinity and low capacity) and albumin (low affinity and high capacity). During development, the binding proteins regulate the availability of thyroid hormones, as suggested by several studies (reviewed by McNabb & Darras 2015). The ontogenic patterns of plasma THs differ between precocial and altricial birds. In precocial species, the function and control of the thyroid gland matures before hatching, which leads to the plasma containing high levels of T4 and low levels of T3 in the latter half of embryonic life. The plasma concentrations of both THs rise considerable to a peak during the perihatch period. After the perihatch peak, the levels in the plasma decrease noticeably, followed by a gradual increase towards adult concentrations (McNabb and Darras, 2015). In altricial species, the concentrations of T3 and T4 in the plasma are very low during the embryonic life and perihatch period. During the first weeks post-hatch, the hormones gradually increase to finally reach adult levels (McNabb and Darras, 2015). The TH status in an organism is best represented by the concentrations of THs in the blood; the plasma concentration is therefore the best measurement of organismal thyroid status (McNabb, 2007). In many vertebrates, maternal THs are available and essential for embryos during development. These hormones are transferred from the mother into eggs in fish (Brown et al., 1987; Kobuke et al., 1987) birds (Groothuis et al., 2005), and across the placenta in mammals (Morreale de Escobar et al., 1988). In teleost eggs the maternal THs have proven to be important in the embryonal neural development (Campinho et al., 2014). In the first part of gestation, prior to the development of thyroid gland function, maternal THs influence fetal development in mammals and avian species, where they play an essential role in ensuring normal development (Patel et al., 2011; Wilson and McNabb, 1997). Substantial amounts of maternal hormones, including THs, are to be found in avian eggs (Groothuis et al., 2005). A study performed by Wilson and McNabb (1997) suggested that maternal THs are transferred into the egg prior to laying in Japanese quails. The same authors also found evidence of some regulation of hormone deposition, where increased amounts of maternal THs were deposited in the yolk of eggs from hens with high T_4 plasma levels. The embryo's thyroid gland appeared to have low capacity for hormone synthesis up until the first half of incubation, and normal

development therefore depends on the maternal hormones (Wilson and McNabb, 1997). The TH status of the hen affects egg laying as well as the thyroid hormone content in the eggs (McNabb and Darras, 2015; Wilson and McNabb, 1997). Wilson and McNabb (1997) demonstrated how Japanese quail hens with decreased thyroid function stopped laying eggs in order not to produce thyroid deficient eggs. Adequate thyroid function is essential for successful egg laying in precocial, altricial and semi-altricial bird species (McNabb and Darras, 2015).

1.6 Effects of endocrine disrupting chemicals on thyroid hormones

EDCs are natural or synthetic compounds that interfere with the hormonal and homeostatic systems by which the organism communicates and responds to its environment (Casarett and Doull, 2013). Endocrine disruptors have the ability to interfere because of structural and/or functional similarities to natural hormones (Casarett and Doull, 2013). EDCs in the environment can affect the thyroid function in birds (and other species) exposed to these chemicals. Endocrine disruptors that have shown to disrupt thyroids are PCBs, perchlorate, and FRs such as PBDEs (McNabb and Darras, 2015). Species differences need to be taken into consideration, and also the fact that avian species have different developmental patterns (precocial and altricial), and a broad range of life histories. Because of this, extent and timing of sensitivity to EDCs varies between species as their vulnerability changes throughout different phases of their lifetime (Ottinger et al., 2014). For example, EDCs that have an impact on thyroid systems are posing a particular risk to migratory avian species. The reason for this is that these species depend on their metabolic and thyroid endocrine systems to be highly functional due to large metabolic demands (Ottinger et al., 2014). Certain flame retardants are endocrine disruptors, and have been detected in avian populations worldwide. They have been shown to alter thyroid function in chickens (Farhat et al., 2013), zebra finches (Eng et al., 2013), wild-caught american kestrels (Fernie et al., 2005) and bald eagles (*Haliaeetus leucocephalus*) (Cesh et al., 2010). The effects of flame retardants on THs have been studied in several different species, and have shown to cause different effects. In American kestrel the plasma T4 levels were lowered in individuals exposed to penta-BDE (Fernie et al., 2005). Another study showed that in the African aquatic frog (*Xenopus laevis*) frog the binding of T3 to TH-transporting protein and receptor was affected by a TBBPA derivate (Kudo et al., 2006). The most significant effect of BFRs in mammals is often a decrease in T4 (Darnerud, 2007). The potential endocrine disrupting effects of (re)emerging FRs are largely understudied.

1.7 Aims and hypotheses of the study

The main aim of the study was to investigate if the *in ovo* exposure to DP and TDCIPP affected development and growth in Japanese quails. In addition, this study investigated if there was an effect of mixture toxicity when both compounds were injected together.

In this study, it was hypothesized that;

1. The emerging flame retardants are able to affect growth and THs in the laboratory-exposed model species (Japanese quail, *Coturnix japonica*)
2. The effects in Japanese quail differ between the two contaminants
3. An interaction effect on growth and development is found in the group exposed to the mixture of the two contaminants.

2. MATERIALS AND METHODS

2.1 Exposure experiment

The exposure study on *Coturnix japonica* was conducted in the spring of 2014 at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. All experiments were approved by the Norwegian Animal Research Authority (NARA) and conducted in accordance to their regulations.

2.1.1 Study design

Eggs from Japanese quail were randomly divided into three experimental groups (DP, TDCIPP and MIX), and within each treatment the eggs were exposed, by injection, to emulsions containing low, medium and high dose (LD, MD, and HD, respectively) of flame retardants. In addition, a control group that was not injected (non-injected), and a control group injected with the emulsions containing lecithin, water and peanut oil only, were also included. Table 1 is an overview of the eleven treatment groups with the ultimate sample size per group, i.e. number of chicks included in the study. In total, 341 eggs were injected, 30-31 within each treatment group. The number of injected eggs was determined based on an expected hatching success of 50% during previous experiments within the research group, and based on the number of chicks aimed for in each treatment group.

Table 1. An overview of the treatment groups in the exposure study of Japanese quails (*Coturnix japonica*), exposed *in ovo* to Dechlorane Plus (DP) and Tris(1,3-dichloroisopropyl)phosphate (TDCIPP), independently and together in a 1:1 mixture. The individuals were exposed to low, medium and high doses, LD, MD, and HD respectively. Two control groups were also included, one injected with the emulsion only (C-I) and one that was not injected (C-NI). The sample size in each treatment group is represented by *n.*

Compound	Dose	Treatment	*n*
DP	low	DP-LD	10
DP	medium	DP-MD	9
DP	high	DP-HD	9
TDCIPP	low	TDCIPP-LD	8
TDCIPP	medium	TDCIPP-MD	9
TDCIPP	high	TDCIPP-HD	9
Mixture	low	MIX-LD	8
Mixture	medium	MIX-MD	10
Mixture	high	MIX-HD	10
Control	injected	C-I	10
Control	non-injected	C-NI	9

2.1.2 Egg injections

The chemicals for the exposure study (DP isomers and TDCIPP) were purchased from Accustandard (New Haven, CT, US A).

The injection site on the top of the egg, the drill, and the needle were sterilized with 70% ethanol before each injection. The syringe was cleaned on suction with MilliQ water, 100% ethanol, and hexane, successively, between the injections of different doses and compounds. Further, the egg injections were done with increasing concentration and the mixture as last.

Injected solutions containing TDCIPP and DP were prepared beforehand. Lecithin (purchased from Sigma-Aldrich L-α-Phosphatidylcholine from egg yolk, ~60% TLC) was dissolved in dichloromethane, followed by addition of peanut oil (purchased from Sigma-Aldrich), and finally the dichloromethane was evaporated in a water bath (35°C) under a steam of clean air. The compounds, dissolved in toluene, was added to the mixture of lecithin and peanut oil. The solution was then evaporated under a stream of clean air using a rotary evaporator (Buchi Rotavapor, Model 205) until there was no more toluene left. The solution with DP consisted of a mixture of *anti*- and *syn*-DP (anti:syn, 70:30%) according to proportions found in wildlife (Sverko et al., 2011 and Xian et al., 2011). A working solution with a mix of the two compounds was also prepared in order to see if there were any interaction effects of the mixture. The working solutions were made to yield concentrations of 5, 50 and 500 ng/µL, resulting in exposure concentrations (doses) of 10, 100, and 1000 ng/g egg, which are referred to as low, medium and high dose, respectively.

Fertilized non-incubated Japanese quail eggs were bought from a commercial quail farm located in Birkeland, Norway. Upon arrival, the eggs were stored in a refrigerated room (12-14 °C) until injection. The eggs were rinsed with water and blotted dry in order to remove dirt. Thereafter they were weighed and pencil marked with an individual identification number. Eggs with visible cracks or lines were not used. The eggshell above the air cell was cleaned with 70% ethanol before a small hole was drilled in the centre through the shell. A 0.5 mm × 26 mm needle together with a Hamilton manual HPLC syringe was used to inject the suitable amount of emulsion (< 2µL/g egg) vertically into the middle of the yolk. After injection, the hole in the eggshell was sealed with a small amount of candle wax. Injections were executed over several days and timed so that, based on a 50% hatching success, approximately 16 eggs would hatch each day (33 eggs injected simultaneously).

2.1.3 Incubation of the eggs

In total 341 eggs were injected and incubated in America A/S incubators (type 180-220V~300W), due to limited space in the incubators and other logistical reasons, not all of the eggs were incubated simultaneously. The temperature in the incubators was held constant at around 37.5°C, and the air humidity was held between 50 and 60%. Eggs from the different treatments were randomized among and within the incubators in order to avoid any possible effect of the incubators. The eggs were expected to hatch after approximately 17 days of incubation, and for this reason, the eggs were placed in hatching boxes (inside the incubator) after 14 days. When transferred to hatching boxes inside the incubators, the eggs were kept in separate compartments to separate the hatchlings from one another. Hatchlings stayed inside the incubator until completely dry. Then they were marked, measured, and transferred to the cages.

2.1.4 Housing of the quails

Figure 2 shows an overview of the cage setup and the placing of the heat lamps. The cages and heat lamps were set up in a way that each cage had the same temperature and the same coverage of light from the lamps. In order to separate chicks within the same cage, a non-toxic permanent marker was used to mark the chick's abdomen.

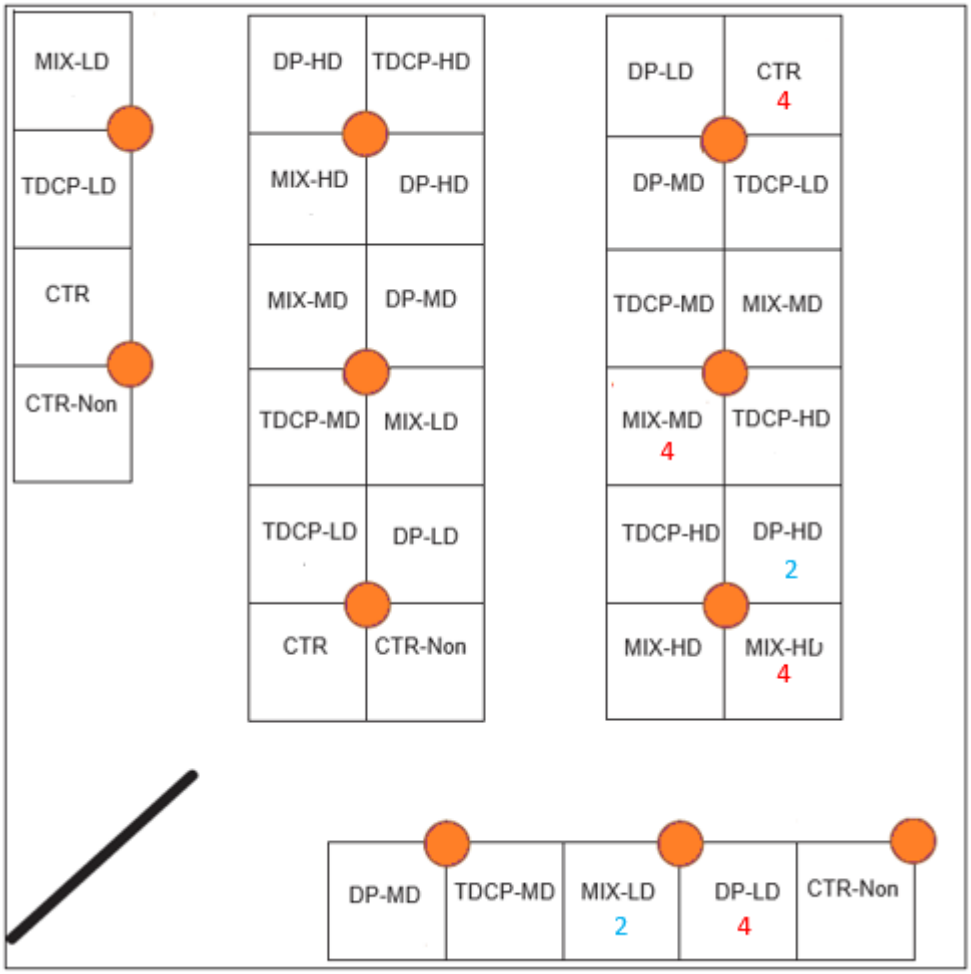

Figure 2. The figure shows a map of the cage setup in the experiment. Except the marked cages with two (2) and four (4) chicks in them, all the cages had three chicks each. The orange circles represent the position of the heat lamps.

There were three cages for each treatment, and cages assigned to different treatment groups were spread randomly among each other. The humidity was 32-40%, and the room temperature was set to 24-25 °C, thus the area in the cage directly under the heat lamps had a temperature of approximately 36 °C. In order to simulate natural day and night cycles, the lights were turned on at 8 AM and turned off again at 10 PM. The original intention was to have three chicks in each cage, but in the end there were four cages with four chicks and two cages with two chicks in them. This was due to differences in hatchability within these treatments.

Due to logistic reasons, the original food obtained for this study had to be replaced after 17 days. As a consequence of this, eighteen birds were fed solely with the original food (Natura – Oppdrett Fjør 1 Mais), and the rest was fed the original food first and then the new food (FJØR Oppdrett Kraft) for different periods of time, depending on when they had hatched.

Supplementary information about the food types is given in appendix A1 and A2. The ratio between original and new food that each bird received throughout their lifetime was calculated and included in the statistics.

2.1.5 Biometric measurements

Head length (from the back of head to the tip of the bill) and both tarsi were measured on each individual using an electronic calliper (to the nearest 0.01 mm), and a digital scale was used to measure the body weight (to the nearest 0.01 g). Measurements of body weight were taken every day, and the remaining measurements were taken approximately every other day depending on the availability of resources and the logistics of the experiment.

2.1.6 Sampling

At 14 days old, the chicks were euthanized by decapitation. They were then dissected, the sex was determined, and tissues and blood were sampled. The blood samples were centrifuged (1164 g, 10 min) to separate plasma from red blood cells, and the plasma was then collected and frozen in liquid nitrogen. The supernatant was discarded and the rest was frozen in liquid nitrogen. The liver was dissected and snap-frozen in liquid nitrogen.

2.2 Chemical analysis

Chemical analyses of the eggs and liver samples for TDCIPP and its metabolite (BDCIPP) and for *syn*-DP and *anti*-DP were conducted at the Toxicological Centre at the University of Antwerp.

2.2.1 Determination of TDCIPP and BDCIPP concentrations in liver and egg

2.2.1.1 Extraction

Approximately 0.5 g of liver and 0.25 g of homogenized egg sample was weighed and homogenised into a powder with ~2 g of anhydrous Na_2SO_4. The powder was transferred to a 15 mL polypropylene Falcon tube and spiked with 50 µL of an internal standard (IS) mix consisting of BCDIPP-d10 (2.5 ng/µL) and TDCIPP-d15 (2 ng/µL). Subsequently, the liver samples were extracted using 5 mL of acetonitrile (ACN) with 1% acetic acid, and the egg samples were extracted with 5 mL of hexane:acetone mixture (Hex:Acet 1:1, v/v) containing

1% acetic acid. The samples were vortexed for 1 min, ultrasonicated for 5 min and finally centrifuged at 3500 g for 5 min. The supernatant was thereafter transferred to a clean glass tube and the same extraction process was repeated on the remaining powder with 3 mL of solvent. This solvent extract was evaporated to dryness at 32 °C under a gentle nitrogen stream.

The samples were dissolved by 1 mL of MilliQ water along with 0.5 mL sodium acetate buffer (pH 4.5, 1M), then vortexed for 30 sec, followed by ultrasonication for 5 min. Prior to solid phase extraction (SPE), the Oasis® WAX cartridges (3 mL, 60 mg, Waters) were prewashed with 2 mL methanol, and conditioned with 2 mL sodium acetate buffer. The liver extracts were transferred onto the cartridges and the glass tubes were washed quantitatively with 2 mL MilliQ water**.** After the water had run through, the cartridges were washed with 1 mL MilliQ water on a Visiprep^{TM} SPE vacuum, and then left on the vacuum for ~10 min to dry**.** The SPE cartridges were then eluted with 4 mL of 5% NH_4OH in methanol into a clean glass tube.

The elution was evaporated until less than 50 µL remained. The samples were reconstituted with 50 µL MilliQ water and then spiked with 50 µL recovery standard (RS) TPHP-d15 (1 ng/µL). The samples were vortexed for 30 sec before transferred onto a VWR centrifugal filter (500 µL, pore size 0.2 µm and 0.45 µm). The samples were centrifuged at 3500 g for 3 min on the filter in order to remove any leftover solvent (methanol). After centrifugation, the filter was removed and the samples were transferred into clear vials with inserts. The food samples were analysed simultaneously with the liver samples, following the same protocol.

2.2.1.2 Detection and quantification

TDCIPP and BDCIPP were measured with an Agilent 1100 series liquid chromatograph coupled with an Agilent 6410 mass spectrometer system (LC-MS/MS). The LC was equipped with a 50 x 2.1 mm, 1.7 µm Kinetex Biphenyl column (Phenomenex). A mobile phase of 5 mM ammonium acetate in 2% acetonitrile in Milli Q water (A), and 5 mM ammonium acetate in 2% MilliQ water in acetonitrile (B) at a flow rate of 200 µL/min, was used. Starting at 20% (B) and 80% (A), then increased linearly to 50% (B) over 6 min, then increased linearly to 95% (B) over 1 min, stayed at 95% (B) for 3 min until the compounds were eluted. BDCIPP, BDCIPP-d10, TDCIPP-d15, TDCIPP, and TPHP-d15 were eluted at 1.0, 1.0, 9.3, 9.3, and 9.4 min, respectively. The MS system was operated in the electrospray negative mode between 0.8 and 4 min (elution of diesters) and in the electrospray positive mode between 4 and 11 min (elution of triesters). MS/MS detection operated in the multiple reaction monitoring (MRM) mode was used for quantitative determination of the analytes. The following transitions were used:

BDCIPP (319 → 35 and 317 → 35), BDCIPP-d10 (329 → 35 and 327 → 35), TDCIPP (433 → 99 and 431 →99), TDCIPP-d15 (446 → 99 and 444 → 99), and TPHP-d15 (342 → 223 and 342 → 82). Drying gas temperature was 350°C and the nebulizer pressure was set at 35 psi (nitrogen).

2.2.1.3 Quality control of the method

Retention times, ion chromatograms and relative abundance of the monitored ions were used as identification criteria. A deviation of ion abundance ratios within 15% of the mean values for calibration standards was considered acceptable. Quantification was based on five-point calibration curves. The peaks were positively identified as target compounds if: (1) the retention time matched that of the standard compound within ± 0.1 min, and (2) the signal-to-noise ratio (S/N) was higher than 3:1. Procedural blanks were analyzed simultaneously with every batch of seven samples to check for interferences or contamination from solvent and glassware. Procedural blanks were consistent (RSD < 30%) and therefore the mean value was calculated for each compounds and subtracted from the values in the samples. The *limit of quantification (LOQ)* was calculated as three times the standard deviation of the mean of the blank measurements and was 2 and 4 ng/g wet weight (ww) for TDCIPP and BDCIPP, respectively.

For estimating recoveries in liver, 0.5 g chicken liver was spiked with 20 and 100 ng of BDCIPP and TDCIPP (as control samples). The experiment was done in five replicates and the control samples were processed as the real exposed samples (see above). Mean ± SD recoveries were 103 ± 7% and 105 ± 4%, for BDCIPP and TDCIPP, respectively. The Toxicological Center has experience with participation in various international proficiency tests for a broad range of analytes.

In order to detect any possible contamination during sample handling, two blanks were included for each round of extraction. The blanks were treated exactly the same way as the liver samples. For the purpose of calculating the accuracy of the method, five samples of chicken liver were spiked with a high concentration (QH, 100 ng) of TDCIPP and BCDIPP and another five were spiked with a low concentration (QL) of TDCIPP and BCDIPP. In addition, one vial was prepared containing QH and RS and another containing QL and RS. The recovery for the 10 samples was calculated in order to see if the method could accurately and precisely measure TDCIPP and BCDIPP in both the lower and the higher range of concentrations.

2.2.2. Determination of DP concentration in liver and egg

2.2.2.1 Extraction

Approximately 0.75 g of liver samples and 0.25 g of homogenized egg samples were weighted and homogenized into a powder with ~2 g of anhydrous Na_2SO_4. The powder was transferred to a 15 mL polypropylene Falcon tube and spiked with 100 µL of internal standard (IS) consisting of 200 pg/µL of each ^{13}C-*syn*-DP and ^{13}C-*anti*-DP. Subsequently, the samples were extracted using 5 mL of hexane:DCM (1:1). The samples were vortexed for 1 min, ultrasonicated for 5 min, and finally centrifuged at 3500 g for 5 min. The supernatant was thereafter transferred to a clean glass tube, and the same extraction process was repeated with 5 mL of solvent. The solvent extract was evaporated to dryness at 30 °C under a gentle nitrogen stream. The samples were reconstituted in 1 mL hexane:DCM (4:1), and vortexed for 20 sec. Prior to SPE, the acid silica (AS) cartridges (~ 2-3 g AS (44%) topped with 0.5 g anhydrous Na_2SO_4 in 6 mL PP cartridge) were prewashed with 6 mL hexane. The liver extracts were transferred to the cartridges and the glass tubes were washed quantitatively with 1 mL hexane, the SPE cartridges were subsequently eluted with 8 mL hexane into a clean glass tube. The elution was evaporated to dryness, re-solubilized in 100 µL iso-octane, vortexed for 20 sec, and finally transferred into clear vials with inserts.

2.2.2.2 Detection and quantification

DPs were measured with an Agilent 6890-5973 gas chromatograph coupled with a mass spectrometer system (GC-MS). The GC was equipped with a 15 m x 0.25 mm x 0.10 µm DB-5ms capillary column (J&W Scientific, Folsom, CA, USA) and the MS was operated in electron capture negative ionisation (ECNI) mode. Methane was used as reagent gas and the ion source, quadrupole and interface temperatures were set at 250, 150 and 300 °C, respectively. The MS was used in the selected ion-monitoring (SIM) mode with two most intense characteristic ions *m/z* = 651.7 and 653.7 for *syn*-DP and *anti*-DP and m/z = 661.7 and 663.7 (for ^{13}C-*anti*-DP and ^{13}C-*anti*-DP). Dwell times were set at 20 ms. One µL of the cleaned extract was injected in solvent vent mode (injector temperature: 90 °C, held for 0.04 min, then with 700 °C/min to 300 °C and kept for 25 min; vent flow was set at 75 mL/min and the purge vent opened at 1.25 min). Helium was used as carrier gas at constant flow (1.5 mL/min). The temperature of the DB-5ms

column was kept at 90 °C for 1.00 min, then increased to 310 °C at a rate of 20 °C/min, kept for 5 min. total run time was 17 min.

2.2.2.3 Quality control of the method

Retention times, ion chromatograms and relative abundance of the monitored ions were used as identification criteria. A deviation of ion abundance ratios within 15% of the mean values for calibration standards was considered acceptable. Quantification was based on five-point calibration curves. The peaks were positively identified as target compounds if: (1) the retention time matched that of the standard compound within ± 0.1 min and (2) the signal-to-noise ratio (S/N) was higher than 3:1. Procedural blanks were analyzed simultaneously with every batch of seven samples to check for interferences or contamination from solvent and glassware. Procedural blanks were consistent (RSD < 30%) and therefore the mean value was calculated for each compounds and subtracted from the values in the samples. The *limit of quantification (LOQ)* was calculated as three times the standard deviation of the mean of the blank measurements and was 0.05 ng/g ww.

For estimating recoveries in liver, 0.7 g chicken liver was spiked with 20 ng of each *syn*-DP and *anti*-DP (control samples). The experiment was done in triplicate and the control samples were processed as the real exposed samples (see above). Mean ± SD recoveries were 103 ± 2% and 102 ± 1%, for *syn*-DP and *anti*-DP, respectively. For estimating recoveries in eggs, 0.25 g chicken egg was spiked with 20 ng of each *syn*-DP and *anti*-DP (control samples). The experiment was done in triplicate and the control samples were processed as the real exposed samples (see above). Mean ± SD recoveries were 105 ± 4% and 103 ± 3%, for *syn*-DP and *anti*-DP, respectively. The Toxicological Center has experience with participation in various international proficiency tests for a broad range of analytes.

2.2.3 Analyses of thyroid hormones in blood plasma

Thyroid hormone concentrations in the plasma samples were quantified by a competitive enzyme-linked immunoassay (ELISA) from MP Biomedicals (LLC, Ohio, USA). ELISA is an immunological technique used to detect the presence of an antibody or an antigen in a sample. The general principle of the technique is that an unknown amount of antigen is fixed onto a surface, followed by the adding of a specific enzyme-bound antibody. The enzyme is activated by adding its substrate, which leads to a detectable colour change of the solution (Sino

biological Inc., 2015). The intensity of the colour formed in the wells at the end of the procedure, is proportional to the amount of enzyme present in the well, and it is inversely related to the amount of unlabelled hormone in the plasma sample. Provided human hormone standard reference sets were used to make a standard curve. The hormone concentrations in the unknown plasma samples were calculated from the standard curve, based on their measured absorbance.

2.2.3.1 Quantification of total triiodothyronine and free triiodothyronine

Total triiodothyronine (TT3) and free triiodothyronine (FT3) concentrations were quantified in plasma samples by a competitive enzyme immunoassay from MP Biomedicals (LLC, Ohio, USA). Provided human TT3 or FT3 standard reference sets were used as quality assurance of the quantification. Ox blood and sample replicates (two on each plate) were used at the beginning and the end of all plates in order to account for intra- and interspecific variation.

For quantification of TT3, 50 µL of the plasma samples was pipetted onto antibody (goat *anti*-mouse IgG) coated wells on a microplate, followed by 50 µL mouse monoclonal *anti*-T3 antibody and 100 µL T3 conjugate. After incubation at room temperature for 60 minutes, and washing with distilled water to remove unbound T3 conjugate, 100 µL tetramethylbenzidine (TMB) reagent was added. The samples were then incubated in darkness at room temperature for 20 min in order for the blue colour to develop. The reaction was stopped after incubation by adding 100 µL of 1 N HCl and as a result the solution turned yellow. The absorbance in the wells was measured spectrophotometrically at 450 nm with a Cytation 5 imaging microplate reader.

For the quantification of FT3, 50 µL of plasma samples was pipetted onto monoclonal T3 antibody-coated wells on a microplate, followed by 100 µL T3-enzyme conjugate. After incubation at room temperature for 60 min, and washing with distilled water to remove unbound T3 conjugate, 200 µL substrate solution (H_2O_2:TMB, 1:1) was added. Following 20 minutes incubation in the dark for the blue colour to develop, 50 µL 3N HCl was added in order to stop the reaction and turn the colour of the solution yellow. The absorbance was measured spectrophotometrically at 450 nm with a Cytation 5 imaging microplate reader within 30 minutes.

Trials were performed, using only half of the plate provided, to test if the kits were suitable for FT3 and total TT3 in quail plasma. The trials showed no apparent problems. When preceding with the analyses, a problem with the kit was discovered. Replicates of the same sample had a

considerably lower absorption at the end of the plate than at the beginning, and in the middle. The calculated plasma concentrations based on the absorptions could therefore not be trusted. New analyses could not be executed due to limited amount of time, and therefore the thyroid hormones are excluded from the current study.

2.3 Data treatment and statistical analysis

The final number of Japanese quails used in the experiment was 101 individuals. The sample size within each treatment (exposure compound and dose) varied from 8-10 individuals (see Table 1).

A statistical significance level of $p < 0.05$ was set. Statistical analyses were performed using R studio (version 0.99.893).

2.3.1 Hatching success

Chi square test was used in order to compare hatching success between the different treatments and the control group. The chi square test showed no significant difference in hatching success between injected and non-injected control. The hatching success in each treatment was compared against control injected in order to eliminate possible effects of the injection.

2.3.2 Growth rate

Due to exponential growth in the quails at the age they were euthanized (14 days), growth rate (GR) per individual was calculated based on the body weight using equation (1).

$$GR = \frac{\log(bw)_{\text{final}} - \log(bw)_{\text{initial}}}{time} \quad (1)$$

, where $\log(bw)_{\text{initial}}$ is the log transformed body weight (in grams) measured at day one, and $\log(bw)_{\text{final}}$ is the log transformed body weight (in grams) measured at day 13, and time is the number of days in between the two measurements, i.e. 13. For one individual from the DP-HD exposure group the body weight from day two was used, and for one individual from the TDCIPP-MD exposure group body weight from day 12 was used.

2.3.3 Biometric measurements

Tukey's honest significance test was performed in order to check if the injected and non-injected controls differed significantly from each other for body weight and head length. Measurements from day 13 were used because the curves of the two controls deviated the most from each other at day 13. Tukey's honest significance test was also performed in order to test for differences in head length and mean tarsus length between the different treatment groups one day after hatching.

2.3.4 Body condition index

In order to get a measure of the size and condition of the chick, a body condition index (BCI) was calculated per chick. One of the most common methods of measuring condition in many vertebrate taxa is by using the residuals from a regression of body mass on a linear measure of body size, called ordinary least squares (OLS) regression (Schulte-Hostedde et al., 2005). In the current study, the BCI is expressed as the residuals from a linear regression with body weight as response variable and a mean of left and right tarsus as explanation variable, using measurements from 12 days old chicks.

2.3.5 Confounding factors

Due to differences in chick hatching time and the experimental setup, the number of chicks in the cages did not stay consistent throughout the experiment. In order to account for this, companionship, i.e. the variation in chick numbers in the cage during an individual's lifetime, was calculated per individual. Companionship was calculated using equation (2).

$$\text{Companionship} = \frac{\sum_{i=1}^{n_{\max}} (n_{\text{chicks}} \cdot k_{\text{days with n chicks}})_i}{t_{\text{total days in cage}}} \qquad (2)$$

, where n is number of chicks in the cage at a certain time, and k is total number of days with n number of chicks, and t is total number of days the chick spent in the cage.

During the experiment, the original food was replaced by a new and different type of food (see information about the two food types in appendix A).

In order to account for this, the proportion of new food was calculated per individual. This was done by dividing hours of new food on the total number of hours the individual was alive.

2.3.6 Mixed effects linear regression

Mixed effects linear regression models with growth rate as a response variable were fitted to the data within each exposure group (TDCIPP, DP, MIX, and controls). The data passed Shapiro-Wilk's test for normality before the models were fitted. The different models were compared using two-way ANOVA (analysis of variance), and the best fitted model was chosen based on the ANOVA output and the AIC (Akaike Information Criterion) values. A full model was fitted with treatment, companionship, and food as fixed effects, and cage as random effect. In addition, simpler models with reduced numbers of fixed effects were fitted. In order to test the assumption of normality of data in linear mixed effects models, the plots of the fitted model against the standardized residual, and a distribution (boxplot) of the residuals within each group of the random factor (cage) were inspected.

2.3.7 Linear regression

2.3.7.1 Growth rate

The output of the mixed effects model showed that the random effect (cage) explains very little of the variation in GR within all the different treatments. Because of this, the random effect was removed and a linear regression model was fitted to the data instead. Before fitting the models, the two control groups (injected and non-injected) were compared with an independent two group Mann-Whitney U test. The test showed statistical equality of the means of the two groups (p-value>0.05), and the GR in the two control groups were pooled into one control in the further analyses.

In order to check for any effects of food and companionship on GR, a linear model was fitted with GR as response variable and the two confounding factors as explanation variables. Summary of the linear model showed that both the confounding factors had a significant effect on GR. The residuals from this model are the data that are not explained by either food or companionship, and therefore the residuals were used in further analyses.

In order to see if treatment alone had any effect on GR, a linear model with the residuals as response variable and treatment as explanation variable was fitted. Tukey's honest significance

test was performed in order to check if the GR differed significantly between any of the treatments, i.e. between the doses for the same exposure, between the two compounds and a mixture of the two, and also if any of the treatments were different from the pooled control.

2.3.7.2 Body condition

The effects of companionship and proportion of new food on body condition were tested by fitting a linear regression model with body condition as response variable and companionship and proportion of new food as explanation variables. In addition, the effect of treatment on body condition was also tested by fitting a linear model. In order to test for any significant differences between the different treatments, i.e. between the doses for the same exposure, between the two compounds and the mixture, and also if any of the treatments were different from the pooled control, Tukey's honest significance tests were performed.

3. RESULTS

3.1 Control of exposure dose

3.1.1 Eggs

A summary of the concentrations of DP and TDCIPP, and its metabolite BDCIPP, in Japanese quail eggs exposed to high doses of DP and TDCIPP, independently and together in a 1:1 mixture, are presented in Table 2.

One egg per treatment was analyzed after one day of incubation, i.e. one day after exposure. The determined ratio between the two isomers *syn*- and *anti*-DP was approximately 1:2 (syn:anti) in the HD-DP and the HD-MIX. Within all treatments, the levels of the compounds were close to the desired concentration for the HD exposures (1000 ng/g). BDCIPP was only detected in MIX-HD, for TDCIPP-HD it was below the LOQ.

Table 2. Summary of the concentrations (ng/g ww) of Dechlorane Plus (DP) and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) quantified in Japanese quail (*Coturnix japonica*) eggs at embryonic day 1. The quails were exposed *in ovo* to high doses of DP (DP-HD, n = 1), TDCIPP (TDCIPP-HD, n =1), and a 1:1 mixture of DP and TDCIPP (MIX-HD, n = 1). The eggs were analyzed after one day of incubation. The data are presented as levels detected above LOQ in each egg, i.e. each value represents one egg. LOQ was 2.0 ng/g for TDCIPP and 4.0 ng/g for BDCIPP, and 0.05 ng/g for DP. BDCIPP was below the LOQ for TDCIPP-HD, and therefore not detected (n.d.).

ng/g ww	DP-HD	TDCIPP-HD	MIX-HD
syn-DP	350.8	---	366
anti-DP	698.6	---	826
∑DP	1049	---	1192
TDCIPP	---	896.9	1046

3.1.2 Liver

A summary of DP and TDCIPP concentrations in liver from 14 days old Japanese quails exposed *in ovo* to high doses (HD) of TDCIPP and DP, independently and together in a 1:1 mixture (MIX-HD), is presented in Table 3.

The metabolite BCDIPP was below the LOQ (4.0 ng/g) in all the samples, both in the non-injected control group and in the exposed groups. Levels of TDCIPP were quantified in six individuals, two individuals within each treatment group (including the control). All quantified

levels were low and close to the LOQ. The chromatogram from the GC-MS shows that the peaks of all the samples, including the blank, overlap (see Appendix B).

Both isomers of DP, *syn*-DP and *anti*-DP, were detected in all liver samples except for one control, and the ratio between *syn*- and *anti*-DP in these samples was approximately 1:2 (syn:anti). Mean concentrations within the two exposure groups (DP-HD and MIX-HD) were similar for both isomers. Concentrations in non-injected controls were low and close to the LOQ (0.05 ng/g egg).

Table 3. Summary of concentrations (ng/g ww) of Dechlorane Plus (DP) and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) quantified in liver samples from Japanese quails (*Coturnix japonica).* The quails were exposed *in ovo* to high doses of DP (DP-HD), and TDCIPP (TDCIPP-HD), independently and in a mixture (MIX-HD). In addition, liver samples from a group of non-injected controls were analyzed (C-NI). The data is presented as mean ± standard error (SE), and range. The sample size within each exposure group is presented as *n*, and the number of individuals with detected concentrations (>LOQ) are presented as *d*.

	syn-DP (ng/g ww)			*anti*-DP (ng/g ww)			∑DP (ng/g ww)			TDCPP (ng/g ww)			
Treatment	Mean ± SE	Range	*d*	Mean ± SE	Range	*d*	Mean ± SE	Range	*d*	Mean ± SE	Range	*d*	*n*
DP-HD	9.49 ± 1.49	6.81-18.4	9	17.9 ± 2.74	3.28-35.8	9	27.4 ± 4.21	5.15-54.2	9	---	---	---	9
TDCIPP-HD	---	---	2	---	---	---	---	---	---	3.50 ± 0.08	3.4-3.6	2	9
MIX-HD	8.35 ± 1.49	3.36-17.3	10	15.3 ± 2.44	6.97-34.01	10	23.6 ± 3.65	10.3-51.3	10	2.60 ± 0.23	2.3-2.9	2	10
C-NI	0.087	---	1	0.11 ± 0.035	0.056-0.16	2	0.17 ± 0.051	0.10-0.24	2	3.09 ± 0.53	2.3-3.9	2	10

3.1.3 Contamination in food

A summary of the quantities of compounds detected in the original and the new food are provided in Appendix C. Most of the detected compounds were below LOQ or at very low concentrations. However, the new food had noticeable higher concentrations than the original food for ∑PCBs and ∑DDXs.

3.2 Hatching

Figure 3 shows the percentage of hatched chicks (hatching success) within each treatment in increasing order. Based on the total number of hatched chicks across all the treatments, the overall hatching success was 40%.

The hatching success was calculated by dividing the number of hatched chicks by the total number of incubated eggs. The treatment group with the highest hatching success was the control non-injected (63%), and the treatment with lowest hatching success was medium dose (MD) DP (29%). There was no significant difference between the two control groups (Chi squared test, $p > 0.05$). However, the non-injected control group had a 16% higher hatching success than the control injected, thus the injection appears to have had an effect on the hatchability. None of the treatments had a significant different hatching success compared to the injected control (Chi squared test, $p > 0.05$). A table with a summary of the number of hatched eggs together with the calculated hatching success within each treatment group is provided in Appendix D.

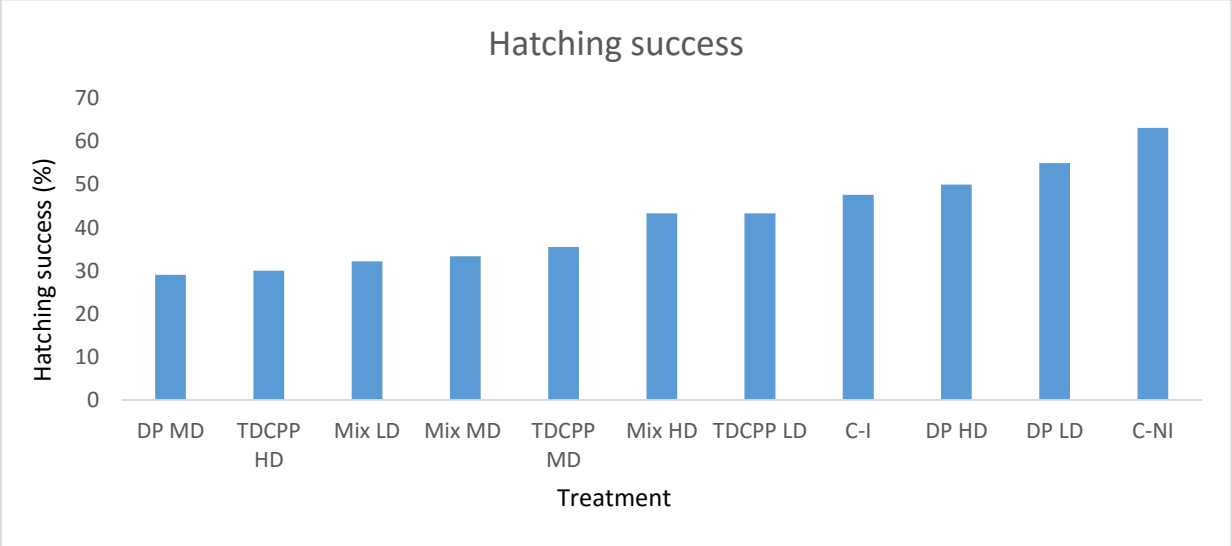

Figure 3. Hatching success within different treatment groups of Japanese quail (*Coturnix japonica*) exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of Dechlorane Plus (DP) and tris(1,3-dichloroisopropyl)phosphate (TDCIPP), independently and together in a mixture. In addition there were two controls, one non-injected control (C-NI) and one control injected with emulsion only (C-I).

3.3 Growth

3.3.1 Biometrical measurements

The graphs in Figures 4-6 and Figures 7-9 demonstrate the increasing body weight and head length over time for all doses within each exposure group (DP, TDCIPP and their mixture, respectively). Similar growth curves for tarsus length are provided in Appendix E. The injected and non-injected control groups are pooled together for both body weight and head length due to lack of significant differences in the two groups **(**Tukey's honest significance test, both $p >$

0.05). For body weight the graphs show an overall increase over time that gets steeper as the chicks get older, and for head length there is linear and constant increase in length over time.

Figure 4 shows similar gain in body weight over time in all the different doses of DP. The fitted lines for the different doses are almost completely overlapping each other and the control, until around day 10 where they differ slightly.

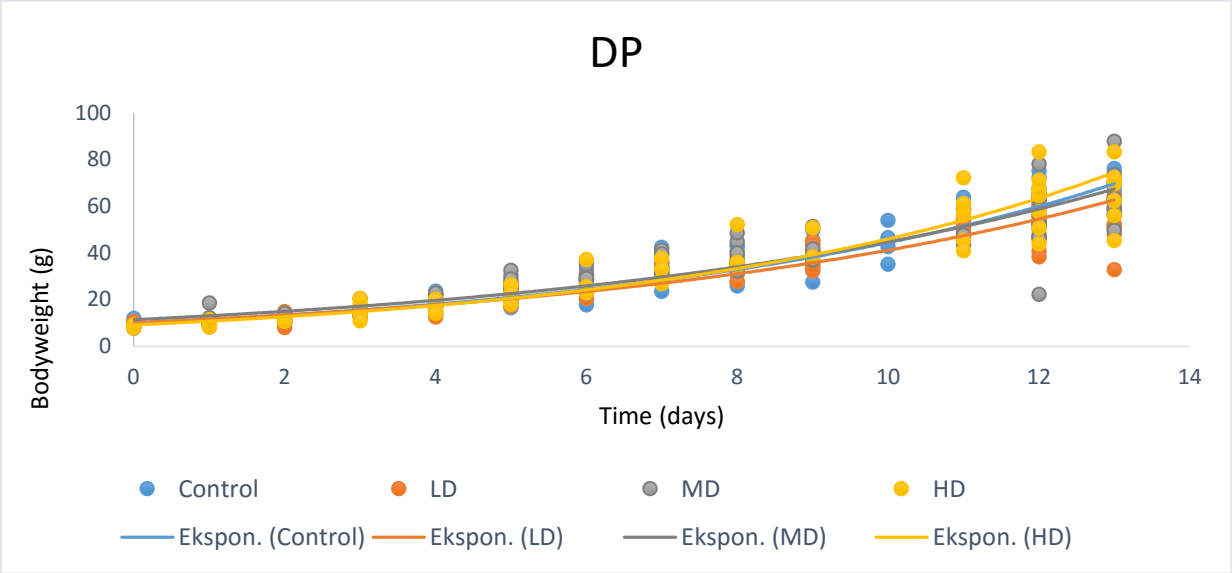

Figure 4. Body weight (g) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of Dechlorane Plus (DP). Injected and non-injected control groups are pooled together because of no significant difference in body weight between the two. The curves are fitted with exponential lines.

A similar trend is found for TDCIPP (Figure 5) with similar gain in body weight over time in all the different doses of TDCIPP. The fitted lines for the different doses are almost completely overlapping each other and the control, until around day 10, where the control deviates slightly from the rest.

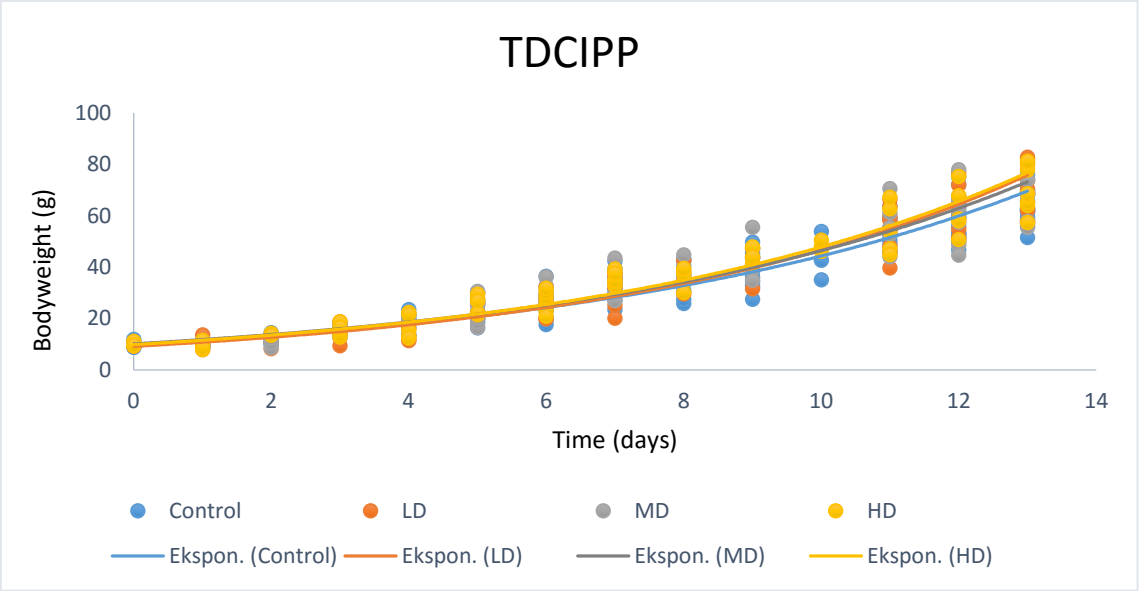

Figure 5. Body weight (g) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) of tris(1,3-dichloro-2-propyl)phosphate (TDCIPP). Injected and non-injected control groups are pooled together because of no significant difference in body weight between the two. The curves are fitted with exponential lines.

Regarding the mixture, figure 6 shows similar gain in body weight over time in all the different doses of the mixture of DP and TDCIPP. The fitted lines for the different doses are almost completely overlapping each other and the control until around day 9 where the LD deviates slightly from the rest. The HD is completely overlapping the control from day 0 until day 13.

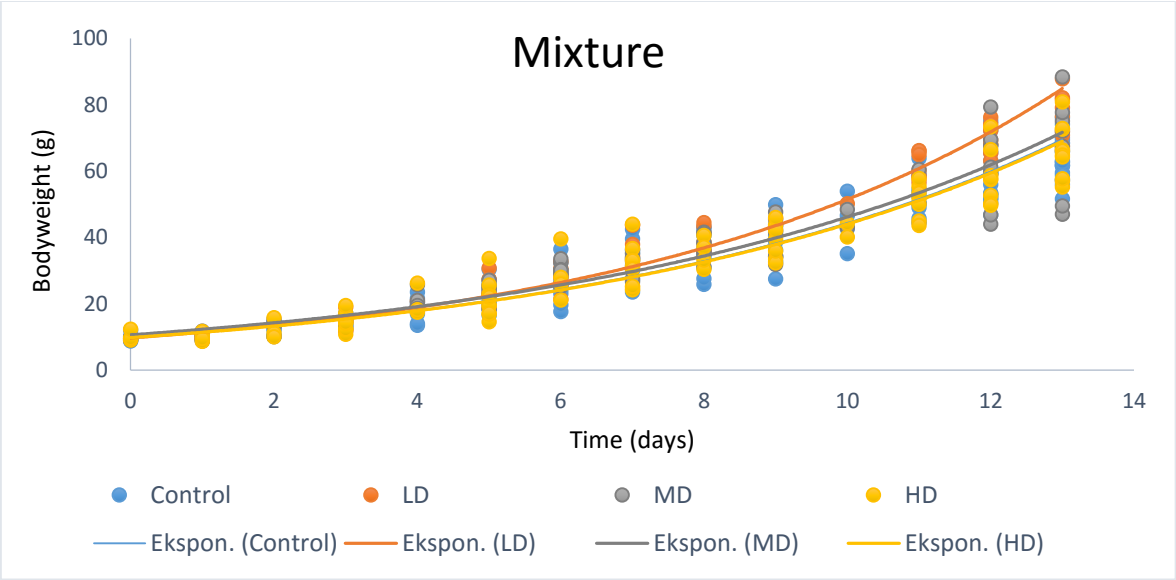

Figure 6. Body weight (g) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) of a mixture of Dechlorane Plus (DP) and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP). Injected and non-injected control groups are pooled together because of no significant difference in body weight between the two. The curves are fitted with exponential lines.

Figures 7-9 show how similar the head length increased in all the exposure treatments, starting at around 25 mm at hatching day and ending up at around 30 mm in all exposure groups. However, the pooled controls deviate from the rest of the doses in all the graphs. The regression line for the pooled control starts at a lower head length, around 15 mm, and ends up at the same head length as the rest of the treatment groups at day 13. No significant difference was found between the pooled controls and the rest of the treatments at hatching day (day 0) or at day one, where the graphs show the greatest difference (Tukey's honest significance test, all $p > 0.05$).

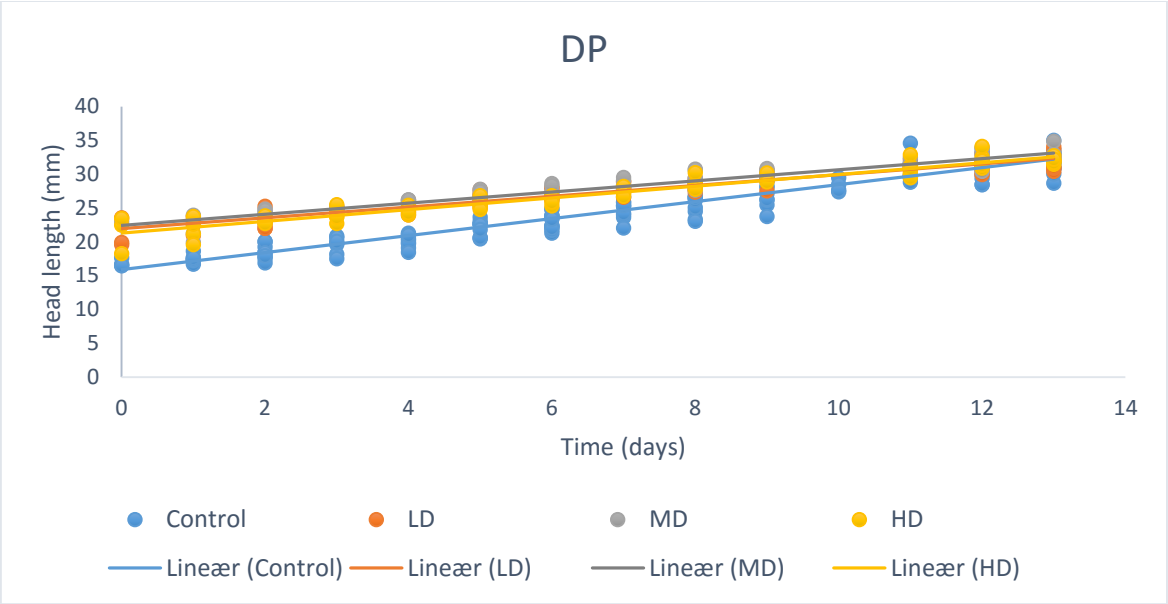

Figure 7. Head length (mm) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) of Dechlorane Plus (DP). Injected and non-injected control groups are pooled together because of no significant difference in tarsus length between the two. The curves are fitted with linear regression lines.

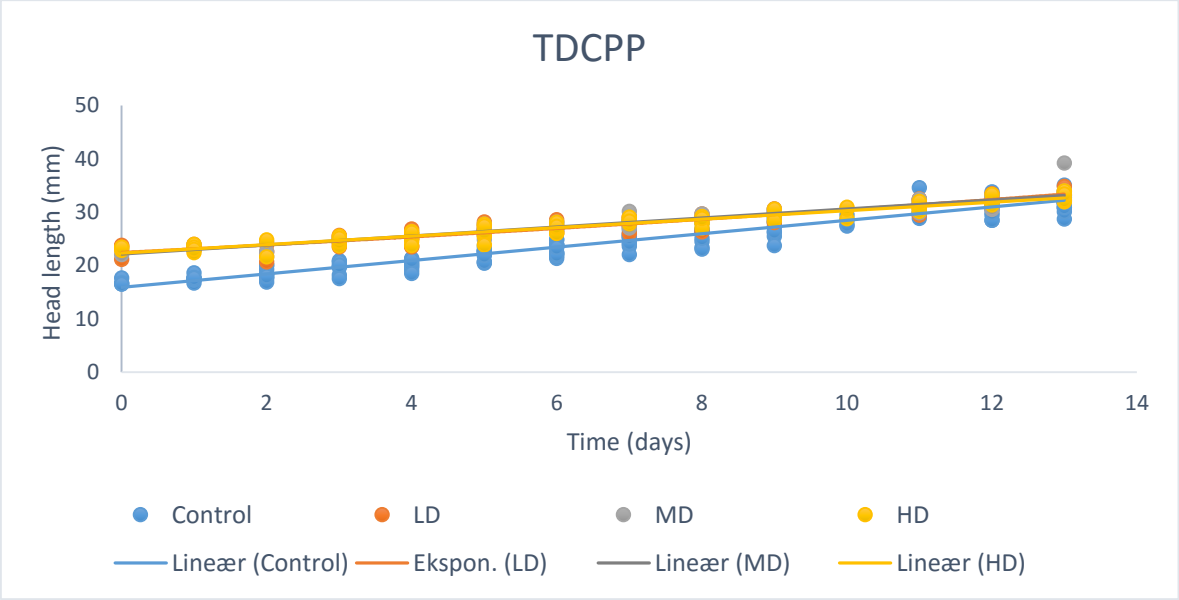

Figure 8. Head length (mm) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) of tris(1,3-dichloro-2-propyl)phosphate (TDICPP). Injected and non-injected control groups are pooled together because of no significant difference in tarsus length between the two. The curves are fitted with linear regression lines.

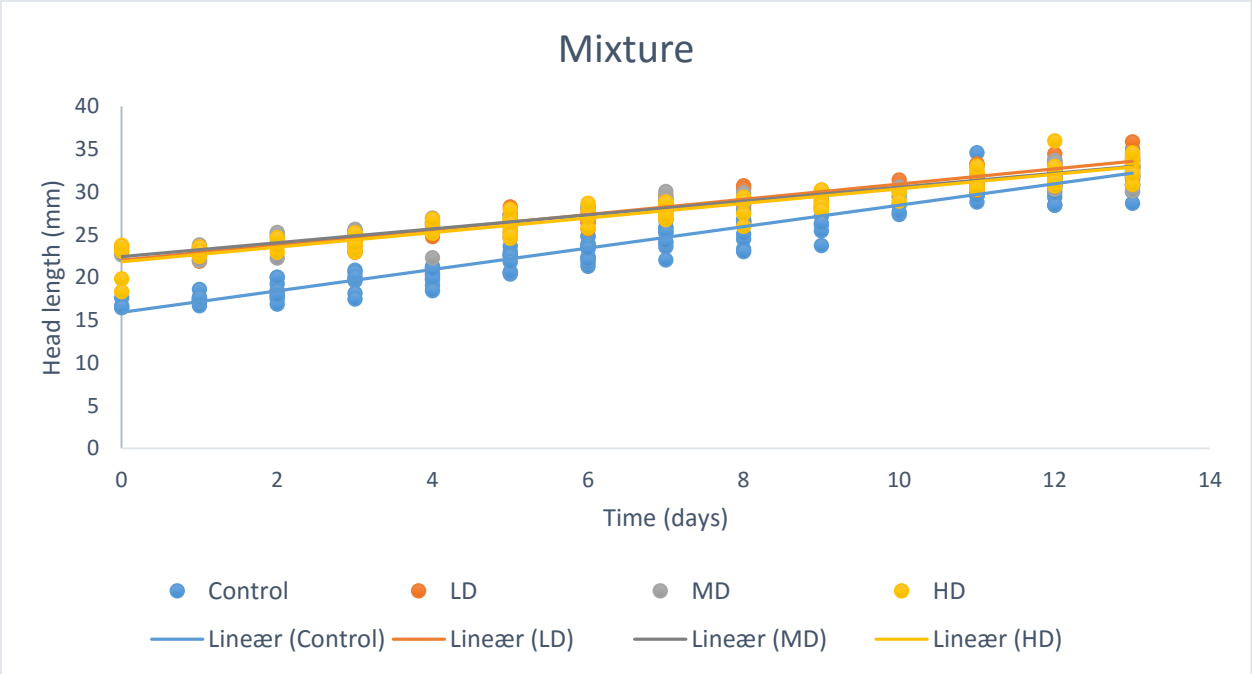

Figure 9. Head length (mm) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) of a 1:1 mixture of Dechlorane Plus (DP) and tris(1,3-dichloro-2-propyl)phosphate (TDCPP). Injected and non-injected control groups are pooled together because of no significant difference in tarsus length between the two. The curves are fitted with linear regression lines.

3.3.2 Growth rate

Figure 10 shows the mean growth rate (GR) in the different doses within all the exposure groups. The two control groups (injected and non-injected) were pooled together due to lack of significant difference in GR between them (independent two-group Mann-Whitney U test, $p > 0.05$). None of the treatments had a significant effect on GR (ANOVA, $p > 0.05$). The figure also demonstrates a similar mean GR in all the treatments, including the control. A boxplot of GR in all treatments, including both controls, is provided in appendix F.

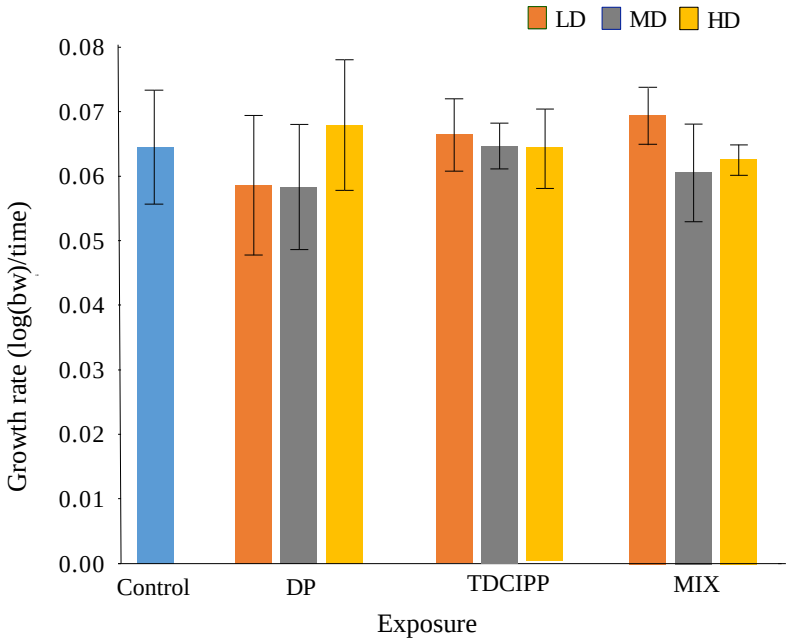

Figure 10. Mean growth rate (GR) in different treatment groups of Japanese quail (*Coturnix japonica*) exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of Dechlorane Plus (DP) and tris(1,3-dichloroisopropyl)phosphate, independently and together in a 1:1 mixture (MIX). Injected and non-injected control are pooled together due to no significant difference in GR between the two. Error bars represent standard deviations.

3.3.3 Body condition

Companionship and proportion of new food did not have any significant effect on the BCI (ANOVA, $p > 0.05$). In addition, there were no significant differences between the two controls, or any of the treatments compared to each other or to the controls (Tukey's honest significance test, $p > 0.05$). None of the treatments had a significant effect on BCI (ANOVA, $p > 0.05$).

3.4 Confounding factors affecting growth

A summary of the calculated companionship and food proportions within each treatment is presented in Table 4. Companionship is a measure of the variation in chick numbers in the cage during an individual's lifetime. The higher the value of companionship for an individual, the bigger portion of its lifetime was spent in company with several other chicks in the cage. Chicks that have companionship values of 3, spent their lifetime with an average of three chicks in their cage, which was the intended number in each cage. Since the number of chicks in the cages varied between one and four throughout the experiment, the majority of chicks have companionship values that are slightly higher or lower than 3 (Table 4). The mean value for companionship varies slightly between treatments, with highest companionship in the injected control (C-I) (3.28), and the lowest in MIX-LD (2.42).

Regarding the ratio of old versus new food, the lowest mean proportion of new food was found in non-injected controls (C-NI) (0.064), and the highest proportion in TDCIPP-LD (0.258). During their lifetime, chicks in C-NI received the smallest amounts of new food, and the chicks in TDCIPP-LD received the largest amounts.

Table 4. Summary of the calculated companionship and proportion of new food within different treatment groups of Japanese quails (*Coturnix japonica*) exposed *in ovo* to Dechlorane Plus (DP) and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP). The quails are exposed to low, medium, and high doses (LD, MD, and HD, respectively) of DP and TDCIPP, independently and together in a 1:1 mixture (MIX). A non-injected control (C-NI), and an injected control (C-I) were also included. The data are presented as mean ± standard error (SE), and range. *n* represents the sample size within each treatment group.

		Companionship		Food	
n	Treatment	Mean ± SE	Range	Mean ± SE	Range
10	C-I	3.28 ± 0.161	2.80-3.93	0.146 ± 0.052	0.00-0.36
9	C-NI	3.01 ± 0.049	2.79-3.27	0.064 ± 0.035	0.00-0.29
10	DP-LD	3.17 ± 0.118	2.79-3.67	0.117 ± 0.048	0.00-0.36
9	DP-MD	3.13 ± 0.170	2.64-3.87	0.138 ± 0.050	0.00-0.36
9	DP-HD	2.84 ± 0.030	2.67-2.93	0.177 ± 0.074	0.00-0.51
9	TDCIPP-LD	2.77 ± 0.048	2.57-3.00	0.258 ± 0.070	0.00-0.51
9	TDCIPP-MD	2.82 ± 0.067	2.47-3.00	0.107 ± 0.057	0.00-0.51
8	TDCIPP-HD	2.67 ± 0.113	2.07-3.00	0.182 ± 0.068	0.00-0.51
8	MIX-LD	2.42 ± 0.110	1.93-2.73	0.226 ± 0.080	0.00-0.51
10	MIX-MD	3.18 ± 0.161	2.53-3.93	0.183 ± 0.054	0.00-0.43
10	MIX-HD	3.15 ± 0.142	2.60-3.86	0.197 ± 0.064	0.00-0.51

The degree of companionship had an overall significant negative effect on GR including all the treatments (p = 0.024), In general, the GR decreased as the companionship increased, i.e. chicks with highest companionship had a slightly lower GR. However, the estimated decrease was close to zero (-0.004 ± 0.002).

Figure 11-13 are graphs of companionship or new food proportion plotted against GR in the exposure groups where there was a significant interaction effect for some of the doses. A pooled control is used in each graph due to no significant difference in the GR between injected and non-injected controls .

Companionship had a significant interaction effect together with DP-MD (p = 0.038) and MIX-MD (p = 0.018) on GR. Figure 11 and 12 illustrates, that the GR, for treatment DP-MD and MIX-MD, increased significantly as the degree of companionship increased. Individuals in these treatments that spent more time with several other chicks had a higher GR. For DP, control showed a slight decrease, while the low dose had a slight increase. For the individuals in the high dose group, the values are close together (between 2.67 and 2.93), still there is a small increase in GR towards individuals with a slightly higher degree of companionship (Figure 11). Regarding the mixture, the low and high dose had almost no change in GR, and the control showed a slight decrease as the degree of companionship increased (Figure 12).

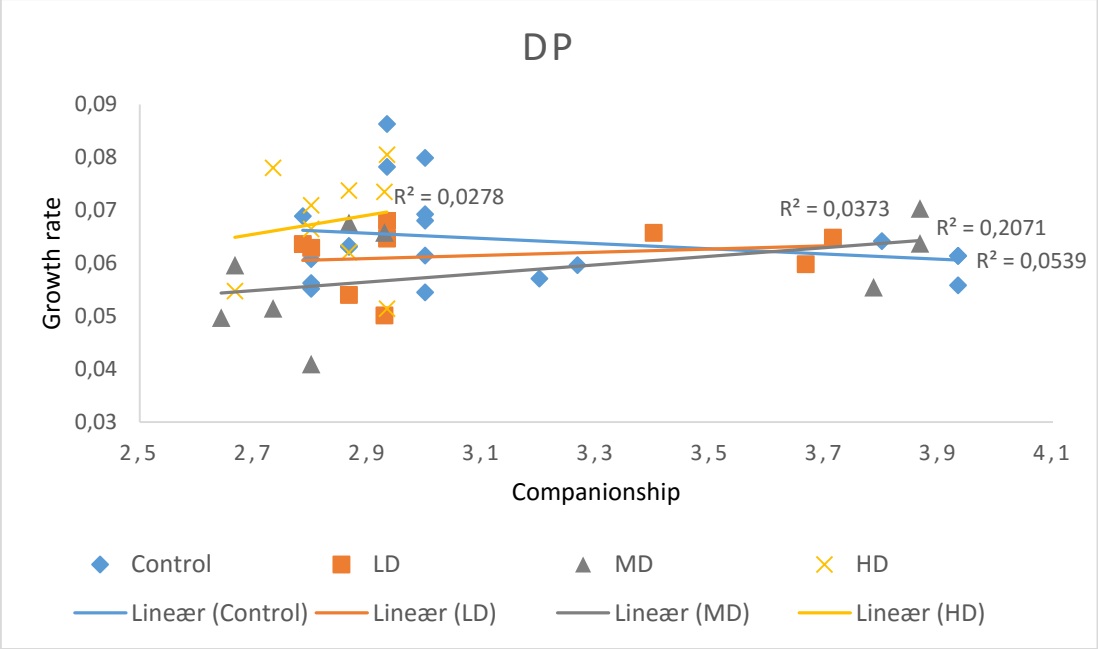

Figure 11. Relationship between growth rate (GR) and companionship, the variation in chick numbers in the cage during an individual's lifetime, in Japanese quail (*Coturnix japonica*). Individuals were exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of Dechlorane Plus (DP). Injected and non-injected control groups are pooled together based on no significant difference between the two. The curves are fitted with a linear regression line.

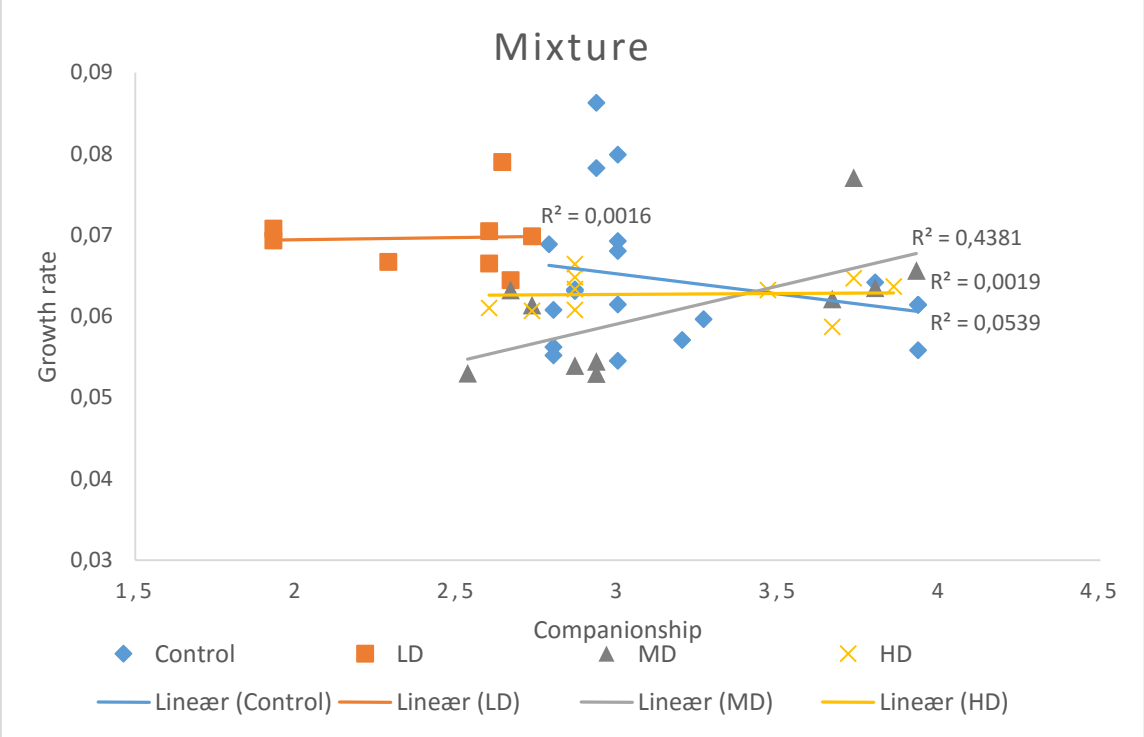

Figure 12. Relationship between growth rate (GR) and companionship, the variation in chick numbers in the cage during an individual's lifetime, in Japanese quail (*Coturnix japonica*). Individuals were exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of a mixture of Dechlorane Plus (DP) and tris(1,3-dichloroisopropyl)phosphate (TDCIPP). Injected and non-injected control groups are pooled together based on no significant difference between the two. The curves are fitted with a linear regression line.

Food in itself did not have any effect on GR, but it had a significant interaction effect on GR together with the treatment MIX-MD (p = 0.0094). For treatment MIX-MD, the GR increased significantly as the proportion of new food increased, i.e. individuals with a higher proportion of new food had a higher GR than individuals that had lower proportions (Figure 13). The control group had a slight decrease, however low and high dose showed no change in GR as the proportion increased.

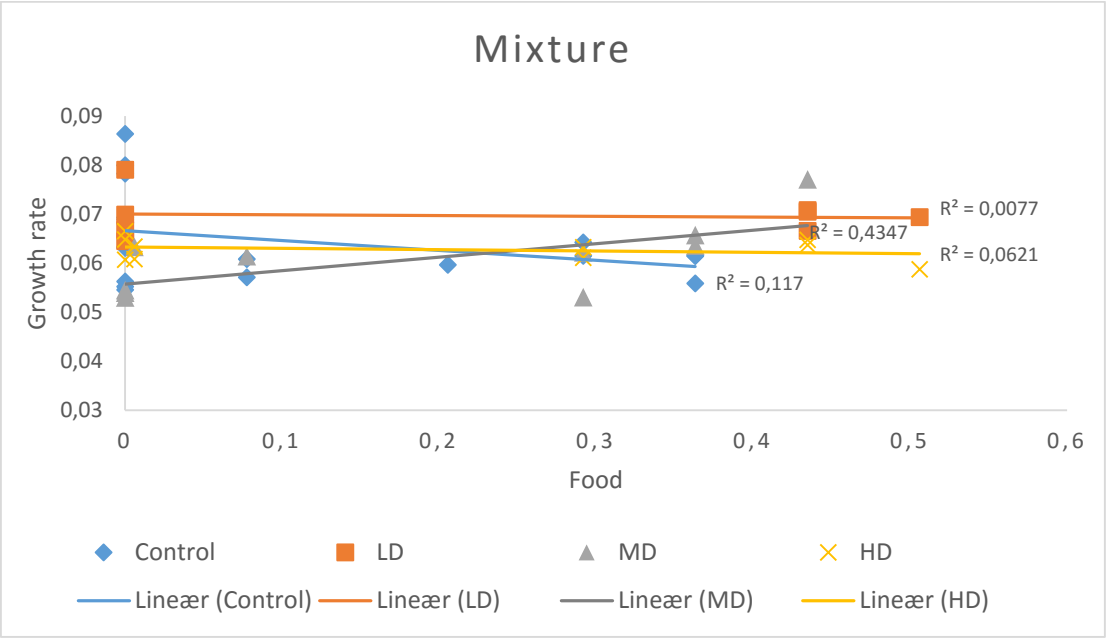

Figure 13. Relationship between growth rate (GR) and proportion of new food received throughout the lifetime in Japanese quail (*Coturnix japonica*). Individuals were exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of a mixture of Dechlorane Plus (DP) and tris(1,3-dichloroisopropyl)phosphate (TDCIPP). Injected and non-injected control groups are pooled together based on no significant difference between the two. The curves are fitted with a linear regression line.

3.4.1 Growth rate corrected for the effect of food and companionship

Based on the residuals for the model fitted with companionship and food, there were no significant differences in GR between the treatment groups, nor between any of the treatments and the pooled control (Tukey's honest significance test, all $p > 0.05$). This indicates that when corrected for the confounding effects of companionship and proportion of new food, the treatments alone did not have any significant effect on the GR.

4.1 DISCUSSION

The current study found that *in ovo* exposure to the flame retardants DP and TDCIPP did not have any significant effects on growth and development in Japanese quail. No significant differences in the results were found between the two compounds or a mixture of the two. Also, there was no significant difference between injected and non-injected control groups.

4.1 Levels of contaminants

4.1.1 TDCIPP

Due to the rapid metabolism of TDCIPP, followed by rapid excretion of the metabolite BCDIPP, it was expected to find very low levels of TDCIPP in the livers of the quails (Lynn et al., 1981; Nomeir et al., 1981). Farhat et al. (2013) demonstrated how rapidly TDCIPP is metabolized in domestic chicken eggs. At the beginning of incubation (day 5) more than 92% of the injected concentration (45 000 ng/g egg) was detectable, and less than 1% was detected at the end of the incubation (day 19) (Farhat et al., 2013). This coincides with the results from the liver analyses in the present study, where no levels of the metabolite and very small amounts of the parent compound were found 14 days post-hatching. Based on these results and the findings by Farhat et al. (2013), it seems likely that the majority of the injected TDCIPP was metabolized prior to hatching.

4.1.1.1 Levels in wild eggs compared to injected doses

Chicken embryos have been shown to metabolize extremely high concentrations of TDCIPP during the incubation period (Farhat et al., 2013). Nevertheless, levels of TDCIPP have been detected in wild avian eggs; 1.9 ng/g wet weight (ww) in Norwegian great-backed gull (*Larus marinus*) (Chen et al. 2012c), and 0.17 ng/g ww in Great Lakes herring gull (*Larus argentatus*) (Leonards et al., 2011). A more recent study reported levels in herring gull eggs with 0.93 ng/g ww in yolk, and 0.47 ng/g ww in albumen (Greaves and Letcher, 2014). Injected doses up until 1000 times higher than levels reported in the latter studies were almost completely metabolized in 14 days old quails, and possibly already during incubation, in the current study.

A possible explanation for detected levels in the wild eggs, despite high metabolism of TDCIPP, could be that a certain exposure threshold is required in order to activate necessary enzymes for biotransformation, and that this threshold may not be reached with the current environmental levels (Farhat et al., 2013). No effects of TDCIPP were found on either hatching success or growth for low dose, or the higher doses, in the current study. This suggests that the considerably lower environmental exposure levels found in the eggs of gulls possibly could have no effect on hatching or growth, although species differences may exist. Documentation of levels of TDCIPP in raptor tissues and eggs is scarce. This is mainly due to the requirements of non-invasive methods in raptors (Espín et al., 2016). Since raptors have a higher trophic position than gulls, they are prone to have higher lipid contamination levels in their tissues (O'Sullivan and Megson, 2014). However, they are altricial species, which means that relatively small quantities of maternal lipids are invested into a clutch of eggs (Drouillard and Norström, 2001). And since contaminants get transferred into the eggs together with the lipids (Verreault et al., 2006), higher concentrations in raptor maternal tissues does not necessary translate to higher contaminant levels in the eggs. Because of different developmental patterns, different trophic positions, and other species differences, in addition to the absence of appropriate literature, it is difficult to compare the results from the current study with egg concentrations in raptors.

4.1.1.2 Levels in liver

In the present study, levels of TDCIPP were detected in the groups exposed to a high dose of TDCIPP, a high dose of the mixture, and in the non-injected control group, with mean concentrations of 3.50 ng/g ww, 2.60 ng/g ww, and 3.09 ng/g ww, respectively. The metabolite, BDCIPP, was not detected. The similarities in concentrations between the two exposure groups and the control, indicate no elevated levels in exposure groups compared to control. This indicates completely metabolism of TDCIPP and excretion of BDCIPP. In the present study, levels were only detected in two individuals per treatment group mentioned above, whereas the rest of the individuals had levels below LOQ, i.e. <4.0 ng/g. Studies of TDCIPP levels in biota, and especially in birds are severely lacking. Liver concentrations of <1.5 ng/g ww were found in kittiwakes (*Genus rissa*) and common eider *(Somateria mollissima)* (Evenset et al., 2009). The levels reported in Evenset et al. (2009) coincide with the expected low levels in the present study due to a rapid metabolism of TDCIPP and excretion of BDCIPP (Lynn et al., 1981; Nomeir et al., 1981).

A possible explanation for the lack of reported levels in wildlife could be due the fact that levels are below LOQ, and therefore not being reported in studies.

4.1.2 DP

Levels of DP were detected in the liver of all birds exposed to high dose of DP independently and the mixture. These results indicate that the metabolism of DP in quails was at least slower compared to TDCIPP. A study on rats demonstrated that the rate of metabolism and excretion of a compound was inversely correlated with its degree of chlorination (Matthews et al., 1974). There is a selective maternal transfer of lower halogenated compounds in eggs, and the highly chlorinated compound DP tends to be retained in the hen (Zheng et al., 2014a). During chick embryo development, selective maternal transfer of *anti*-DP and a stereoselective metabolism of s*yn*-DP has been observed (Zheng et al., 2014a). This stereoslective metabolism was not observed in the present study as the ratio between the isomers in eggs one day after exposure was the same as in the livers of 14 days old chicks. However, the biotransformation of DP during embryonic development is poorly studied (Zheng et al., 2014b).

4.1.2.1 Levels in wild eggs compared to injected doses

In the present study, concentrations of DP were quantified in eggs after one day of incubation in chicks *in ovo* exposed to high doses of DP or the mixture, with mean concentrations of 1049 ng/g ww and 1192 ng/g ww, respectively.

Concentrations of DP have been found in wild avian eggs. The majority on reported levels are from sites in close proximity to point sources like manufacturing plants in North America Great Lake basin and China (Chen et al. 2012a). Most of the reported concentrations of DP in avian species are in aquatic ecosystems. Regarding precocial species, studies on gull species are most common. Muñoz-Arnanz et al. (2012) found a mean ∑DP concentration of 0.21 ng/g ww in eggs of yellow-legged gulls (*Larsus michahellis*), and a mean ∑DP concentration of 0.027 ng/g ww in Audouin's gull eggs (*Ichthyaetus audouinii*). In the latter study, the differences in DP concentrations in eggs from the two species of gulls could most likely be explained by the birds' different exploitation of food resources. The yellow-legged gull's diet includes marine, terrestrial and human-derived resources, while the Audouin's gull feeds almost exclusively on marine food resources. (Muñoz-Arnanz et al., 2012). Higher levels than in Chen et al. (2012a) were found in eggs of herring gull colonies in Great Lakes (<5 ng/g ww), and in Niagara Falls

(15 ng/g ww) (Gauthier and Letcher, 2009), although these levels are still lower or comparable to the low dose exposure in the current study. Levels of DP have been detected in fish and sediment in both the Niagara Falls and the Great lakes areas (Hoh et al., 2006). A possible source to the elevated contaminant levels in the Niagara Falls colony could be a DP's manufacturing facility located in Niagara Falls (Hoh et al., 2006). Chen et al. (2012b) reported concentrations in eggs from colonies of four different gull species, spanning the Atlantic to Pacific Canada, covering both marine and freshwater ecosystems. Overall, the marine colonies had lower concentrations in their eggs. The highest detected mean concentration of ∑DP was detected in a freshwater colony of herring gulls (5.5 ng/g ww), and the second to highest was in a freshwater colony of ring-billed gull (*Larus delawarensis*) (1.0 ng/g ww). The colonies with the lowest mean concentrations were a freshwater colony of Californian gulls (*Larus californicus*) (0.3 ng/g ww) and a marine colony of glaucous-winged gull (*Larus glaucescens*) (0.2 ng/g ww). The gulls had a higher contamination load of other flame retardants than DP (Chen et al. 2012b). In the latter study, the gulls' diet along with human population density was explaining the significant differences in egg concentrations between the colonies, and between the two ecosystems. In the studies mentioned above, concentrations in eggs seems to be mainly affected by the bird's foraging habits and its proximity to pollution sources, i.e. human activities. Concentrations in eggs of a few altricial bird species have also been reported. Spanish white stork (*Ciconia ciconia*) eggs had even lower ∑DP concentrations with a median of 0.3 ng/g ww and 0.08 ng/g ww from two different locations (Muñoz-Arnanz et al., 2011). Peregrine falcons (*Falco peregrinus*) of Canadian specimens had a median egg concentration of 2.11 ng/g ww (36.4 ng/g lw), and eggs of Spanish specimens had a median concentrations of 0.10 ng/g ww (1.78 ng/g lw) (Guerra et al., 2011). These studies indicates low levels even in raptor species, despite them being at a high trophic level and thus being prone to biomagnification of DP.

The concentrations in all studies mentioned were mostly below the low dose in the current study. One exception was gull eggs from the Niagara Falls colonies in Gauthier and Letcher et al. (2009), which had concentrations slightly above the low dose in the present study (10 ng/g ww). One of the major prey fish species in the area (Lake Ontario) is alewife, which had a reported mean ∑DP of 0.12 ng/g lw (Tomy et al., 2007). The same authors reported ∑DP levels in other prey fish, like rainbow smelt (*Osmerus mordax*) and lake herring (*Coregonus artedi*), to be comparable or lower.

Based on the findings of Tomy et al. (2007), Gauthier & Letcher (2009) estimated a biomagnification factor (BMF) of 600 or greater from the prey fish to herring gull egg. This suggests that DP is highly biomagnified in herring gull, and could therefore explain the elevated levels in gull eggs from Niagara Falls. Since the hen only deposits a proportion of her contaminant load into the eggs, the levels in the hens could possibly be higher than the eggs. As the results of the current study showed no effects on growth for all investigated doses, including the high dose (1000 ng/g), this indicates that DP alone (or in a mixture with TDCIPP) will likely have no significant effects on embryonic development in any of the species from the above mentioned studies. Even though both the gulls and the Japanese quail are precocial species, it is important to keep in mind species differences when extrapolating from the quails in the current study to the gulls. The altricial species have a different developmental pattern than the quails, starting with the development of the embryo. Thus, even though the egg concentrations in the altricial species are lower than the quail in the current study, the different developmental pattern in addition to species differences could play a role in how DP affects growth and development.

4.1.2.2 Levels in liver tissue

In the current study, concentrations of DP was quantified in the liver in chicks at 14 days old in the groups *in ovo* exposed to high doses of DP and the mixture, with mean concentrations of 27.4 ng/g ww and 23.6 ng/g ww, respectively. Little data is available on liver concentrations of DP in birds and other terrestrial species (Sun et al., 2012).

Sun et al. (2012) studied accumulation of DP in three terrestrial passerine bird species (altricial species) from a river in South China. The study showed a preferentially accumulation of DP in liver tissue rather than muscle, and significantly higher concentrations at urban sites. The median levels at the urban areas were 15.6 ng/g ww (330 ng/g lw) for the light-vented bulbul (*Pycnonotus sinensis*), 23.8 ng/g ww (280 ng/g lw) for the long-tailed shrike (*Lanius schach*), and 15.2 ng/g ww (270 ng/g lw) for the oriental magpie-robin (*Copsychus saularis*) (Sun et al., 2012). These levels are similar to the concentrations found in the quail livers after 14 days in the high dose exposure group in the current study. Chen et al. (2012a) detected concentrations in liver and muscle tissues of six terrestrial raptors. The highest liver concentrations of ∑DP were in Japanese sparrowhawk (*Accipiter gularis*) with a median of 18.4 ng/g ww (160 ng/g lw), and the second to highest concentration was in common kestrels with a median of 71.5 ng/g ww (550 ng/g lw).

The lowest liver concentrations were in long-eared owl (*Asio otus*) with a median of 0.000603 ng/g ww (0.603 ng/g lw), and the second to lowest in scops owl (*Otus scops*) with a median of 0.36 ng/g ww (71 ng/g lw). The raptor with liver concentrations closest to the liver concentrations in the current study was Eurasian sparrowhawk (*Accipiter nisus*) with a median of 20.3 ng/g ww (203 ng/g lw) (Chen et al. 2012a). When comparing concentrations in Chen et al. (2012a) with exposure doses in the current study, two of the raptors have levels close to the injected exposure doses. The Japanese sparrowhawk has concentrations close to the medium dose (100 ng/g), and the little owl (*Athene noctua*), with concentrations of 7.2 ng/g ww (480 ng/g lw) have concentrations close to the low dose (10 ng/g). There is a large variation in DP concentrations between the raptor species in Chen et al. (2012a), demonstrating different liver accumulation of DP despite all species being raptors and living in a terrestrial ecosystem. In addition to being at a higher trophic level than the quails, these raptors have most likely accumulated DP throughout their lifetime, and so we cannot differentiate between exposure from maternal transfer and from the diet. In the current study there were no significant effects on growth in the quails exposed to any dose of DP, and the results from the current study could possibly indicate that there will be no effects on growth in the raptors either. As already mentioned, the different developmental patterns need to be taken into consideration, and the degree of exposure through diet and trophic position could have an effect, and also species differences may have an impact on accumulation and possible toxicological effects. Further, wild birds living in contaminated areas are probably exposed to DP through their environment post-hatching in addition to a possible *in ovo* exposure. Neither Sun et al. (2012) nor Chen et al. (2012a) state the age of the birds, which makes it difficult to know theoretically how long the birds could have had to metabolize or excrete one or potentially multiple exposures of DP. It has been suggested that metabolism of DP increases with increasing trophic level in birds (Zhang et al., 2011). This could possibly contribute to explain the different liver concentrations of DP in the studies mentioned above.

4.2 Injection and hatching success

The first days of incubation are the first of three critical periods in embryonic development, and it involves initial differentiation and primary organogenesis (DeWitt et al., 2005). When injecting the quail eggs prior to incubation in the current study, it is difficult to know if a non-developing embryo is due to the exposure of contaminants, the injection volume, or natural causes.

DeWitt et al. (2005) investigated the effect of egg injection day in developmental toxicology by evaluating mortality as an endpoint. In the study, a comparison in mortality was made between chicken eggs injected in the air sac with 1.0 µL/g egg of vehicle oil (corn oil) at day zero and at day four of incubation. Their study concluded, in line with other studies, that survival of the embryo is strongly influenced by what day of incubation the egg is injected. They concluded that overall mortality will most likely be increased when the eggs are injected at day zero of incubation, however this approach better simulates environmental exposure situations related to maternal transfer (DeWitt et al., 2005). DeWitt et al. (2005) demonstrated that eggs injected at day zero had an overall mortality of 54%, while the non-injected group had a mortality of 23.2%. The non-injected eggs were handled the same way as the injected eggs, where a hole was made into the center of the air sac before it was sealed again (DeWitt et al., 2005). In the current study, the non-injected eggs (63%) had a 15% higher hatching success than the eggs injected with the emulsion only (48%). In the study of DeWitt et al. (2005), they injected 1 µL/g egg and in the present study 2 µL/g egg was injected. Since DeWitt et al. (2005) found an effect of the injection volume, it could possibly have had an effect in the present study as well, i.e. disturbed normal development during the first days of incubation.

However, the exposed quails did not show any significant differences in hatching success compared to the eggs injected with the emulsion only. When comparing the treatments against the injected group, the effect of injection volume is accounted for. There were also no significant differences in hatching success between the treatments. This is in accordance with a study by Farhat et al. (2013) where TDCIPP did not show any effects on pipping time at a concentration of 1000 ng/g egg. Pipping time is the time from the first visible cracks in the eggshell until the shell is pierced, while hatching is the process from the pierced hole until the chick is out of the egg. A significant decrease in pipping time did not become apparent until a concentration of 10 000 ng/g egg (Farhat et al. 2013). A study investigating the concentration-dependent effects of DP, exposed *in ovo* in chicken observed no effects of pipping success up to the highest dose of 500 ng/g egg, i.e. half of the high dose in the current study (Crump et al., 2011).

4.3 Growth curves

Karaman et al. (2013) took weekly measurements of body weight in 89 male and 89 female Japanese quails. The growth curve with body weight against time in the current study matches the one in Karaman et al. (2013), where the chicks have a mean weight around 25 g at day 7 and around 75 g at day 13. However, in the current study the mean body weight at day 13 varied from 65 g to 80 g. The sample size in each treatment in the current study is much smaller than in Karaman et al. (2013), so the variation may possibly be explained by individual differences. Overall, the comparison shows that the quails in the current study had normal gain in body weight throughout their lifetime. Female quails are in general heavier than males, and in Karaman et al. (2013) a slightly higher body weight can be observed in the curve for the females on day 13. In the current study both the sexes were incorporated into the same growth curves, because the difference in growth does not becomes apparent until three weeks of age (Karaman et al., 2013). In the current study, regarding the increase in head length over time, the growth curves showed a trend where the pooled controls started at a lower head length in all the treatments. The difference in head length between the controls and the rest of the treatments were not significant at day one, and at day 13 the curve for the controls was overlapping with the curves from the rest of the treatments. This also indicates that the control group had a larger and more rapid increase in head length during their lifetime than the rest of the treatments. However, regarding gain in body weight and increase in tarsus length, the controls did not deviate noticeably from the treatments.

4.4 Growth rate

Growth rate in the current study is the rate of increase in body weight during the first two weeks of development post-hatching (Ricklefs, 1979). The characteristic growth curve for Japanese quail has the sigmoid shape when plotting body weight as a function of time (i.e. age). The first phase of a sigmoid shape is the self-accelerating phase, which is linear and starting from the hatching weight. During the linear phase the growth rate reaches its maximal value in a certain period, before it decreases and approaches zero during the decelerating phase of the curve (Kýzýlkaya et al., 2005). Since the chicks in the current study were euthanized at 14 days of age, they were still growing linearly (Karaman et al., 2013). Therefore, it seemed appropriate to use growth rate in order to compare growth between the treatment groups. The purpose of doing this was to see if any of the chicks grew slower as an effect of the exposure. There was

no difference in growth rate between the treatments, which means that all the chicks from different treatments gained weight at a similar rate.

The study by Ricklefs (1979) supports the hypothesis that functional development in birds affects the growth rate post-hatch. As a result, precocial chicks (gulls, quails) grow slowly, while altricial chicks (starlings, eagles, hawks) grow fast. Species with an altricial developmental pattern might respond differently to the flame retardants than precocial quails do. It is important to bear in mind developmental patterns when comparing growth. It is easier to compare results from the quails in the current study to other precocial species, even though species differences also need to be considered.

4.5 Body condition

A study by Schulte-Hostedde et al. (2005) tested several assumptions regarding the use of residuals from an OLS regression, and found no reason to reject OLS residuals as legitimate indices of body condition. However, they specify that residuals should be used with some caution.

In the present study, a BCI was calculated in order to give a more complete measure of size than body weight alone. Since the quails were in captivity, their general condition (health) was closely monitored during the experiment. The body condition index of quails was calculated based on measurements from day 12, that is two days prior to being euthanized. There was no significant difference in BCI between the treatment groups. This indicates that there was no effect of *in ovo* exposure to TDCIPP, DP or their 1:1 mixture on the body condition in Japanese quails chicks.

4.6 Effects of food and companionship

It seemed that the highest or lowest values of companionship and proportion of new food over the different treatment groups were random. The degree of companionship per chick depended on when the chicks in the same cage hatched in relation to each other, which varied because of different hatching days for chicks within the same treatment. The proportion of new food each chick received throughout its lifetime, depended solely on how old they were when the new food was introduced, i.e. at what time they hatched.

Chicks that had a lower degree of companionship were typically in cages with age gaps, meaning also that they were alone in the cage for certain periods. Duval et al. (2012) stated that

food competition could favor dominant individuals when quails are grouped, i.e. more dominant individuals would eat larger portions of food. This theory could possibly be extrapolated to the quails in the present study, especially in the cages that had four chicks in them, or cages with larger age gaps of five or six days between the chicks. In the present study, older chicks were more active than the newly hatched, and therefore dominating more of the space inside the cage. Furthermore, typically no competitive behavior is shown in recently hatched chickens until three days of age (Wood-Gush, 1955). This again implicates different behavior patterns in chicks of different ages, and similar observations as Wood-Gush (1955) was observed in the present study. With both species being poultry, it would seem likely that the quails show similar behavior as the chicken.

Analyses of the two different types of food showed that the new food contained higher levels of certain contaminants (see appendix C). A study conducted by Jacobsen et al. (in prep.) found that the proportion of new food had a highly significant effect on 12 of 18 measured blood clinical-chemical parameters (BCCP) in tissues of the quails in the present study. In the study by Jacobsen et al. (in prep.), the BCCPs reflect the health and homeostasis of the liver, kidney function, possible diseases in digestion and pancreas, electrolyte homeostasis and dehydration, and energy metabolism in the quails (Sonne et al., 2012). This demonstrates the importance of food, and why it was important to correct for the effect of food when investigating for any effects of the treatments in the present study. Calculated BCI gives a more overall indication of size, and no significant difference in body condition between the treatments ensures that the quails have similar shapes and sizes at the end of the experiment, despite eating different proportions of new food and having different degree of companionship.

An important difference between the current experimental study and wildlife is that the quails had unlimited access to food, while this is not the case in wild bird populations. Young birds may reduce growth and metabolism when experiencing poor feeding conditions during their post-hatching period (Rønning et al., 2009). Food restriction has shown to reduce metabolism and structural growth in Japanese quail, in addition thyroid hormone levels in plasma decreased (Rønning et al., 2009). Based on the study by Rønning et al. (2009), there is a possibility that the flame retardants might induce effects in wild birds at concentrations that did not lead to any effects in the quails in the current study, because of differences in food availability between our experiment and natural conditions.

4.7 Potential developmental toxicity of DP and TDCIPP

Toxicological information about the endocrine disrupting capabilities of TDCIPP is limited (Wang et al., 2015). However, some studies have documented endocrine effects of TDCIPP. *In vitro* studies have shown that TDCIPP can act as an agonist for the androgen receptor and the estrogen receptor α (Kojima et al., 2013; Zhang et al., 2014). *In vivo* studies have shown an upregulation in gene expression of estrogen receptors and associated genes in zebrafish larvae (Liu et al. 2013b) altered sex hormone levels in zebrafish (Liu et al. 2013a), and an increase in developmental abnormalities in zebrafish embryos exposed to TDCIPP (McGee et al., 2012). Wang et al. (2015) observed an impairment of reproduction in zebrafish exposed to long-term low concentration exposure of TDCIPP. Farhat et al. (2013) found several *in ovo* effects of TDCIPP in chickens. Growth was affected at several levels in exposed chickens to very high doses of TDCIPP; reduced tarsus length (at 51 600 ng/g egg), and a significant decrease in head plus bill length, embryo mass, and gallbladder size (at 45 000 ng/g egg) (Farhat et al., 2013). In the current study there were no differences in body weight and head length at day 1 between any of the treatments and the pooled control. On the other hand, Farhat et al. (2013) found no adverse effects on growth and development in chickens exposed to environmental relevant levels (12 ng/g egg). The high doses used by Farhat et al. (2013) were extremely high compared to environmental levels, and also much higher than the exposure doses in the present study (HD: 1000 ng/g egg). The latter study found that TDCIPP delayed pipping at 9240 ng/g egg, but not at environmentally relevant levels (12 ng/g egg).

Since thyroid hormones have an important role in growth and development, their disturbance in an organism can lead to effects on growth (McNabb and Darras, 2015). Farhat et al. (2013) showed reduced T4 levels in chicken plasma at an exposure of 7640 ng/g egg. TDCIPP has also been associated with a decline in free T4 levels in humans (Meeker and Stapleton, 2010), and a decrease in T3 and T4 levels in female zebrafish (Xu et al., 2015). Kojima et al. (2013) demonstrated *in vitro* the potential agonistic activity of TDCIPP on the pregame x receptor (PXR). The activation of PXR leads to induction of multiple detoxification enzymes, including T4 metabolizing enzymes (CYP3A) (Kojima et al., 2013). Based on these studies, it is hypothesized to find lower levels of T4 in the quails exposed to TDCIPP in the current study, although this could not be confirmed so far due to difficulties with the method. Several studies have found induction of cytochrome P450 mRNA levels as an effect of TDCIPP exposure (chicken: Farhat et al. 2013; human: Kojima et al. 2013). In the present study, it could be possible that some effects are present on hormonal or genetic levels, even though no effects

were observed in growth in the quails. It would therefore have been interesting to see if there were any alterations in hormonal levels or mRNA expression in exposed quails in the current study. In contrast, DP has shown not to lead to any changes in mRNA transcripts in chicken exposed both *in vivo* and *in vitro* (Crump et al., 2011)

Similar to TDCIPP, information on the toxicity of DP is lacking (Feo et al., 2012). Several studies have found very low toxicity in several different species exposed to concentrations greater than levels detected in biota (Feo et al., 2012). Hepatic oxidative damage, disturbance of metabolism and signal transduction were induced by oral exposure to DP in mice (Wu et al., 2012). Also in zebrafish, DP's oxidative damage potential has been demonstrated (Kang et al., 2016). However, in bluegill sunfish (*Lepmis macrochirus*) no acute toxicological effects were found at a concentration of 100 mg/L (Review: Feo et al. 2012). In rats, there were no effects at a high-exposure dosage of 500 mg/kg per day (Brock et al., 2010). No effects of DP on vitality were found on pipping success in chicken embryos up to exposure levels of 500 ng/g egg (Crump et al., 2011). Similar to TDICPP, a study has shown that DP might lead to alterations in the metabolic and xenobiotic activities of metabolizing enzymes (CYP3A) in quails, which might contribute to toxic effects of DP (Li et al. 2013).

It would have been useful to be able to investigate the thyroid hormone levels in the DP exposed quails in the current study, especially since no experimental studies on the endocrine disrupting potential of DP have been reported (Kang et al., 2016). In addition, it would have been interesting to look at possible effects on hormonal and genetic levels in the quails exposed to the mixture, since a thyroid disrupting potential already has been reported for TDICPP, and also since both flame retardants seem to have an effect on metabolic enzymes.

4 Conclusions

The present study suggests that both environmental levels, and100 times higher levels, of both DP and TDCIPP are unlikely to cause effects on embryonal development and post-hatch growth in Japanese quails. However, other studies have shown effects on levels much higher than environmental levels, indicating toxic potential of the flame retardants investigated in this study. Based on the results from other studies the concentrations used in the present study could possibly lead to disruptions on genetic and hormonal levels, which ultimately could lead to effects on growth and development. Based on the fact that these flame retardants are increasingly and frequently used in industries, and also ubiquitous in the environment and biota,

the potential effects need to be further investigated, in particular in complex mixtures with other contaminants.

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Appendix A1

Oppdrett Fjør 1 Mais[1]

Blandingsnr 331716505 Varenr 11353

FULLFÔR

TIL OPPDRETTSFÔR FJØRFE.

ANALYTISK INNHOLD

Råprotein	21.9%	Kalsium	1.00%
Trevler	7.3%	Fosfor	0.81%
Råfett	7.4%	Natrium	0.16%
Råaske	6.5%	Lysin	1.07%
Selen	0.40 mg/kg	Methionin	0.34%

INGREDIENSER

Mais økologisk, Åkerbønner økologisk, Solsikke ekspeller økologisk, Soya økologisk, Rapsfrø økologisk, Maisgluten, Monokalsiumfosfat, Potetprotein, Kalksteinsmel, Natriumbikarbonat, Salt

TILSETNINGSSTOFFER (PER KG)

Vitaminer:

E672 Vitamin A 10000 ie, E671 Vitamin D3 4500 ie, Vitamin E 80 mg

Mikromineraler:

E1 Jern som jern (II) fumarat 53 mg, E2 Jod som kalsiumjodat 1.1 mg, E4 Kopper som kopper (II) sulfat 15 mg, E5 Mangan som mangansulfat 128 mg, E6 Sink som sinksulfat 83 mg, E8 Selen som natriumselenitt 0.36 mg

Enzymer:

E1602 Endo-1,4 -betaglukanase EC 3.2.1.4 800 U, E1602 Endo-1,3 (4)-betaglukanase EC 3.2.1.6 1800 U, E1602 Endo-1,4 -betaxylanase EC 3.2.1.8 2600 U

BRUKSANVISNING

Landbruksopprinnelse i tørrstoff: 86%

Økologisk tørrstoff andel: 95% Økologisk oppdrettsfôr til slaktekylling med moderat proteininnhold. Blandingen inneholder minst 95% økologiske råvarer på tørrstoffbasis og kan benyttes i økologisk produksjon i samsvar med økologiforskriften. Natura Oppdrett Fjør 1 Mais er godkjent av Debio for bruk i økologisk drift. Inneholder kolinklorid. Drikkevann med kolinklorid unngås.

Holdbarhet: BULK, se best før dato i utleveringsseddel.
Holdbarhet: SEKK, se best før dato trykt på sekk. Nettovekt: Se utleveringsseddel.

ANSVARLIG Godkjenningsnr.

FELLESKJØPET AGRI NO10050272

Flyporten

2060 Gardermoen

Appendix A2

FJØR Oppdrett Kraft

Blandingsnr 308215501 Varenr 12507 **TIL OPPDRETT AV**

KOMBINASJONSRASER/KJØTTFULLE FJØRFERASER.

ANALYTISK INNHOLD

Råprotein	23.0%	Kalsium	0.73%
Trevler	4.8%	Fosfor	0.55%
Råfett	7.8%	Natrium	0.17%
Råaske	4.9%	Lysin	1.24%
		Methionin	0.54%
Selen	0.40 mg/kg		

INGREDIENSER

Havre, Hvete, Soya ekstrahert, Maisgrits, Maisgluten, Fiskemel, Vegetabilsk fett, Animalsk fett, Kalksteinsmel, Monokalsiumfosfat, Aminosyrepremiks, Natriumbikarbonat, Vitaminpremiks, Smakspremiks

TILSETNINGSSTOFFER (PER KG)

Vitaminer:

E672 Vitamin A 10000 ie, E671 Vitamin D3 4950 ie, Vitamin E 120 mg

Mikromineraler:

E1 Jern som jern (II) fumarat 53 mg, E2 Jod som kalsiumjodat 1.1 mg, E4 Kopper som kopper (II) sulfat 15 mg, E5 Mangan som mangansulfat 128 mg, E6 Sink som sinksulfat 83 mg, E8 Selen som natriumselenitt 0.22 mg

Enzymer:

4a1640 6-fytase EC 3.1.3.26 500 FTU, E1641 Endo-1,4 -betaxylanase EC 3.2.1.8 70 AXC, E1634 Endo-1,3 (4)-betaglukanase EC 3.2.1.6 100 AGL

BRUKSANVISNING

Fjør Oppdrett Kraft er et kraftig og proteinrikt vekstfôr som gir en god start i livet til for eksempel kalkuner og vaktler. Inneholder fiskeprodukter, forbudt å bruke til drøvtyggere.

Holdbarhet: BULK, se best før dato i utleveringsseddel.
Holdbarhet: SEKK, se best før dato trykt på sekk. Nettovekt: Se utleveringsseddel.

ANSVARLIG Godkjenningsnr.

FELLESKJØPET AGRI alfa NO10050160
Depotgata 22
2000 Lillestrøm

Felleskjøpet

Appendix B

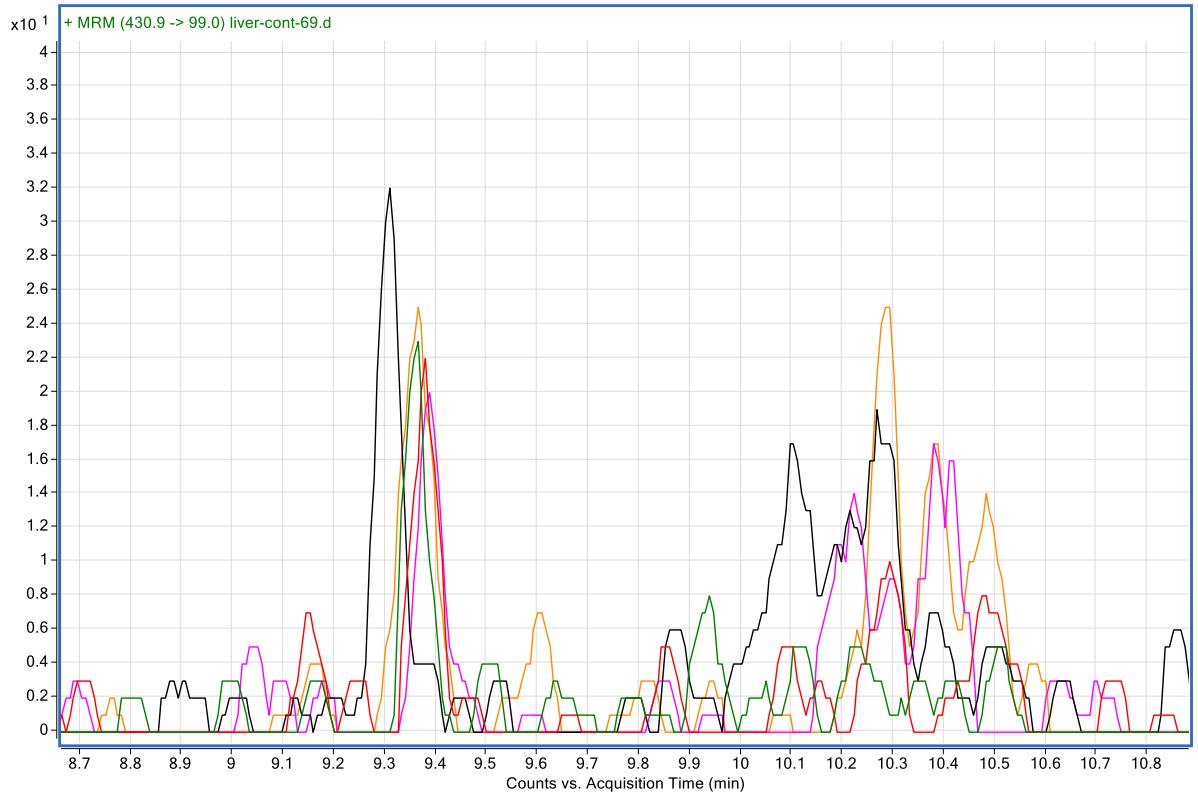

Figure B1. The figure shows a chromatogram from the GC-MS analysis of TDICPP and BDICPP in livers from Japanese quails (*Coturnix japonica*) exposed *in ovo* to high doses of either tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) or Dechlorane Plus (DP), or a mixture of both. A blank sample was analyzed together with the liver samples.

Appendix C

Table C1. A summary of the quantities of compounds detected in the original and the new food used in a study on Japanese quail (*Coturnix japonica*) injected *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of either Dechlorane Plus (DP) or tris(1,3-dichloroisopropyl)phosphate (TDCIPP), or a 1:1 mixture of both.

Compound	Original food	New food	Compound	Original food	New food
CB 28	<0.02	<0.02	**OxC**	<0.01	<0.01
CB 49	<0.02	<0.02	**TN**	<0.01	0.02
CB 52	<0.02	<0.02	**CN**	<0.01	0.01
CB 74	<0.02	<0.02	**∑CHLs**	<0.01	0.03
CB 95	<0.01	<0.01			
CB 99	<0.01	<0.01	***α*-HCH**	0.02	<0.01
CB 101	<0.01	0.05	***β*-HCH**	0.02	<0.01
CB 105	<0.01	0.01	***γ*-HCH**	0.05	0.03
CB 118	0.01	0.02	**∑HCHs**	0.09	0.03
CB 138	0.01	0.01			
CB 153	0.01	0.04	**BDE 28**	<0.01	<0.01
CB 156	<0.01	<0.01	**BDE 47**	0.01	0.02
CB 170	0.01	<0.01	**BDE 100**	<0.01	0.01
CB 171	<0.01	<0.01	**BDE 99**	0.01	0.01
CB 177	<0.01	<0.01	**BDE 154**	<0.01	<0.01
CB 180	<0.01	0.01	**BDE 153**	<0.01	<0.01
CB 183	<0.01	<0.01	**BDE 183**	<0.01	<0.01
CB 187	<0.01	<0.01	**∑PBDEs**	0.02	0.05
CB 199	<0.01	<0.01			
CB 194	<0.01	<0.01	**BEH-TEBP**	<0.05	<0.05
CB 196/203	<0.01	<0.01	**BTBPE**	<0.01	<0.01
CB 206	<0.01	<0.01	**EH-TBB**	<0.02	<0.02
CB 209	<0.01	<0.01	**∑nBFRs**	<0.05	<0.05
∑PCBs	0.03	0.16			
			***syn*-DP**	<0.01	<0.01
***p,p'*-DDE**	<0.01	0.21	***anti*-DP**	0.01	0.01
***p,p'*-DDT**	0.02	0.03	**∑DPs**	0.01	0.01
∑DDXs	0.02	0.24			
			BDCIPP	< 4.00	< 4.00
HCB	0.01	0.03	**TDCIPP**	< 2.00	3.09

Appendix D

Table D1. An overview of the hatching success (%) in different treatment groups of Japanese quail (*Coturnix* japonica) exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of either Dechlorane Plus (DP) or tris(1,3-dichloroisopropyl)phosphate (TDCIPP), or a 1:1 mixture of both. In addition there were two controls; non-injected control (C-NI) and a control injected with emulsion only (C-I). The hatching success was calculated by dividing the number of hatched eggs on the number of injected eggs.

Treatment	Injected	Hatched	Not hatched	Hatching success (%)
C-I	21	10	11	48
C-NI	19	12	7	63
DP LD	20	11	9	55
DP MD	31	9	22	29
DP HD	22	11	11	50
TDCIPP LD	30	13	17	43
TDCIPP MD	31	11	20	35
TDCIPP HD	30	9	21	30
Mix LD	28	9	19	32
Mix MD	30	10	20	33
Mix HD	30	13	17	43
Overall	293	118	175	40

Appendix E

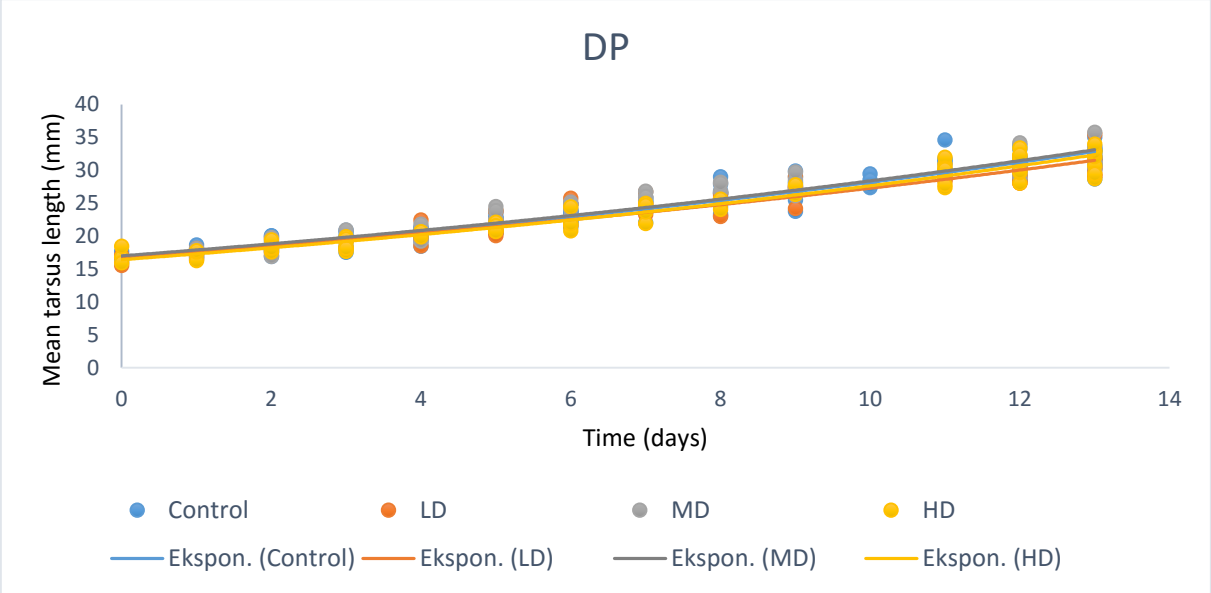

Figure E1. Mean tarsus length (mm) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) of Dechlorane Plus (DP). Injected and non-injected control groups are pooled together based on no significant difference in tarsus length between the two. The curves are fitted with exponential lines.

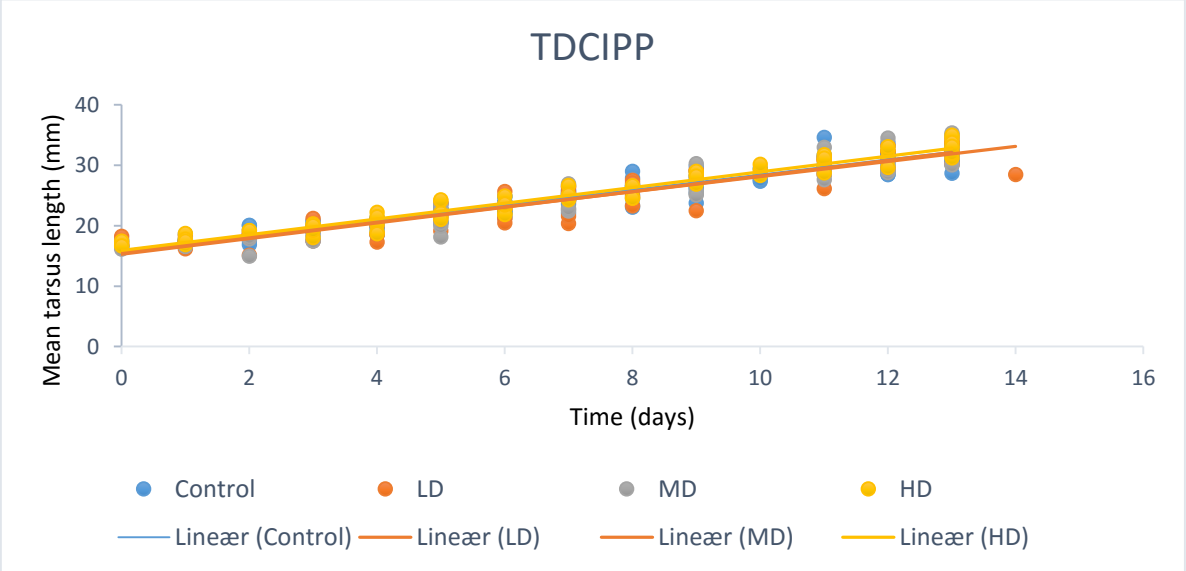

Figure E2. Mean tarsus length (mm) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) of tris(1,3-dichloro-2-propyl)phosphate (TDCIPP). Injected and non-injected control groups are pooled together based on no significant difference in tarsus length between the two. The curves are fitted with exponential lines.

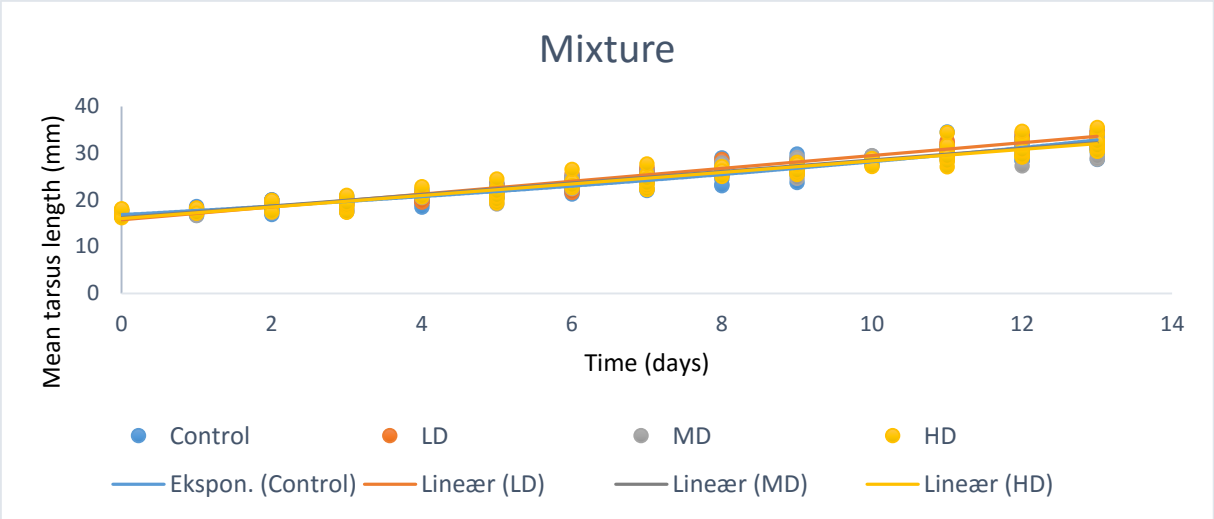

Figure E3. Mean tarsus length (mm) measured in Japanese quails (*Coturnix japonica*) from newly hatched chicks until 14 days of age. Individuals were exposed *in ovo* to low, medium and high dose (LD, MD, HD, respectively) to a mixture of Dechlorane Plus (DP) and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP). Injected and non-injected control groups are pooled together based on no significant difference in tarsus length between the two. The curves are fitted with exponential lines.

Appendix F

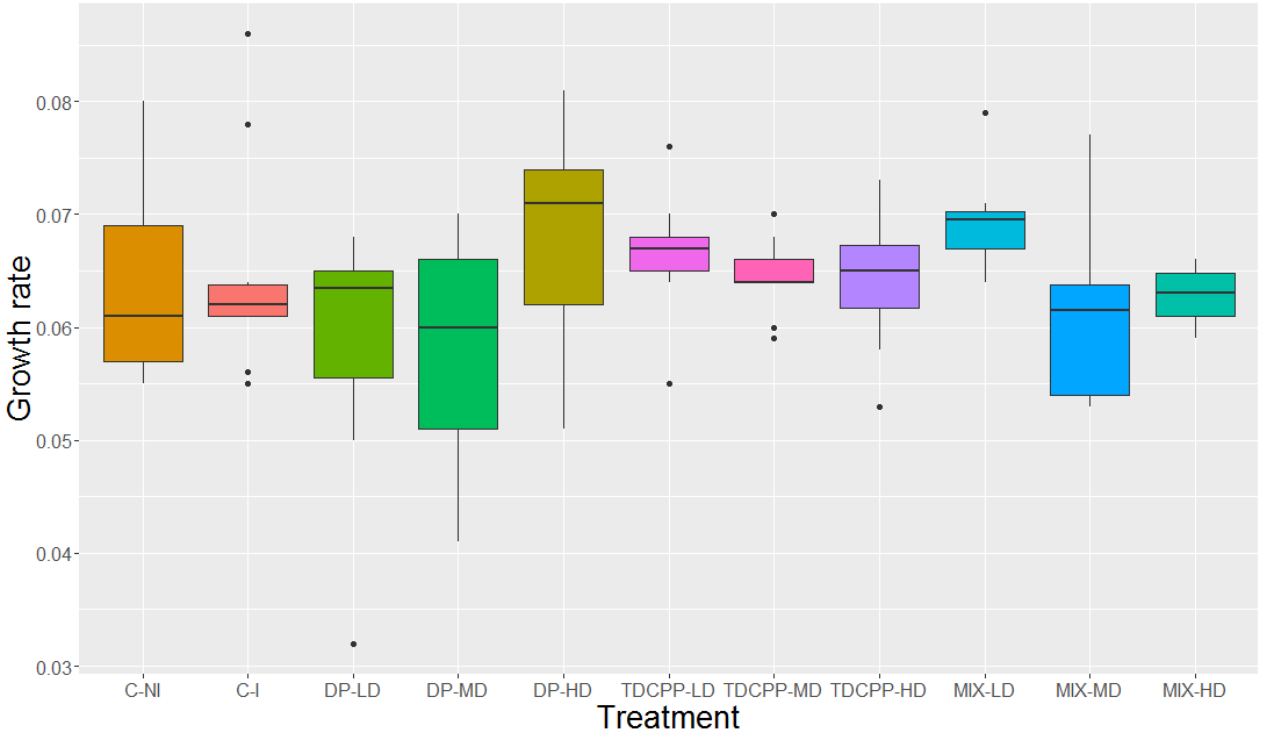

Figure F1. Growth rate (GR) in different treatment groups of Japanese quail (*Coturnix japonica*) exposed *in ovo* to low, medium and high doses (LD, MD, HD, respectively) of Dechlorane Plus (DP) and Tris(1,3-dichloroisopropyl)phosphate, independently and in a 1:1 mixture. In addition there were two controls; non-injected control (C-NI) and a control injected with emulsion only (C-I). The median within each treatment are marked as a thicker line going across the boxes. The bottom and top line of the box represent the 25th and 75th percentiles, respectively (i.e. the location of the middle 50% of the data, also called the first and third quartiles). For the boxes with outliers, the upper whiskers are the largest data point less than 1.5 times the first and third quartile. The outliers in this case are points more than 1.5 times the interquartile ranges above and below the first and third quartile. Thus, when there are no outliers the whiskers simply show the minimum and maximum values individually. Asymmetry in the size of the upper and lower parts of the box indicates skewness in the data.