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## Hatching failure and accumulation of organic pollutants through the terrestrial food web of a declining songbird in Western Europe

H. Herman van Oosten<sup>a,b,c,\*</sup>, Arnold B. van den Burg<sup>d</sup>, Debora Arlt<sup>e</sup>, Christiaan Both<sup>f</sup>, Nico W. van den Brink<sup>g</sup>, Suzanne Chiu<sup>h</sup>, Doug Crump<sup>h</sup>, Tobias Jeppsson<sup>e,i</sup>, Hans de Kroon<sup>b</sup>, Wim Traag<sup>j</sup>, Henk Siepel<sup>b</sup>

<sup>a</sup> Bargerveen Foundation, Toernooiveld 1, Postbox 9010, 6500 GL Nijmegen, the Netherlands

<sup>b</sup> Institute for Water and Wetland Research, Animal Ecology and Physiology & Experimental Plant Ecology, Radboud University, PO Box 9100, 6500 GL Nijmegen, the Netherlands

<sup>c</sup> Oenanthe Ecologie, Hollandseweg 42, 6706 KR Wageningen, the Netherlands

<sup>d</sup> Biosphere Science Foundation, Onderlangs 17, 6731 BK Otterlo, the Netherlands

<sup>e</sup> Department of Ecology, Swedish University of Agricultural Science, Box 7044, 75007 Uppsala, Sweden

<sup>f</sup> Center for Ecological and Evolutionary Studies, University of Groningen, Nijenborgh 7, Groningen 9747 AG, the Netherlands

<sup>g</sup> Department of Toxicology, Wageningen University, Postbox 8000, 6700 EA Wageningen, the Netherlands

<sup>h</sup> Environment and Climate Change Canada, National Wildlife Research Centre, Ottawa, Ontario K1A 0H3, Canada

<sup>i</sup> CEES, Department of Biosciences, University of Oslo, Postbox 1066, Blindern, NO-0316 Oslo, Norway

<sup>j</sup> RIKILT Wageningen UR, Akkermaalsbos 2, 6708 WB Wageningen, the Netherlands

### HIGHLIGHTS

- Many migratory songbirds decline without reasons being known.
- Egg failure in Northern Wheatears high locally in Europe, but why
- We measure different toxins as dioxin-like compounds in the foodweb and determine receptor-type.
- Despite clear accumulation, DLCs not only cause of egg failure since species is not very sensitive.
- Egg failure and embryo malformation mystery: other toxins, nutrient deficiency or inbreeding

### GRAPHICAL ABSTRACT



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### ABSTRACT

Population growth in passerine birds is largely driven by fecundity. If fecundity is affected, for instance by hatching failure, populations may decline. We noted high hatching failure of up to 27% per year in relict populations of the Northern wheatear (*Oenanthe oenanthe*) in The Netherlands, a strongly declining, migratory passerine in Europe. This hatching failure itself can cause population decline, irrespective of other adverse factors. Additionally, we investigated the cause of hatching failure. Unhatched eggs showed egg yolk infections or embryonic malformations, part of which is associated with the actions of dioxin-like compounds (DLCs). Indeed, DLCs appear to bioaccumulate in the local foodweb, where the soil contained only background concentrations, similar to those found at many other locations. DLC concentrations in Dutch eggs were six-fold higher than those in a reference population in Sweden, where egg failure was only 6%. However, Northern wheatears appear to be only moderately sensitive to the actions of DLCs, because of their specific Ah-receptor type which may moderate the receptor mediated effects of DLCs. This indicates that the concentrations of DLCs, although elevated, may

\* Corresponding author at: Bargerveen Foundation, Toernooiveld 1, Postbox 9010, 6500 GL Nijmegen, the Netherlands.

E-mail address: [herman\\_vanoosten@yahoo.co.uk](mailto:herman_vanoosten@yahoo.co.uk) (H.H. van Oosten).

## 1. Introduction

Population-dynamics of short-lived passerines are to a large extent driven by fecundity (Arcese and Smith, 1988; Saether and Bakke, 2000), which may be affected by different factors. For instance, songbirds may be less inclined to produce a second brood in times of food shortage, resulting in a lower number of offspring (Nagy and Holmes, 2005; White, 2008; Seward et al., 2013). Fecundity may also be affected more directly by predation of nestlings or of breeding females (Martin, 1995; Pärt, 2001; Low et al., 2010). Another important factor which potentially influences population growth in a negative way is hatching failure. This occurs in many species and with varying intensity in different populations. It has been estimated that on average for all bird species about 10% of eggs produced do not hatch (Koenig, 1982; Spottiswoode and Møller, 2004). Causation is not often clear and unhatched eggs are typically dismissed as being infertile, although eggs without any visual signs of embryonic development may, in fact, have been fertilized after all (Birkhead et al., 2008). Hatching failure is also regularly attributed to inbreeding (Van Noordwijk and Scharloo, 1981; Bensch et al., 1994; Keller, 1998) or to pollutants, most famously perhaps the organochlorine insecticides DDT and aldrin (Newton, 1998).

One of the most strongly declining songbirds in Western Europe is the Northern wheatear (*Oenanthe oenanthe*) (Gregory et al., 2009; PECBMS, 2013). This insectivorous migrant was widespread until the 1980s but over large parts of Western Europe it nowadays occurs only very locally, probably mainly as a result of adverse changes in breeding habitat over large spatial scales (Glutz von Blotzheim and Bauer, 1988; Arlt et al., 2008). With decreasing population size, the synchrony in population dynamics may decrease because of increasing demographic stochasticity (Koenig, 2002; Sæther et al., 2007, 2011). Therefore, factors driving population growth in endangered species, which occur in small and isolated populations, may be affected differentially at local scales due to site-specific conditions (Van Oosten et al., 2015). In the case of the Northern wheatear, hatching failure may play an important role in population growth on a local scale. In The Netherlands, monitoring over six years revealed consistently high hatching failure of eggs in one of the remnant populations situated in a coastal nature reserve where on average 20% of eggs failed to hatch. However, despite the small population size and low population growth, genetic variation (heterozygosity) is still high in this population (Van Oosten et al., 2016), suggesting that inbreeding alone is not likely the main cause of the observed high hatching failure. Although the extent to which heterozygosity, based on markers and as proxy for individual inbreeding coefficients (rather than pedigree-based estimates inbreeding coefficients), is debated (Chapman et al., 2009; Szulkin et al., 2010; Taylor et al., 2010), it seems likely that other factors may be involved in the decreased hatchability of the eggs.

Preliminary work in the above mentioned and another remnant breeding population in the Netherlands demonstrated the presence of embryonic malformations in the unhatched eggs of Northern wheatears, which might potentially be caused by exposure to organic pollutants as dioxin-like compounds (DLCs) as described in other studies (Gilbertson, 1983; Brunström and Andersson, 1988; Grasman et al., 1998). In this study we 1) evaluated to what extent low hatching rates may affect population growth; 2) quantified which embryo anomalies are present in the population; 3) analysed organic pollutants in eggs; 4) investigated their bioaccumulation from soil to birds; and 5) determined the relative sensitivity of Northern wheatears to DLCs based on

the amino acid sequence of the aryl-hydrocarbon (Ah)-receptor (AhR1) ligand-binding domain (LBD) (Farmahin et al., 2013). Most, if not all, of the toxic effects of DLCs are mediated by the Ah-receptor (Denison et al., 2011). Finally, we discuss how likely pollutants are causal to hatching failure in the Northern wheatear.

## 2. Methods

### 2.1. Study species and study sites

The Northern wheatear is an insectivorous long-distance migrant, which breeds from Eastern Canada and Greenland across Eurasia to Western Alaska and overwinters in the African Sahel (Glutz von Blotzheim and Bauer, 1988). Northern wheatears occur in open habitats where they mainly forage by running on the ground for which they need arthropod-rich, short vegetation. The European population has declined by over 50% since 1990 (PECBMS, 2013). In the Netherlands, population numbers have plummeted from 1900–2500 breeding pairs in 1980 to <300 in 2012 (Boele et al., 2014), most likely due to declining rabbit (*Oryctolagus cuniculus*) populations and increased atmospheric nitrogen deposition which both change species-rich short-grown and open grasslands into mono-specific stands of tall grasses, (Bobbink et al., 2010), and hence reduce the amount of suitable habitat and additionally reduce nest site (rabbit burrows) availability. Small and isolated populations of a few dozen breeding pairs are today limited to heathlands and oligotrophic grasslands in coastal dunes, where they breed in abandoned burrows of rabbits or in decaying stumps of felled trees.

Since 2007 we have been studying the breeding ecology and population dynamics of two remaining Dutch populations of the Northern wheatear. One studied population inhabits a coastal dune area (Vogelduin, Noord-Hollands Dune Reserve, NHD 52° 55' N 06° 55' E). The second population is situated 120 km inland (Aekingerzand 52° 33' N 04° 36' E). Both areas have been designated Natura2000 sites, i.e. are part of the pan-European network of protected nature reserves. For selected comparisons, we use reference data from a population of Northern wheatears in Sweden near Uppsala where birds inhabit pastures, crop fields, farmyards and unmanaged grasslands (Arlt et al., 2008). We anticipated lower DLC pollution in Sweden, as Sweden is less industrial, and DLC concentrations in locally produced foods for human consumption (butter) are lower in Sweden than in the Netherlands (Weiss et al., 2005).

### 2.2. Effects of egg hatchability on population growth

To evaluate the potential effects of the difference in hatching success between the Dutch Vogelduin population and the Swedish Uppsala reference population on population growth rate ( $\lambda$ ), we used a simple deterministic unstructured, single-sex population model, based on the female population. The model was defined as:

$$N_{t+1} = N_t(F + S) = N_t\lambda$$

$$F = p_{\text{breeding}} \times \frac{\text{clutch size}}{2} \times \text{hatching rate} \times \text{survival}_{1y}$$

with  $F$  = net reproduction,  $S$  = survival<sub>female</sub>. We calculated  $\lambda$  for two scenarios, (1) 'affected' using the 80% hatching rate from Vogelduin (Van Oosten, 2016) and (2) 'reference' using the reference 94% hatching rate from Uppsala (Arlt, unpublished results). All the other parameters characterised the Dutch Vogelduin population (estimated using an

integrated population model following Van Oosten et al., 2015) and were identical between the two scenarios, namely:

$$\text{Probability of breeding } (p_{\text{breeding}}) = 1$$

Clutch size = 4.13

$$\text{First-year survival } (\text{survival}_{1y}) = 0.315$$

$$\text{Adult female survival} = 0.46$$

Note that the purpose of this population model is only to evaluate the effects of hatching success on potential population growth based on the current demography of the Vogelduin population. This model is not meant to provide accurate projections of the Vogelduin population, since it ignores various factors, such as density dependence as the population size increases, variability in vital rates between years and immigration into the population.

### 2.3. Hatching failure and embryonic anomalies

We collected detailed demographic data: each year from 2008 to 2013 we monitored 95–100% of all nests at Vogelduin ( $n = 105$  nests) and Aekingerzand ( $n = 129$  nests; Table A.2). Eggs that did not hatch in an otherwise successful nest were counted as failed. This procedure excludes nests where females were predated upon, or nests that were disturbed by other external causes (e.g. burrow collapse). These procedures were similar for the Swedish reference population, using data on hatching failure for 2008–2013. In the two Dutch populations, when the nestlings were ringed around day 8, eggs which failed to hatch were collected and stored at 4 °C. Eggs were later opened and visually inspected. In all failed eggs, which were not decayed too much to establish conclusive results, anomalies were observed. Another cause reducing breeding success was occasional mortality of hatched nestlings without any externally visible trauma. Due to the time-lag between time of death and time of collection, the rapid onset of decay of organs prevented conclusive post-mortem autopsy. After three years of repeated observations we were able to discern a priori which nestlings were dying, since they were transferred outside the nest by the parents. We collected  $n = 4$  dying nestlings from  $n = 3$  females in 2011 and  $n = 1$  dying nestling in 2013 for subsequent fresh autopsy and histological study of their livers. To compare the histology of the necrotic Northern wheatear livers to healthy livers, we opted to use an apparently healthy juvenile Eurasian blackbird (*Turdus merula*), a common breeding bird in The Netherlands (Hustings and Vergeer, 2002). This individual was freshly collected as prey of Northern sparrowhawk (*Accipiter nisus*), which one of the authors studies closely. Since liver function, anatomy and structure are similar among bird species (Hickey and Elias, 1954; Romer and Parsons, 1986), comparison of livers of two songbirds within the same family is valid, and allowed us not to euthanize a healthy nestling of a Dutch red-list bird species. Comparable data on embryonic anomalies or autopsy of dying nestlings are not available for the Swedish reference population. Nevertheless, contents of unhatched eggs were normally checked and recorded (whether any embryo was visible and its developmental stage). While there was no detailed search for

malformations like the externally visible growth abnormalities mentioned in Table 1, no obvious malformations were ever noticed.

### 2.4. Contaminant analyses: dioxins, PCBs and pesticides

To test whether dioxins, polychlorinated biphenyls (PCBs) and organochlorinated pesticides (OCPs) could be involved in causing the hatching failures and embryo and liver anomalies, we collected egg and nestling samples from different individual females. In the two Dutch populations, we collected  $n = 35$  eggs from  $n = 29$  females. Of these eggs,  $n = 8$  from  $n = 6$  females were collected as freshly laid eggs and  $n = 27$  from  $n = 23$  females were collected as failed eggs. We collected a further 3 failed eggs and nestlings of  $n = 3$  females from the Swedish reference population. Concentrations of dioxins and PCBs were determined by applying WHO-TEFs (World Health Organisation Toxic Equivalent Factors; Van den Berg et al., 1998). Concentrations are reported as 2, 3, 7, 8 – tetrachlorodibenzo *p* dioxin equivalents of dioxins, non-ortho and mono-ortho PCBs (WHO-PCDD/F-PCB-TEQ).

Pesticides, such as DDT, are also known to be deleterious to avian wildlife and their mode of action may be similar to dioxins and PCBs (Fry, 1995). Therefore, the reported detrimental effects may be caused by pesticides because DDT and derivatives are still used in the African wintering areas. We therefore also determined the concentration of OCPs belonging to the ‘dirty dozen’ (Stockholm Convention; UNEP, 2008) in eggs and nestlings from Vogelduin, Aekingerzand ( $n = 5$  eggs each) and the Swedish reference site Uppsala ( $n = 10$  nestlings).

Upon receipt, the samples were all stored at  $-20$  °C. After thawing, the contents of the eggs without the shells were thoroughly homogenized and a homogeneous (sub) sample was taken for analysis of dioxins, PCBs and OCPs. Mass labelled internal standards were spiked to the sub-sample. The lipids and contaminants were extracted from the samples by the Smedes method (Smedes, 1999). This method allows extraction of the non-bound lipids (triglycerides) and the bound lipids (e.g. phospholipids). The latter are particularly relevant in the case of non-fatty biota and soil sample matrices, as was the case in current study. After extraction of the samples, the lipid yield was determined and lipids were dissolved in a 1/1 (v/v) mixture of ethylacetate/cyclohexane (15 ml) of which 12.5 ml was injected on a gel permeation chromatography (GPC) system for lipid removal. The fraction containing the dioxins, PCBs and OCPs was collected and the solvent was reduced to 1 ml. Some extracts were cleaned through lipid removal using  $\text{Al}_2\text{O}_3$  column chromatography and afterwards the volume was reduced to 1 ml. Fifty microliter out of 1 ml extract was taken for analysis of OCPs by comprehensive multidimensional gas chromatography coupled to mass spectrometry (GC  $\times$  GC-MS). The details of the applied method can be found elsewhere (Van der Lee et al., 2008). The dioxins, dioxin-like (dl-) PCBs and the non-dioxin-like (ndl-) PCBs were determined by GC coupled to high resolution mass spectrometry (HRMS). The details of this method are published elsewhere (Van Leeuwen et al., 2009; Uçar et al., 2011). The quality of the applied methods is controlled by analysis of blank samples, the use of (mass labelled) internal standards, recovery experiments and the participation in proficiency tests. The detection limit for dioxins and PCBs was  $0.10 \text{ pg g}^{-1}$ . RIKILT is ISO 17025 accredited for the analysis of dioxins and PCBs in biota and soil samples and RIKILT performed the analyses. Table A.1 provides

**Table 1**

Overview of the number of embryonic anomalies and egg yolk infections found in unhatched Dutch Northern wheatear eggs from two populations (Vogelduin, Aekingerzand) inspected between 2008 and 2013. Of all eggs collected ( $n = 156$ ), 34% were decayed too much to establish conclusive results. All other eggs were aberrant with defined embryonic anomalies. Eggs often contain multiple anomalies. Fluorescence microscopy showed that the yolk membrane of all inspected eggs with egg yolk infections contained sperm, indicating that all eggs were fertilized.

	Amnion feather growth	Egg yolk infection	Curled toes/crossed bill/coelosoma/microphtalmia/no eyes	Heart deformities/double heart	Other morphological anomalies	Decayed
Vogelduin ( $n = 118$ )	9	30	15	4	36	41
Aekingerzand ( $n = 38$ )	0	7	7	0	15	12

information on which dioxins, PCBs and organochlorine pesticides were analysed.

### 2.5. Local uptake in food chain: from soil to arthropod to egg

To investigate how DLCs bioaccumulate in the food chain on the Dutch breeding grounds we analysed soil and arthropod (Northern wheatear prey) samples. In both Vogelduin and Aekingerzand we collected  $n = 3$  soil samples, each of which consisted of 4 subsamples taken in a  $2 \times 2 \text{ m}^2$  and containing all soil between 0 and 10 cm depth. Standing crop, mosses and other debris were removed, and the samples were dried in a stove at  $70 \text{ }^\circ\text{C}$  for 48 h. Prey samples were collected only in Vogelduin since we have detailed knowledge regarding the local nestling diet for this population (Van Oosten et al., 2014; Van Oosten, 2016). Of the main prey Orthoptera: Acrididae (imagos), Lepidoptera (larvae), Coleoptera: *Phyllopertha horticola* (imagos) and Elateridae (larvae) we collected  $n = 3$  samples each consisting of at least 10 individuals collected in territories of Northern wheatears. Animals were stored at  $-17 \text{ }^\circ\text{C}$ . Concentrations of dioxins and PCBs were determined by HRMS (as above) and final concentrations were corrected by applying WHO-TEFs.

### 2.6. Ah-receptor type characterization

DLC sensitivity varies up to 1000-fold and is determined by the type of aryl hydrocarbon receptor (Ah-receptor; Brunström, 1988; Hoffman et al., 1998; Head et al., 2008). The Ah receptor can be characterised by sequencing of the aryl hydrocarbon receptor 1 ligand-binding domain (AhR1 LBD).

#### 2.6.1. Source of northern wheatear sample for AHR1 LBD sequencing

The Northern wheatear liver was obtained from a collected, runt chick much behind in development compared to fellow nestlings. The specimen was collected in accordance with Dutch law, permit FF/75A/2015/004. The liver sample was collected with solvent-rinsed utensils, preserved in a  $-80 \text{ }^\circ\text{C}$  freezer at Wageningen University (Wageningen, The Netherlands).

#### 2.6.2. AHR1 LBD sequencing

Total RNA was isolated from liver ( $\sim 10 \text{ mg}$ ) using RNeasy Mini kits according to the manufacturer's instructions (Qiagen, Toronto, ON). RNA was transported on dry ice to the National Wildlife Research Centre (Ottawa, Ontario) for AHR1 LBD sequencing. The cDNA samples were PCR amplified with forward primer 5' CCAGACCAACTTCTCCAGA 3' and reverse primer 5' CACCAGTGGCAAACATGAAG 3', for 35 cycles using  $95 \text{ }^\circ\text{C}$  for 30 s,  $60 \text{ }^\circ\text{C}$  for 30 s, and  $72 \text{ }^\circ\text{C}$  for 45 s (Head et al., 2008; Head and Kennedy, 2010; Farmahin et al., 2013). The E-Gel iBase system and 0.8% SYBR Safe precast agarose gels (Invitrogen, Burlington, ON) were used to separate PCR products for sequencing on an Applied Biosystems 3730 DNA Analyzer at the Ottawa Hospital Research Institute (OHRI, Ottawa, ON). Detailed analysis of each chromatogram and translation from nucleotide to amino acid sequence were performed using Sequencher version 4.9 software (Gene Codes Corporation, Ann Arbor, MI). The AHR1 LBD sequences corresponded to amino acid residues 235–402 in chicken.

## 3. Results and discussion

### 3.1. Hatching failure and population growth

Detailed monitoring over 6 years (2008–2013) revealed consistently high hatching failure of Northern wheatears in Vogelduin (average 20%/year; range 11–27%) and Aekingerzand (average 14%/year; range 9–18%) ( $n = 230$ ; Table A.2). These are significantly higher values compared to the reference population breeding near Uppsala, Sweden, where 6% (range 4–9% per year, 2008–2013,  $n = 54$ ; Table A.2) of

eggs failed [generalized linear mixed model (GLMM) with a binomial distribution, a logit link function, and year as random factor: Vogelduin versus Uppsala  $Z = 6.35$ ,  $P < 0.001$ ; Aekingerzand versus Uppsala  $Z = 4.23$ ,  $P < 0.001$ ;  $n = 355$ ].

In Germany, between 3 and 5% of eggs failed to hatch in several adjacent populations of the Northern wheatear (Buchmann, 2001). In Sweden, hatching failure in the Uppsala control population at an earlier time period (1982–1985) was also low (5%) as was that in one other population at the island of Öland (8%) (Moreno, 1987). Those rates are similar to the 4–9% failure rates for the Swedish reference population in 2008–2013. There is one other report of relatively high failure rate comparable to the Dutch populations; 20% of eggs failed to hatch in one population in the UK of which 89% contained dead early embryos, which were arrested in their development (Currie et al., 1998). Unfortunately, no information on the occurrence of embryonic malformations, or on possible causes for the hatching failures was provided in that study. In other Northern Wheatear studies it remained unclear how hatching failure was determined, which makes it impossible to compare the data (Conder, 1989).

High hatching failure is often reported for island species or highly endangered species (Briskie and Mackintosh, 2004). For not particularly inbred populations on the mainland hatching failure is less prevalent. However, occasional high degrees of hatching failure have been reported, as summarized by Spottiswoode and Møller (2004, and references therein), although the cause for hatching failure in those studies remains unknown. For example, up to 19% of eggs failed to hatch in House Finches (*Carpodacus mexicanus*), and 19.3% in the equally granivorous corn bunting (*Emberiza calandra*). Two insectivorous passerines were prone to high hatching failure: willow tit (*Parus montanus*) with 17.5% hatching failure and the Eastern bluebird (*Sialia sialis*) with 17%. Spottiswoode and Møller (2004) report 10.9% as the average hatching failure in 99 examined species. Thus, the examined populations of Dutch Northern wheatears show high, but not exceptional degrees of hatching failure. Nevertheless, as the Northern wheatear is a strongly declining songbird, elucidating the causes of hatching failure is of importance from a conservation point of view.

### 3.2. Effects of egg hatchability on population growth

Population growth in the 'affected' scenario was negative with  $\lambda_{\text{affected}} = 0.98$  at Vogelduin, indicating a declining population. In contrast, the 'reference' scenario showed an increasing population with  $\lambda_{\text{reference}} = 1.07$ . If these two growth rates are used to deterministically project the current population of 17 breeding females (the average number of breeding females 2007–2011 in the Vogelduin population) over 10 years, the 'affected' scenario results in 14 breeding females while the 'reference' scenario results in 34 females, a strong increase. This simple simulation indicates that reduction of hatching success can be strong enough to change a growing population into a declining population, given the current values of demographic parameters of the Dutch Northern wheatear population.

### 3.3. Embryonic anomalies

All inspected failed eggs that were not decayed too much to establish conclusive results showed embryonic anomalies or egg yolk infections (Table 1; De Buck et al., 2004; Van den Burg, 2017). Visual inspection of dead embryos from the Dutch populations showed growth abnormalities, including crossed bills, curled toes, microphthalmia, underdeveloped extremities, shortened lower mandibles, mottled livers, cerebral haemorrhage, dilated veins, intestine loops outside the body, and feather growth on the inside of the amniotic sac (Table 1, Fig. A.1). Egg yolk infections (Fig. A.2) appear at first glance to be normal, fresh eggs with transparent and fluid egg white and a normal, globular egg yolk. However, whereas normal egg yolk flows out upon breaking the yolk membrane, in yolk infections the egg yolk is very

thick. Fluorescence microscopy showed that the yolk membrane of all inspected eggs with egg yolk infections contained sperm, indicating that all eggs were fertilized.

The variety of embryonic malformations seen in the current study may be related to exposure to DLCs, as similar effects have also been described in birds suffering dioxin toxicity (Gilbertson, 1983; Brunström and Andersson, 1988; Grasman et al., 1998). For instance, curled toes and crossed bills were reported in a variety of bird species because of dioxin toxicity (Gilbertson, 1983; Brunström and Andersson, 1988; Grasman et al., 1998). Further indication of potential risks on toxic effects in the Dutch populations was the observed mortality of nestlings without apparent external anomalies and without signs of starvation (18% of all nests in the period 2008–2013 had at least one dead nestling, range 7–43% of total number of chicks per year). Inspection of the internal organs of five dead nestlings revealed the presence of mottled livers due to necrosis in all nestlings (Fig. A.3). Histological examination showed large and pale swollen hepatocytes, and occasional highly vacuolated hepatocytes featuring pycnotic nuclei, a sign of cell death which leads to liver necrosis (Fig. A.4). Liver necrosis may be caused by PCBs and dioxins (Brooks et al., 1988; Gilbertson et al., 1991). All these observations may indicate the actions of pollutants, perhaps dioxin-like compound (DLCs) most prominently.

### 3.4. DLCs and organochlorine pesticides in eggs and the terrestrial foodweb

To assess the potential role of DLCs in the occurrence of the malformation in the failed eggs, DLC concentrations were analysed in failed eggs. Overall concentrations of DLCs in eggs from the Dutch populations showed significantly elevated levels, more than six times higher than those in Sweden (Kruskal-Wallis test,  $\chi^2 = 22.70$ ,  $P < 0.001$ ), whereas Dutch populations did not differ among each other (Table 2) (Mann-Whitney test,  $U = 135.00$ ,  $P = 0.686$ ).

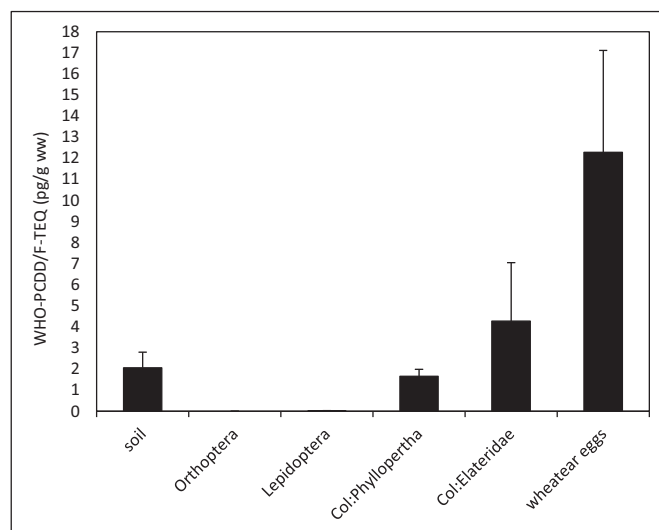
Most of the organochlorine pesticides occurred in low concentrations in eggs of Northern wheatears, according to EU standards (European Commission, 2008). Only pp'-DDE showed a slightly elevated concentration (Table A.3) but the concentration did not differ between the three sites (Kruskal-Wallis test,  $\chi^2 = 4.552$ ,  $P = 0.208$ ). pp'-DDE levels were similar to maximum allowed residue levels in consumer eggs in the European Union (European Commission, 2018). The observed low OCP concentrations led us to conclude that the effects were not caused by organochlorine pesticides.

Analysis of dioxin concentrations in the local food chain showed low concentrations in the soil (Table A.4) that were comparable to those in many other sites (Table A.5). However, as expected, the dioxins accumulated in some of the major prey species and concentrations were still higher in Northern wheatear eggs (Fig. 1). DLCs generally only accumulate to a limited extent in plants from the soil pore water. Therefore, arthropods feeding on above-ground foliage are not expected to contain elevated concentrations of DLCs, especially since the emission of dioxins in The Netherlands decreased strongly due to governmental regulations since 1990 (Bremmer et al., 1993). Indeed, aboveground herbivorous

**Table 2**

Concentrations of DLCs in eggs of Northern wheatears from three locations. Concentration values for dioxins (WHO-PCDD/F-TEQ) and PCBs (WHO-PCB-TEQ) are expressed in pg TEQ/g fat per egg per location (mean  $\pm$  standard deviation). Overall toxicity was estimated by applying WHO-TEFs (WHO-PCDD/F-PCB-TEQ) in pg TEQ/g fat. Number of eggs analysed and number of individual females: 'coastal Netherlands'  $n = 14$  eggs of  $n = 9$  females, 'inland Netherlands'  $n = 2$  eggs of  $n = 2$  females, 'Sweden'  $n = 3$  eggs of  $n = 3$  females. The three Swedish eggs were analysed together as one sample, which precludes standard deviation.

	Dioxins and furans (pg TEQ/g lw)	PCBs (pg TEQ/g lw)	WHO-TEFs (pg TEQ/g lw)
Coastal Netherlands	285 $\pm$ 109	71 $\pm$ 64	356 $\pm$ 127
Inland Netherlands	208 $\pm$ 79	267 $\pm$ 343	476 $\pm$ 421
Uppsala, Sweden	43	9	51



**Fig. 1.** Concentrations of dioxins (WHO-PCDD/F-TEQ in  $\text{pg g}^{-1}$  wet weight, mean  $\pm$  SD) in the food chain of Northern wheatears in the coastal Vogelduin population. Concentrations were below the detection limit of  $0.10 \text{ pg g}^{-1}$  in Orthoptera. Sample sizes are 3 for soil (pooled samples consisting of 4 subsamples each) and insects (pooled samples consisting each of at least 10 individual insects) and 21 for wheatear eggs.

prey (*Orthoptera* images, *Lepidoptera* larvae) only contained very low amounts of DLCs, whereas arthropods dwelling in the top-soil (Coleoptera: Elateridae larvae and *Phyllopertha* larvae) accumulated higher amounts (amounts of DLCs in aboveground herbivorous prey versus soil-dwelling prey, Mann-Whitney test,  $U < 0.001$ ,  $P = 0.002$ ). A consequence of the different amounts of pollutants in the different prey species is that the actual diet of a Northern wheatear determines the amounts of poison ingested. Unfortunately, there are not data available on the adult diet during egg formation. Nevertheless, it seems likely that soil dwelling arthropods form a relatively large part of the diet because of their availability in that period of the year.

### 3.5. Ah-receptor type characterization

Despite clear bioaccumulation of DLCs on the Dutch breeding site, concentrations in Northern wheatear eggs and chicks are relatively low, and below generally known threshold levels of embryonic malformations. However, differences in sensitivity to DLCs across many avian species have been observed and are associated with specific amino acid identities (sites 324 and 380) of the AHR1 LBD (Head et al., 2008; Farmahin et al., 2013). Three broad sensitivity classes were identified: type 1 or highly sensitive (Ile324\_Ser380), type 2 or moderately sensitive (Ile324\_Ala380), and type 3 or least sensitive (Val324\_Ala380) (Farmahin et al., 2013). These sensitivity classes refer to the affinity of DLCs to bind to the AHR1 and elicit downstream effects. The amino acid sequence determined for the Northern wheatear revealed that it has a type 2, subtype E AHR1, and therefore falls into the 'moderately sensitive' type in terms of the embryotoxic effects of DLCs (Fig. A.5.A and B; Farmahin et al., 2013).

Others bird species with a type 2 Ah-receptor are only affected at much higher concentrations of one of the DLCs (TCDD). The LOAEL (lowest-observed-adverse-effect level) of the Eastern Bluebird, for instance, is  $1000 \text{ pg/g egg}$  (Thiel et al., 1988), whereas we found only  $12 \text{ pg/g egg}$  for total WHO-PCDD/F-TEQ. In addition, LD<sub>50</sub> values of TCDD are predicted to be  $1700$  ( $730$ – $3400$ )  $\text{pg/g egg}$  for type 2 species (Farmahin et al., 2013). Species-specific data on the lower toxicity limits within the type 2 Ah-receptor are still very limited. Nevertheless, these few studies indicate that the concentrations of dioxins and DLCs we reported in eggs of Northern wheatears may be too low to cause the malformations or high degree of hatching failure.

### 3.6. Alternative causes for hatching failures and embryonic malformations

Our results regarding the Ah-receptor indicate that DLCs are not the only cause for the hatching failure in the Northern wheatear populations. Other, as yet unidentified pollutants may cause hatching failure, concentrations of which must then be higher than in Sweden. Residues of OCPs in eggs were at low concentrations and similar for the Dutch and Swedish populations. Brominated flame retardants (PBDEs, 18 congeners; Table A.6) were detected in the eggs but not at concentrations associated with malformations or hatching failure (e.g. Daso et al., 2015).

Although the observed embryonic malformations and liver anomalies have been seen linked to DLCs, similar malformations have been described also for other causes. It is possible that other mechanisms (e.g. nutrient deficiencies, such as thiamine deficiency; Balk et al., 2009) could lead to the observed hatching failure and increased malformation rate. Nutrient deficiencies are known to cause embryo malformations similar to the ones found in the current study (Romanoff, 1972).

Lastly, hatching failure is an often reported outcome of inbreeding depression in birds (Kempnaers et al., 1996; Briskie and Mackintosh, 2004). The correlation between heterozygosity in certain markers and fitness of an individual is often weak (Chapman et al., 2009; Szulkin et al., 2010). This could mean that fitness of Dutch Northern Wheatears may still be low, despite the relatively high heterozygosity values (Van Oosten et al., 2016). Inbreeding may be suspected because of small contemporary population size and because exchange of colour-ringed individuals between populations is rare (Van Oosten et al., 2015).

To conclude, at this stage we do not know what causes the elevated hatching failure in Dutch Northern wheatears. However, it is not likely that DLC exposure is the only cause, if at all. This uncertainty is worrisome since this songbird is one of the songbird species in Europe that have showed strongest population decline, and a decline that continues. We show that egg failure is, at least locally, contributing to this decline. Future work should investigate the possibility of other pollutants affecting hatchability by means of a broad scan of pollutants, which would also evaluate the combined effects of toxins on hatching success.

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