Article (refereed) - postprint

Pereira, M. Gloria; Murk, A.J.; Van den Berg, H; Walker, Lee A.; Shore, Richard F. 2014. **How much do PCB toxic equivalents account for PHAH toxicity in predatory birds?**

How much do PCB toxic equivalents account for PHAH toxicity in predatory birds?

M. Glória Pereira[1*], A.J. Murk[2], H. Van den Berg[2], Lee A. Walker[1] & Richard F. Shore[1]

[1]*NERC Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster, LA1 4AP, UK.*

[2]*Division of Toxicology, Wageningen University, The Netherlands.*

*Corresponding author:
Dr M. Glória Pereira
NERC Centre for Ecology and Hydrology,
Lancaster Environment Centre,
Library Avenue,
Bailrigg,
Lancaster,
LA1 4AP,
UK

Tel: +44 (0) 01524 595963
Fax: +44 (0) 01524 61536
e-mail: mdgds@ceh.ac.uk

Abstract

Various diffuse polyhalogenated aromatic hydrocarbons (PHAHs) exert common toxicity through the aryl hydrocarbon receptor (*Ah*R). Apex predators spatially and temporally integrate diffuse contamination and simultaneous exposure can cause additive toxicity. We investigated the extent to which PCBs, still amongst the most prevalent PHAHs accumulated by predators, accounted for total PHAH toxicity in raptors and fish eating birds from Britain. We analysed egg or liver extracts from six species and compared chemically determined ΣPCB-TEQs concentrations with total *Ah*R-mediated toxicity determined using the chemical- activated luciferase gene expression bioassay (CALUX-TEQ). Dioxin-like PCB profiles in eggs and livers were dominated by congeners 118, 105 and 167. ΣPCB-TEQ and CALUX-TEQ concentrations were positively associated but not in a 1:1 relationship. ΣPCB-TEQ were broadly similar to CALUX-TEQ concentrations in eggs and livers with CALUX-TEQ concentrations >50-80 and 160-320 pg g^{-1} lipid respectively, but were lower than CALUX-TEQ concentrations in less contaminated samples.

Keywords- Dioxin-like PCBs, TEQs, Ah receptor, CALUX-assay, sparrowhawk, kestrel, herons, gannet, merlin and peregrine falcon.

Introduction

Polyhalogenated aromatic hydrocarbons (PHAHs) include polychlorinated dibenzo-*p*-dioxins (PCDDs) and furans (PCDFs), polychlorinated biphenyls (PCBs), polychlorinated terphenyls and naphthalenes (PCNs) and polycyclic aromatic hydrocarbons (PAHs). They are ubiquitous, persistent, lipophilic and bioaccumulative compounds, characteristics that enhance the risk of exposure in a diverse array of apex predators. Many PHAHs induce effects that are mediated through a common mode of action. This involves the binding of the parent compound or their metabolites to the aryl hydrocarbon receptor (*Ah*R) in the cytoplasm and results in a rise or fall of the transcription of specific genes via dioxin-responsive elements in the DNA (Safe, 1990; Denison and HeathPagliuso, 1998). The consequences can be adverse effects such as reproductive impairment (disruption of hormone status, embryotoxicity, teratogenicity), hepatotoxicity, immunotoxicity and neurotoxicity (Denison and HeathPagliuso, 1998; Harper et al., 2006).

PHAHs are typically found in complex mixtures in environmental matrices. The commonality in their mode of action means that the toxicity of individual PHAHs can be additive. The chemical-activated luciferase gene expression (CALUX) bioassay has been developed to quantify the overall toxicity caused by simultaneous exposure to multiple compounds that exert toxicity via the *Ah*R. This reporter gene assay is based on *Ah*R-mediated firefly (*Photinus pyralis*) luciferase expression in genetically modified cell lines (Aarts et al., 1995; Garrison et al., 1996). A vector containing the luciferase gene under transcriptional control of dioxin-responsive elements has been stably transfected into a number of cell lines, including the rat H4IIE hepatoma cell line (Aarts et al., 1995; Garrison et al., 1996; Richter et al., 1997). CALUX has been used widely for screening and quantifying the toxicity of dioxin-like compounds in blood (Van Wouwe et al., 2004; Ziccardi et al., 2000), sediments (Behnisch et al., 2002; Stronkhorst et al., 2002), various marine matrices (Tsutsumi et al., 2003; Windal et al., 2005) and bovine milk (Bovee et al., 1998).

The occurrence of PCB concentrations in the livers and eggs of many different bird species has been well documented (Harris and Elliott, 2011). Some PCB congeners (non-*ortho*, and to a lesser extent some mono-*ortho* substituted congeners) have a coplanar dioxin-like configuration. These have a high affinity for the *Ah*R and so exert similar toxic effects to other PHAHs that act via the *Ah*R. The potencies of different dioxin-like PCB congeners are between one and five orders of magnitude lower than that of 2,3,7,8 -tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic dioxin (Ahlborg et al.,

1992; Van den Berg et al., 1998, 2006). Because accumulation of PCBs typically exceeds that of other more potent PHAHs, such as dioxins and furans (Jemenez et al., 2007), it is often considered that PCBs are likely to be the main contributors to *Ah*R mediated toxicity unless there are particular localised sources of other compounds. However, there is a general lack of quantitative evidence to support this contention, not is it clear how variation or future changes in PCB contamination are likely to affect *Ah*R mediated toxicity. The aim of the present study was therefore to investigate the extent to which dioxin-like PCBs in predatory birds account for their total TEQ concentrations. This involved measuring PCB concentrations chemically in the eggs and livers of various predatory birds and comparing the resultant calculated summed toxic equivalence (∑ΣPCB-TEQ) with that estimated for all PHAHs as measured by CALUX assay (CALUX-TEQ)

1. Materials and Methods

1.1. Sample collection

Livers were obtained from bird carcasses sent in by members of the public to the Predatory Bird Monitoring Scheme (PBMS: http://pbms.ceh.ac.uk/), a long-term UK chemical surveillance and monitoring programme that uses birds of prey as sentinels of exposure (Walker et al., 2008). In all, 48 livers were used for the present study and were from 16 sparrowhawk (*Accipiter nisus*), 16 kestrel (*Falco tinnunculus*) and 16 heron (*Ardea cinerea*) carcasses that had been found at various locations from across Britain. A collection of 44 eggs, each from a separate nest and collected for the PBMS by licensed egg collectors, were also analysed. The sample was comprised of 16 addled peregrine falcon (*Falco peregrinus*) eggs from throughout Britain, 11 addled merlin (*Falco columbarius)* eggs from various locations largely in northern Britain and 16 fresh gannet (*Morus bassanus*) eggs, half from Ailsa Craig (West Scotland) and half from Bass Rock (East Scotland). All samples were from the PBMS tissue archive and had been collected in 2002 or 2003. Samples from these years were used because this was a period for which we had a representative selection of samples for these different species, the samples had already been characterised for PCB concentrations as part of the PBMS monitoring, and 2002-3 was a period when other major PHAHs, such as polybrominated diphenyl ethers, were also still widely accumulated by piscivorous and terrestrial predatory birds (Crosse et al., 2012a,b; 2013). Livers were excised from carcasses and kept at -20^{0}C until analysed. The weight, length and

breadth of collected eggs were measured and the eggs were then blown or cracked open. The eggshells were washed, air-dried and reweighed, while the egg contents were homogenised.

1.2. Analysis

1.2.1. Extraction and clean-up

A sub- sample of each egg and liver (1-2 g) was thawed, weighed accurately, ground with sand, dried with anhydrous sodium sulphate, and cold extracted in 50 ml of 1:1 acetone:hexane (v/v). The solvent in half of the extract was evaporated and the lipid content determined gravimetrically. The other half of the extract was then re-dissolved in hexane and the lipids were removed from the extract using an alumina glass column packed with pre-treated alumina (4 h at 800°C) that had been deactivated with 5% deionised water (w/w). This clean extract was then subdivided and was used for the determination of TEQ concentrations, by chemical determination of PCBs and by CALUX assay.

1.2.2. PCB analysis

The suite of the 12 dioxin-like PCB congeners (77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, 189) was analysed using gas chromatography with electron capture detection (GC-ECD). Prior to the GC analysis, dichlobenil was added as an internal standard. A 4 µl aliquot of each extract was injected into the gas chromtograph (Agilent, Wokingham, UK) using a splitless injector, and a 50m HT8 column (0.22mm internal diameter and 0.25µm film thickness, SGE Milton Keynes, UK). The injector temperature was set at 250°C and the carrier gas was helium (2.0 ml min^{-1}). The temperature programme was: isothermal at 50°C for 2 min, 45°C min^{-1} to 200°C, 1.5°C min^{-1} to 240°C, 2°C min^{-1} to 285°C, 50°C min^{-1} to 325°C and isothermal at 325°C for 10 min. The detector temperature was set at 335°C. Residues were quantified using the internal standard method and also using calibration curves with standards of the 12 PCBs (Greyhound Ltd, Birkenhead, UK).

For quality control and assurance purposes, a blank, a sample of an uncontaminated chicken egg (or liver) and second sample of chicken egg (or liver) were spiked with a known concentration of PCB congeners with each batch of egg or liver samples, respectively. Recovery values determined from the spiked samples varied between 75% and 110%. Limits of detection (LoD), determined from the calibration curve and based on the mean weight of the sample contents that were analysed, varied between 0.776 and 4.203 ng g^{-1} lipid.

1.2.3. Calux assay

The cleaned-up extract was evaporated to dryness and transported to the Wageningen University for analysis by the CALUX assay. The dried extracts were re-dissolved in 100 µl of 1:1 acetone:hexane and mixed for one minute, after which 15 or 20 µl aliquots of DMSO were added to each egg or liver extract, respectively. The acetone:hexane was then evaporated. DR CALUX® gene assay was performed as described elsewhere (Murk et al., 1998). In short, *in-vitro* cultivated rat hepatoma cells (H4IIE-luc), transfected with a stable plasmid which carries luciferase gene of fireflies (*Photinus pyralis*) as a reporter gene, were seeded on a 96-well Packard ViewPlates in medium supplemented with hormone-stripped serum. The next day, the medium was replaced with medium containing the egg or liver extracts (0.1% DMSO). After 24 hours exposure, the medium was removed and the cells were lysed. The substrate luciferin was then added to the wells to quantify the amount of luciferase produced by the cells by measuring the amount of light. Bio-luminescence was measured using a luminometer, Luminoskan RS (Thermo Life Science), equipped with two internal injectors to inject the luciferine containing FlashMix, and the NaOH for signal quenching. All samples were corrected for the solvent DMSO signal. The measured luminescence was converted into a CALUX toxic equivalent (CALUX-TEQ) value by the direct comparison of the response for a given samples to a dose –response obtained with 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (see Supporting Information). One single global biological response is thus measured by CALUX® for all AhR ligands present in the extract. The limit of detection for the assay was 0.30 pM TEQ and all samples exceeded this.

1.3. Data analysis

Individual coplanar PCB congener TEQs were calculated as the product of their designated Toxic Equivalence Factor (TEF) (Van den Berg et al., 2006) and their concentration; non-detected concentrations of individual congeners were assigned a zero value. ΣPCB-TEQ concentrations were calculated as the sum of the TEQs for the individual congeners. ΣPCB-TEQ concentrations and CALUX-TEQ concentrations are expressed in pg ΣPCB-TEQ g^{-1} lipid. Concentrations in eggs were not adjusted for desiccation as the purpose of this study was to compare ΣPCB-TEQs and CALUX-TEQs, not focus on reporting absolute concentrations, and any desiccation would have affected each measurement equally.

Summary data for ΣPCB-TEQs and CALUX-TEQs concentrations are presented as medians and inter-quartile ranges, and differences between ΣPCB-TEQs and CALUX-TEQs concentrations for each species were assessed by Mann-Whitney U tests. The relationships between the ΣPCB- and CALUX-TEQs concentrations in samples were determined using linear regressions on log-transformed data; the residuals of the analyses were all normally distributed.

2. Results

Between five and eight of the 12 PCB congeners that we analysed for were detected in the livers and eggs of each species. Congeners 118, 105, 156 and 167 were found in all six species (Figure 1) and were generally the congeners detected most frequently in all samples independently of the species; PCB 118 was detected in more than 80% of the samples analysed. In contrast, PCB congeners 81, 126 and 169 were never detected. All of the congeners that were detected in livers were also found in eggs except congener 77 which was only found in some sparrowhawk livers (Figure 1).

In terms of concentrations, ΣPCB-TEQs ranged between non-detected (ND) and 248 pg g^{-1} lipid in eggs and between ND and 2102 pg g^{-1} lipid in livers, equivalent to wet weight (ww) concentrations of ND-11.5 pg g^{-1} and ND-130.6 pg g^{-1}, respectively. The corresponding ranges of the measured CALUX-TEQs in eggs and livers were 7-241 pg g^{-1} lipid and 10-467 pg g^{-1} lipid respectively, equivalent to 0.32-12 pg g^{-1} ww and 0.6-29 pg g^{-1} ww. There was no significant difference between the median ΣPCB-TEQ and CALUX TEQ (Figure 2) for gannet eggs, merlin eggs or heron livers (Mann-Whitney U tests, $P>0.05$ in each case), but the ΣPCB-TEQ was significantly lower than the CALUX-TEQ in peregrine eggs and in kestrel and sparrowhawk livers (Mann-Whitney $U \leq 36$, $P<0.001$ in all cases). The dispersion in the data appeared to differ between eggs and livers (Figure 2). When the variances in the data were examined, there were differences between species for both CALUX-TEQs (Levene's test = 5.64, $P<0.001$) and, to a lesser extent, for ΣPCB-TEQs (Levene's test = 2.03, $P=0.083$). These differences may have been more related to matrix type than species as variances in both ΣPCB-TEQs and CALUX-TEQs were generally greater in livers than eggs.

When individual ΣPCB-TEQ and corresponding CALUX-TEQ measurements were compared, the relationship between the two was always statistically significant in the eggs (Figure 3). The gradient of the relationship was significantly less than one (upper 95% confidence limit: 0.329) with the result that ΣPCB-TEQ concentrations were similar to or exceeded CALUX-TEQs in more heavily contaminated

eggs but CALUX-TEQs exceeded ΣPCB-TEQs in eggs with lower levels of contamination. For example, ΣPCB-TEQs were some 63% (median value) higher than their corresponding CALUX-TEQs concentrations in gannet eggs that were relatively contaminated (log ΣPCB-TEQ >1), but made up only 10-14% of CALUX-TEQs in less contaminated samples (Figure 3). There was a similar but less marked pattern in merlin eggs, although ΣPCB-TEQs made up on average (median value) 82% of the CALUX-TEQ. PCB concentrations in the peregrine falcon eggs were lower than those in the eggs of the other two species (Figure 3) and, as with the less contaminated gannet eggs, ΣPCB-TEQs in the peregrine eggs comprised only a small proportion (median: 7%) of the CALUX-TEQ concentration. When the data for all the eggs were pooled and analysed together with species included in the model as a factor, there was a highly significant positive relationship between ΣPCB- and CALUX-TEQ concentrations ($F_{(1,39)}$ = 17.1, P<0.001) but species was not a significant factor in the model ($F_{(2,39)}$ = 2.18, P=0.13). The ΣPCB-TEQ concentrations explained 66% of the variance in the CALUX-TEQ concentrations and the relationship between ΣPCB- and CALUX-TEQ concentrations was similar between species (Figure 3). Overall, ΣPCB-TEQs were lower than CALUX-TEQs in the eggs of all three species until ΣPCB-TEQ concentrations were approximately >65 pg g^{-1} lipid.

When liver CALUX- and ΣPCB-TEQ concentrations were compared for each species separately (Figure 4), linear regression models could not be fitted to the data. However, there was a significant positive correlation between the two measurements for sparrowhawks (Spearman Rank correlation coefficient r = 0.697, P=0.027) and weaker (0.05<P<0.1) positive associations for herons and kestrels (Figure 4). The associations between CALUX- and ΣPCB-TEQ concentrations were weaker in livers than eggs, partly as a result of the relatively high number of livers (mainly sparrowhawk and kestrel) with detectable CALUX-TEQ concentrations but no detectable ΣPCB-TEQs. These livers comprised 69% and 56% of the sparrowhawk and kestrel samples respectively although only 12% of the heron sample (Figure 4). When the liver data for all three species were pooled and were analysed by ANCOVA, there was a significant relationship between the two TEQ measurements (r^2 = 0.294, $F_{(1,43)}$ = 10.6, P=0.002) and species was a significant factor ($F_{(2,43)}$ = 3.79, P=0.13; Figure 4). As with the eggs, the gradient of the relationship was significantly less than one (upper 95% confidence limit: 0.256) and the extent of correspondence between the ΣPCB-TEQ and CALUX-TEQ concentrations increased with the level of PCB contamination (Figure 4). ΣPCB-TEQs and CALUX-TEQs reached parity when ΣPCB-TEQ concentrations were approximately 160 pg g^{-1} lipid (kestrel and heron) and 320 pg g^{-1} lipid (sparrowhawk). Although the relationship between the two TEQ measurements was significant,

233 ΣPCB-TEQs explained only 29.4 % of the variation in CALUX-TEQs, and 47% of the livers had non-
234 detected ΣPCB-TEQs but CALUX-TEQs that ranged up to 355 pg g^{-1} lipid.
235

236 ## 4. Discussion

237 The results from our study, in terms of the congener prevalence and TEQ concentrations, are
238 similar to those reported in other species elsewhere. Generally, non dioxin-like PCB congeners,
239 principally 153, 138 and 180 predominate in birds from a range of habits and trophic status (Alcock et
240 al., 2002; Borlakoglu et al., 1990; Caccamise et al., 2012; Jemenez et al., 2007; Norstrom, 1988;
241 Pereira et al., 2009a) and this was also the case in the present samples (data not shown). The
242 prevalence of non-*ortho* and mono-*ortho,* dioxin-like, congeners varies with source, and possibly with
243 trophic strategy and species-specific metabolism (Harris & Elliott 2011). The prevalence of congeners
244 118, 105, 156 and 167 in the present study was consistent with observations in 17 bird species from
245 Japan (Senthilkumar et al., 2002), although we did not detect the two PCB congeners (81, 126) with
246 the highest TEFs (0.1) and only found PCB 77 (TEF: 0.05) in about 10% of the sparrowhawk livers;
247 these three congeners have been found in bald eagles *(Haliaeetus leucocephalus*) (Elliott et al., 1996),
248 in various piscivorous bird species (Bosveld et al., 1995; Kubiak et al., 1989; Yamashita et al., 1993)
249 and in Chinese birds of prey (Chen et al., 2009). The observed ΣPCB -TEQ concentrations in the current
250 study were of the same order of magnitude of those measured in owl livers and American kestrel
251 (*Falco sparverius)* eggs in the United States (Coady et al., 2001) and in albatross chicks from Pacific
252 (Caccamise et al., 2012). However, concentrations in the present study were lower than those
253 reported in some piscivorous species (Guruge et al., 2000; Schmutz et al., 2009), in raptors from SE
254 Asia (Chen et al., 2009) and in belted kingfisher (*Ceryle alcyon*), spotted sandpiper (*Actitis macularius*),
255 and tree swallow (*Tachycineta bicolor*) eggs in the USA (Custer et al., 2010). The lower concentrations
256 in the present study perhaps reflect the fact that birds did not come from locations known to be
257 particularly contaminated and also our samples did not contain detectable levels of high TEF
258 congeners. CALUX-TEQ concentrations in our samples were a similar order of magnitude to those in
259 kestrel eggs and owl livers from the USA (Coady et al., 2001) and similar to TEQ concentrations
260 (determined by chemical analysis of multiple PHAHs) in fish eating birds such as white-tailed sea
261 eagles (*Haliaeetus albicilla*) from Germany (Kannan et al., 2003) and double-crested cormorants

(*Phalacrocorax auritus*) and caspian terns (*Hydroprogne caspia*) from the USA (Yamashita et al., 1993).

It would not be expected that ΣPCB-TEQ and CALUX-TEQ concentrations would exactly correspond, even in the same sample, and this was the case in the present study in which median CALUX-TEQ concentrations were significantly greater than ΣPCB-TEQs in peregrine falcon eggs and in kestrel and sparrowhawk livers (Figure 2). PHAHs other than PCBs may contribute to the CALUX-TEQ. However, there can also be differences in the relative potency of congeners to the species used to generate the cells lines in the CALUX assay and the avian TEFs assigned to PCB congeners (Windal et al., 2005a) which adds some uncertainty to direct comparisons of the two measures. Examination of how variation in ΣPCB-TEQ concentrations affected CALUX-TEQ concentrations (Figures 3 and 4) was arguably more informative than simple comparisons of mean values. When data for eggs, were pooled, we found a strong and significant relationship between PCB- and CALUX-TEQs, as has been reported in blood plasma for both individual congener and summed TEQs (Murk et al., 1997, 1998), but there was not a 1:1 correspondence between ΣPCB-TEQ and CALUX-TEQ concentrations. When egg ΣPCB-TEQ concentrations were relatively low (taken as <65 pg g^{-1} lipid) the median contribution of ΣPCB-TEQs to the CALUX-TEQ concentration was only 10% (inter-quartile range: 5-29%). This could have been because other PHAHs may have been present in the eggs and exerted *Ah*R mediated toxicity. It is also possible that dioxin-like PCBs were present below the analytical limit of detection and additively contributed to the detected CALUX-TEQs. The most potent congeners exert a relatively large influence on the ΣPCB-TEQ concentration because of their high TEFs and so uncertainties and errors in calculation of ΣPCB-TEQs are likely to be relatively large in samples with low concentrations of the more potent PCBs. In contrast to eggs with relatively low levels of PCB contamination, CALUX-TEQs in the more contaminated eggs were almost completely attributable to dioxin-like PCBs. In fact, ΣPCB-TEQs exceeded CALUX-TEQs in eggs with CALUX-TEQs >100 pg g^{-1} lipid. CALUX-TEQs can be influenced by antagonistic, additive and synergetic interactions (Schroijen et al., 2004) and one possibility is that antagonism between different PHAHs may have reduced the measured CALUX response but would not have been accounted for in the additive model used to calculate ΣPCB-TEQs (Sanderson et al., 1996). However, it would be necessary to measure all PHAHs in the samples to determine whether such interactions may have occurred.

The pattern of the relationship between ΣPCB-TEQs and CALUX-TEQs in livers was broadly similar to that in eggs. When data for all livers were pooled, there was a significant positive

293 relationship between the two measurements and ΣPCB-TEQs explained a relatively low percentage of
294 the CALUX-TEQs in less contaminated samples. The relationship between ΣPCB-TEQs and CALUX-TEQs
295 was weaker in livers than in eggs. This may have been because, compared with eggs, livers may
296 contain a greater concentration of other PHAHs that exert Ah*R* mediated toxicity and/or very low
297 levels of a range of the more potent coplanar PCBs. Although comprehensive analysis of other PHAHs
298 would have to be done to confirm this, the fact that predicted (log_{10}) CALUX concentration in samples
299 that contained no detectable ΣPCB-TEQ concentration was higher in livers (Figure 4, 95% Confidence
300 Limits: 1.80-2.06) than in eggs (Figure 3, 1.28 - 1.61 pg g^{-1} lipid) is consistent with livers containing
301 more non-PCB PHAHs than eggs. Higher non-PCB related CALUX-TEQ concentrations in livers than
302 eggs may be due to differences between the two matrix types in accumulation and retention of
303 different xenobiotics. It may also be because concentrations in eggs reflect a limited period of
304 exposure and metabolism in a restricted portion of the population (adult females during laying)
305 whereas liver concentration can be the result of exposure over a different and wider time period and
306 reflect metabolism by individuals that may vary in nutritional state, age and sex (Wienburg and Shore,
307 2004). The observed greater variability in CALUX and PCB-TEQ concentrations in livers than in eggs
308 (Figure 2) is also consistent with the concept that liver CALUX-TEQ concentrations reflect more
309 heterogenous exposure and accumulation of PHAHs.
310 Overall, for the species that we examined, egg and liver CALUX-TEQ concentrations greater
311 than approximately 50-80 and 160-320 pg g^{-1} lipid respectively (equivalent approximately 2.5-4 and
312 10-20 pg g^{-1} ww) could be largely attributed to dioxin-like PCBs. Lowest Observable Effect TEQ
313 concentrations in eggs of 10 -2200 pg g^{-1} ww have been suggested (AMAP, 1998) while more recently,
314 a threshold egg concentration of 400-1600 pg g^{-1} ww has been mooted for the sensitive endpoint of
315 ethoxyresorufin-O-deethylase (EROD) induction in wild bird hatchlings (Cohen-Barnhouse et al., 2008;
316 Harris and Elliott, 2011). A liver LOEL of 25,000 pg g^{-1} lipid for induction of cytochrome P450 enzymes
317 and reduction in plasma thyroxin levels, has been described for common tern (*Sterna hirundo*) chicks
318 (Bosveld et al., 2000). These concentrations are between one and two orders of magnitude higher
319 than the concentration above which coplanar PCBs became the major contributors to CALUX-TEQs in
320 our samples. Our data therefore suggest that, of the compounds that are assimilated and retained
321 within tissues or eggs in predatory birds and that mediate toxicity through the A*h*R, it is the dioxin-
322 like PCBs that appear to be associated with TEQ concentrations that approach toxicologically
323 significant levels. Other studies have likewise attributed CALUX activity in birds largely to dioxin-like

PCBs (Jemenez et al., 2007). However, we recognise that exposure to more readily metabolised compounds from diffuse sources, such as PAHs, may also result in toxicity mediated through the the A*h*R. These compounds may largely have gone undetected in the present study because residues are poorly assimilated in liver and eggs (Malcolm and Shore 2003, Pereira et al., 2009b), and so would not have been present in the tissue and egg extracts that we analysed. Furthermore, it is also possible that contamination from point sources by PHAHs may result in effects in birds that are spatially localised and so are difficult to detect without targeting sampling for analysis. However, where such localised impacts have been found and linked to PHAHs in Britain (Thompson et al., 2006), dioxin-like PCBs were still found to account for most (77-92%) of the ∑TEQ concentrations.

In conclusion, our results are consistent with the premise that, in predatory birds sampled at a broad spatial scale across Britain, low level TEQ concentrations in eggs and livers may largely arise from exposure to non-PCB PHAHs and/or trace levels of dioxin-like PCBs that are present below analytical detection limits. However, dioxin-like PCB congeners appear to be the major contributors to TEQ concentrations in the more heavily contaminated eggs and livers. Although samples used in this study were collected in 2002 and 2003, there has been no clear decline since then in PCB contamination across Britain in many of the species we examined (Pereira et al., 2009a; Walker et al., 2011). Thus, there is no *a priori* reason to believe conclusion drawn from these samples do not remain valid. We conclude that measurement of dioxin-like PCBs, as part of wide-scale monitoring in Britain, is likely to be adequate for detecting toxicologically significant TEQ concentrations in bird eggs and carcasses. Such measurements are likely to underestimate TEQ concentrations in less contaminated samples however. A full suite of chemical analysis for PHAHs, coupled with bioassay measurements such as CALUX, would be necessary to determine actual exposures and their contribution to *Ah*R mediated toxicity in such samples and in samples where there has been contamination from major point sources of unkown PHAHs.

Acknowledgements *- This work is part of the Predatory Bird Monitoring Scheme (http://pbms.ceh.ac.uk) which is currently funded by the NERC Centre for Ecology & Hydrology (CEH), Natural England (NE), the UK Department for Food and Rural Affairs (Defra), the Campaign for Responsible Rodenticide Use (CRRU), the Royal Society for the Protection of Birds (RSPB), Scottish Natural Heritage (SNH) and the Scottish Environmental Protection Agency (SEPA). We thank all the*

354 *volunteer collectors who have submitted bird carcasses and the licensed egg collectors who have*
355 *submitted eggs to the PBMS.*
356

357 **References**

358 Aarts, J., Denison, M.S., Cox, M.A., Schalk, M.A.C., Garrison, P.M., Tullis, K., Dehaan, L.H.J., Brouwer,
359 A., 1995. Species- specific antagonism of the AH receptor action by 2,2',5,5'-tetrachlorobiphenyl
360 and 2,2',3,3',4,4'-hexachlorobiphenyl. European Journal of Pharmacology-Environmental
361 Toxicology and Pharmacology Section 293, 463-474.
362 Ahlborg, U.G., Brouwer, A., Fingerhut, M.A., Jacobson, J.L., Jacobson, S.W., Kennedy, S.W., Kettrup,
363 A.A.F., Koeman, J.H., Poiger, H., Rappe, C., Safe, C.H., Seegal, R.F., Tuomisto, J., van den Berg, M.,
364 1992. Impact of polychlorinated dibenzo-p-dioxins, dibenzofurans and biphenyls on human and
365 environmental health, with special emphasis on the toxic equivalency factor concept. European
366 Journal of Pharmacology 228, 179-199.
367 Alcock, R.E., Boumphrey, R., Malcolm, H.M., Osborn, D., Jones, K.C., 2002. Temporal and spatial
368 trends of PCB congeners in UK Gannet eggs. Ambio 31, 202-206.
369 AMAP, 1998. AMAP asssessment report: arctic pollution issues. Arctic monitoring and assessment
370 programme (AMAP), Oslo, Norway.
371 Behnisch, P.A., Hosoe, K., Brouwer, A., Sakai, S., 2002. Screening of dioxin-like toxicity equivalents for
372 various matrices with wildtype and recombinant rat hepatoma H4IIE cells. Toxicological Sciences
373 69, 125-130.
374 Borlakoglu, J., Wilkins, J., Walker, C., Dils, R., 1990. Polychlorinated biphenyls (PCBs) in fish-eating sea
375 birds. III. Molecular features and metabolic interpretations of PCB isomers and congeners in
376 adipose tissues. Comparative Biochemistry and Physiology 97, 173-177.
377 Bosveld, A.T.C., Gradener, J., Murk, A.J., Brouwer, A., Vankampen, M., Evers, E.H.G., Vandenberg, M.,
378 1995. Effects of PCDDS, PCDFS and PCBS in Common Tern (*Sterna hirundo*) breeding in estuarine
379 and coastal colonies in the Netherlands and Belgium. Environmental Toxicology and Chemistry 14,
380 99-115.
381 Bosveld, A.T.C., Nieboer, R., de Bont, A., Mennen, J., Murk, A.J., Feyk, L.A., Giesy, J.P., van den Berg,
382 M., 2000. Biochemical and developmental effects of dietary exposure to polychlorinated biphenyls

126 and 153 in common tern chicks (*Sterna hirundo*). Environmental Toxicology and Chemistry 19, 719-730.

Bovee, T.F.H., Hoogenboom, L.A.P., Hamers, A.R.M., Traag, W.A., Zuidema, T., Aarts, J., Brouwer, A., Kuiper, H.A., 1998. Validation and use of the CALUX-bioassay for the determination of dioxins and PCBs in bovine milk. Food Additives and Contaminants 15, 863-875.

Caccamise, S.A.L., Wang, J., Wu, L., Woodward, L.A., Li, Q.X., 2012. Accumulation and toxicity assessment of polychlorinated biphenyls in black-footed albatross (*Diomedea nigripes*) from Midway Atoll, North Pacific Ocean. Ecological Indicators 20, 75-81.

Chen, D., Zhang, X.L., Mai, B.X., Sun, Q.H., Song, J., Luo, X.J., Zeng, E.Y., Hale, R.C., 2009. Polychlorinated biphenyls and organochlorine pesticides in various bird species from northern China. Environmental Pollution 157, 2023-2029.

Coady, K.K., Jones, P.D., John, P., 2001. 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents in tissue samples from three species in the Denver, Colorado, USA, metropolitan area. Environmental Toxicology and Chemistry 20, 2433-2442.

Cohen-Barnhouse, A., al, e., 2008. Effects of TCDD,TCDF and PeCDF injected into the air cell of Japanese quail (*Coturnix japonica*) prior to incubation. Abstracts of the 29th Society of Environmental Toxicologyand Chemistry North America Meeting. Abstract WP210.

Crosse, J.D., Shore, R.F., Jones, K.C., Pereira, M.G., 2012a. Long term trends in PBDE concentrations in gannet (*Morus bassanus*) eggs from two UK colonies. Environmental Pollution 161, 93-100.

Crosse, J.D., Shore, R.F., Wadsworth, R.A., Jones, K.C., Pereira, M.G., 2012b. Long-term trends in PBDEs in sparrowhawk (*Accipiter nisus*) eggs indicate sustained contamination of UK terrestrial ecosystems. Environmental Science & Technology 46, 13504-13511.

Crosse, J.D., Shore, R.F., Jones, K.C., Pereira, M.G., 2013. Key factors affecting liver PBDE concentrations in sparrowhawks (*Accipiter nisus*). Environmental Pollution 177, 171-176.

Custer, C.M., Custer, T.W., Dummer, P.M., 2010. Paterns of organic contaminants ineggs of an insectivorous, an omnivorous and a piscivorous bird nesting on the Hudson River, New York, USA. Environmental Toxicology and Chemistry 29, 2286-2296.

Denison, M.S., HeathPagliuso, S., 1998. The Ah receptor: A regulator of the biochemical and toxicological actions of structurally diverse chemicals. Bulletin of Environmental Contamination and Toxicology 61, 557-568.

413 Elliott, J.E., Norstrom, R.J., Lorenzen, A., Hart, L.E., Philibert, H., Kennedy, S.W., Stegeman, J.J.,
414 Bellward, G.D., Cheng, K.M., 1996. Biological effects of polychlorinated dibenzo-p-dioxins,
415 dibenzofurans, and biphenyls in bald eagle (*Haliaeetus leucocephalus*) chicks. Environmental
416 Toxicology and Chemistry 15, 782-793.
417 Garrison, P.M., Tullis, K., Aarts, J., Brouwer, A., Giesy, J.P., Denison, M.S., 1996. Species-specific
418 recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-
419 dioxin-like chemicals. Fundamental and Applied Toxicology 30, 194-203.
420 Guruge, K.S., Tanabe, S., Fukuda, M., 2000. Toxic assessment of PCBs by the 2,3,7,8-
421 tetrachlorodibenzo-p-dioxin equivalent in common cormorant (*Phalacrocorax carbo*) from Japan.
422 Archives of Environmental Contamination and Toxicology 38, 509-521.
423 Harper, P.A., Riddick, D.S., Okey, A.B., 2006. Regulating the regulator: Factors that control levels and
424 activity of the aryl hydrocarbon receptor. Biochemical Pharmacology 72, 267-279.
425 Harris, M.L., Elliott, J.E., 2011. Effects of polychlorinated biphenyls, bibenzo-*p*-dioxins and
426 dibenzofurans, and polybrominated diphenyl ethers in wild birds, in: Beyer, W.N., Meador, J.P.
427 (Eds.), Environmental contaminants in wildlife: interpreting tissue concentrations, 2nd Edn. Taylor
428 & Francis, pp. 477-528.
429 Jemenez, B., Merino, R., Abad, E., Rivera, J., Olie, K., 2007. Evaluation of organochlorine compounds
430 (PCDDs, PCDFs, PCBs and DDTs) in two raptor species inhabiting a Mediterranean island in Spain.
431 Environmental Science and Pollution Research 14, 61-68.
432 Kannan, K., Hilscherova, K., Imagawa, T., Yamashita, N., Williams, L.L., Giesy, J.P., 2001.
433 Polychlorinated naphthalenes, -biphenyls, -dibenzo-p-dioxins, and -dibenzofurans in double-
434 crested cormorants and herring gulls from Michigan waters of the Great Lakes. Environmental
435 Science & Technology 35, 441-447.
436 Kubiak, T.J., Harris, H.J., Smith, L.M., Schwartz, T.R., Stalling, D.L., Trick, J.A., Sileo, L., Docherty, D.E.,
437 Erdman, T.C., 1989. Microcontaminats and reproductive impairment of the Forsters Tern on Green
438 Bay, Lake Michigan 1983. Archives of Environmental Contamination and Toxicology 18, 706-727.
439 Malcolm, H.M. & Shore, R.F. 2003. Effects of polycyclic aromatic hydrocarbons (PAHs) on terrestrial
440 and freshwater birds and mammals, Douben, P.E.T. (Ed), PAHs: an ecotoxicological perspective,
441 John Wiley & Sons, Chichester, pp. 225-241.
442 Murk, A.J., Leonards, P.E.G., Bulder, A.S., Jonas, A.S., Rozemeijer, M.J.C., Denison, M.S., Koeman, J.H.,
443 Brouwer, A., 1997. The CALUX (chemical-activated luciferase expression) assay adapted and

validated for measuring TCDD equivalents in blood plasma. Environmental Toxicology and Chemistry 16, 1583-1589.

Murk, A.J., Leonards, P.E.G., van Hattum, B., Luit, R., van der Weiden, M.E.J., Smit, M., 1998. Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). Environmental Toxicology and Pharmacology 6, 91-102.

Norstrom, R., 1988. Bioaccumulation of polychlorinated biphenyls in Canadian wildlife., in: Crine, J. (Ed.), Hazards, decontaminations and replacement of PCB. Plenum New York, NY, pp. 85-100.

Pereira, M.G., Walker, L.A., Best, J., Shore, R.F., 2009a. Long-term trends in mercury and PCB congener concentrations in gannet (*Morus bassanus*) eggs in Britain. Environmental Pollution 157, 155-163.

Pereira, M.G., Walker, L.A., Wright, J., Best, J., Shore, R.F. 2009b. Concentrations of polycyclic aromatic hydrocarbons (PAHs) in the eggs of predatory birds in Britain. Environmental Science & Technology 43, 9010-9015.

Richter, C.A., Tieber, V.L., Denison, M.S., Giesy, J.P., 1997. An in vitro rainbow trout cell bioassay for aryl hydrocarbon receptor-mediated toxins. Environmental Toxicology and Chemistry 16, 543-550.

Safe, S., 1990. Polychlorinated-biphenyls (PCBS), dibenzo-para-dioxins (PCDDS), dibenzofurans (PCDFS), and related-compounds - environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFS). Critical Reviews in Toxicology 21, 51-88.

Sanderson, J.T., Aarts, J., Brouwer, A., Froese, K.L., Denison, M.S., Giesy, J.P., 1996. Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-O-deethylase induction in H4IIE cells: Implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicology and Applied Pharmacology 137, 316-325.

Schmutz, J.A., Trust, K.A., Matz, A.C., 2009. Red-throated loons (*Gavia stellata*) breeding in Alaska, USA, are exposed to PCBs while on their Asian wintering grounds. Environmental Pollution 157, 2386-2393.

Schroijen, C.; Windal, I.; Goeyens, L.; Baeyens, W. 2004. Study of the interference problems of dioxin-like chemicals with the bio-analytical method CALUX. Talanta 63, 1261-1268

Senthilkumar, K., Iseki, N., Hayama, S., Nakanishi, J., Masunaga, S., 2002. Polychlorinated dibenzo-p-dioxins, dibenzofurans, and dioxin- like polychlorinated biphenyls in livers of birds from Japan. Archives of Environmental Contamination and Toxicology 42, 244-255.

475 Stronkhorst, J., Leonards, P., Murk, A.J., 2002. Using the dioxin receptor-calux in vitro bioassay to
476 screen marine harbor sediments for compounds with a dioxin-like mode of action. Environmental
477 Toxicology and Chemistry 21, 2552-2561.
478 Thompson, H.M., Fernandes, A., Rose, M., White, S., Blackburn, A., 2006. Possible chemical causes of
479 skeletal deformities in grey heron nestlings (*Ardea cinerea*) in North Nottinghamshire, UK.
480 Chemosphere 65, 400-409.
481 Tsutsumi, T., Amakura, Y., Nakamura, M., Brown, D.J., Clark, G.C., Sasaki, K., Toyoda, M., Maitani, T.,
482 2003. Validation of the CALUX bioassay for the screening of PCDD/Fs and dioxin-like PCBs in retail
483 fish. Analyst 128, 486-492.
484 Van den Berg, M., Birnbaum, L., Bosveld, A.T.C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J.P.,
485 Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X.R., Liem,
486 A.K.D., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes,
487 M., Waern, F., Zacharewski, T., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for
488 humans and wildlife. Environmental Health Perspectives 106, 775-792.
489 Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H.,
490 Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A.,
491 Tuomisto, J., Tysklind, M., Walker, N., Peterson, R.E., 2006. The 2005 World Health Organization
492 Re-evaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-like
493 Compounds. Toxicological Sciences.
494 Van Wouwe, N., Windal, I., Vanderperren, H., Eppe, G., Xhrouet, C., Massart, A.C., Debacker, N., Sasse,
495 A., Baeyens, W., De Pauw, E., Sartor, F., Van Oyen, H., Goeyens, L., 2004. Validation of the CALUX
496 bioassay for PCDD/F analyses in human blood plasma and comparison with GC-HRMS. Talanta 63,
497 1157-1167.
498 Walker, L.A., Beith, S.J., Lawlor, A.J., Moeckel, C., Pereira, M.G., Potter, E.D., & Shore, R.F. , 2011.
499 Persistent Organic Pollutants (POPs) and inorganic elements in predatory bird livers and eggs 2007
500 to 2009: a Predatory Bird Monitoring Scheme (PBMS) Report.
501 http://pbms.ceh.ac.uk/docs/AnnualReports/PBMSreport_2009_2010_FINAL.pdf. Centre for
502 Ecology & Hydrology, Lancaster, p. 30.
503 Walker, L.A., Shore, R.F., Turk, A., Pereira, M.G., Best, J., 2008. The Predatory Bird Monitoring
504 Scheme: Identifying chemical risks to top predators in Britain. Ambio 37, 466-471.

505 Wienburg, C.L., Shore, R.F., 2004. Factors influencing PCB concentrations in the livers of predatory
506 birds in Britain. Environmental Pollution 132, 41-50.
507 Windal, I., Denison, M.S., Birnbaum, L.S., Van Wouwe, N., Baeyens, W., Goeyens, L., 2005a.
508 Chemically-activated luciferase gene expression (CALUX) cell bioassay analysis for the estimation of
509 dioxin like activity: critical parameters of the CALUX procedure that impact assay results.
510 Environmental Science & Technology 39, 7359-7364.
511 Windal, I., Van Wouwe, N., Eppe, G., Xhrouet, C., Debacker, V., Baeyens, W., De Pauw, E., Goeyens, L.,
512 2005. Validation and interpretation of CALUX as a tool for the estimation of dioxin-like activity in
513 marine biological matrixes. Environmental Science & Technology 39, 1741-1748.
514 Yamashita, N., Tanabe, S., Ludwig, J.P., Kurita, H., Ludwig, M.E., Tatsukawa, R., 1993. Embryonic
515 abnormalities and organochlorine contamination in double-crested cormorants (*Phalacrocorax*
516 *auritus*) and caspian terns (*Hydroprogne caspia*) from the upper great-lakes in 1988. Environmental
517 Pollution 79, 163-173.
518 Ziccardi, M.H., Gardner, I.A., Denison, M.S., 2000. Development and modification of a recombinant
519 cell bioassay to directly detect halogenated and polycyclic aromatic hydrocarbons in serum.
520 Toxicological Sciences 54, 183-193.
521
522
523 ***Figure Legends***
524
525 Figure 1. Percentage of gannet (n=16), merlin (n=11) and peregrine falcon (n = 16) eggs and kestrel,
526 sparrowhawk and heron livers (n=16 for each species) in which each coplanar PCBs was detected.
527
528 Figure 2. Median (±) inter-quartile range concentrations in (pg/g lipid) gannet (n=16), merlin (n=11)
529 and peregrine falcon (n=16) eggs and in kestrel, sparrowhawk and heron livers (n=16 for each species)
530 as determined by CALUX assay and by chemical measurement of planar PCB congeners. **** P<0.001.*
531
532 Figure 3. Comparison of log (+1) ΣPCB-TEQ concentrations and log CALUX-TEQ concentrations for
533 gannet, merlin and peregrine falcon eggs. The relationships between the two measures, as
534 determined by linear regression models, are indicated by the regression lines and their 95%
535 confidence limits. When data for all eggs was pooled, analysis was by ANCOVA (see text for details)

Figure 4. Comparison of log (+1) ΣPCB-TEQ concentrations and log CALUX-TEQ concentrations for sparrowhawk, kestrel and heron livers. The relationship between the two measures, as determined by linear regression models, was only statistically determined for pooled data (see text for details).

Figure 1

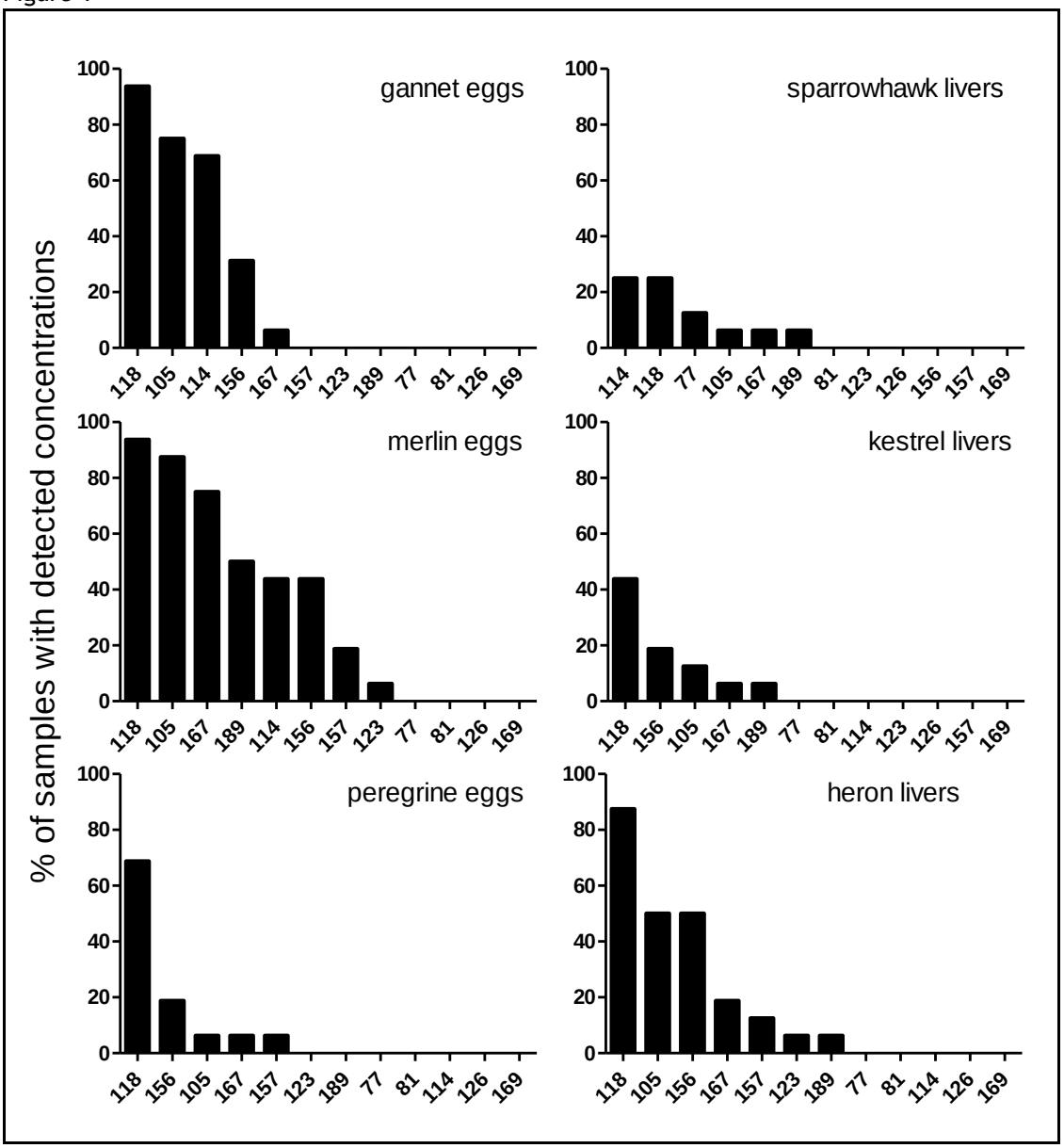
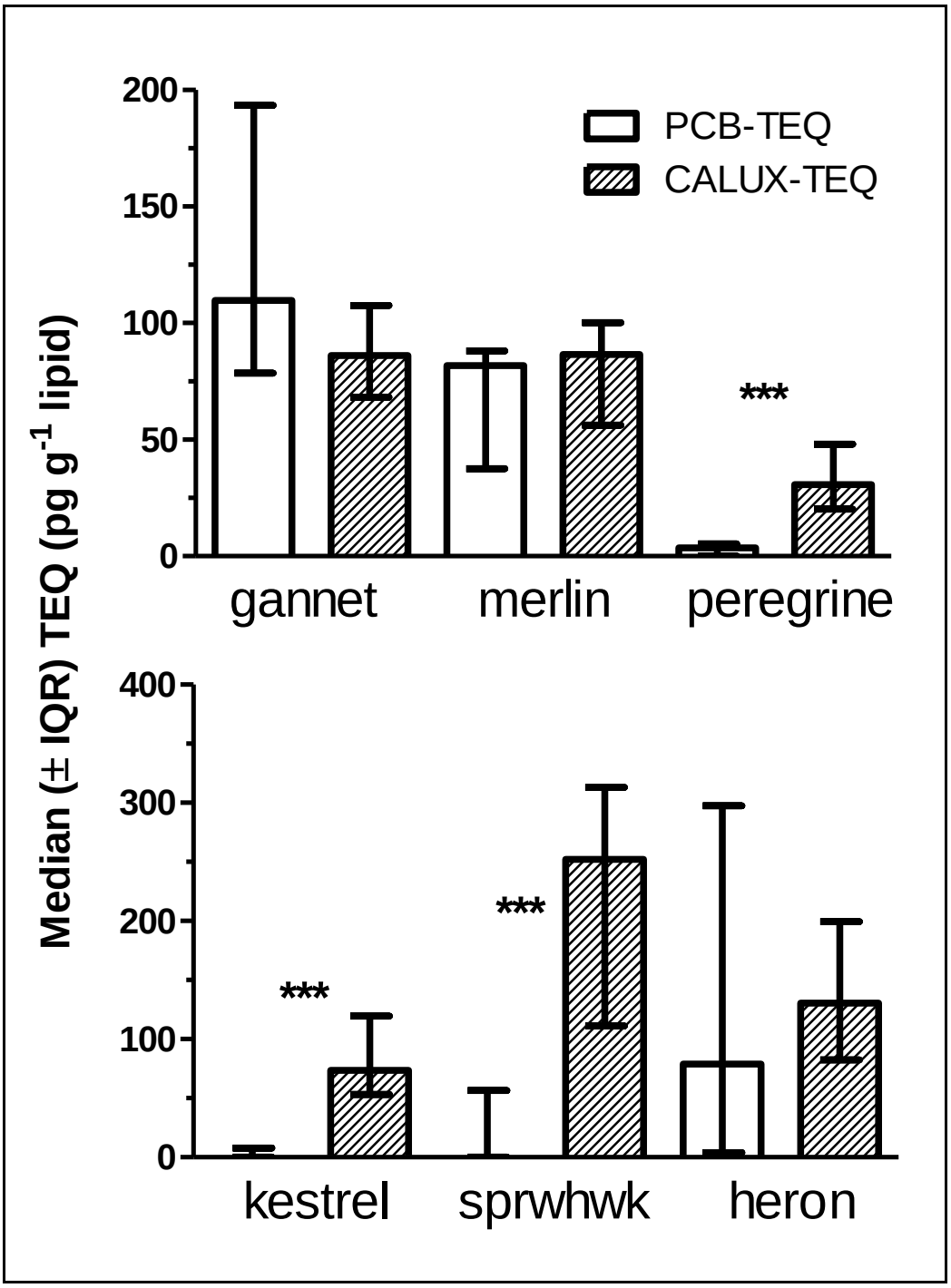
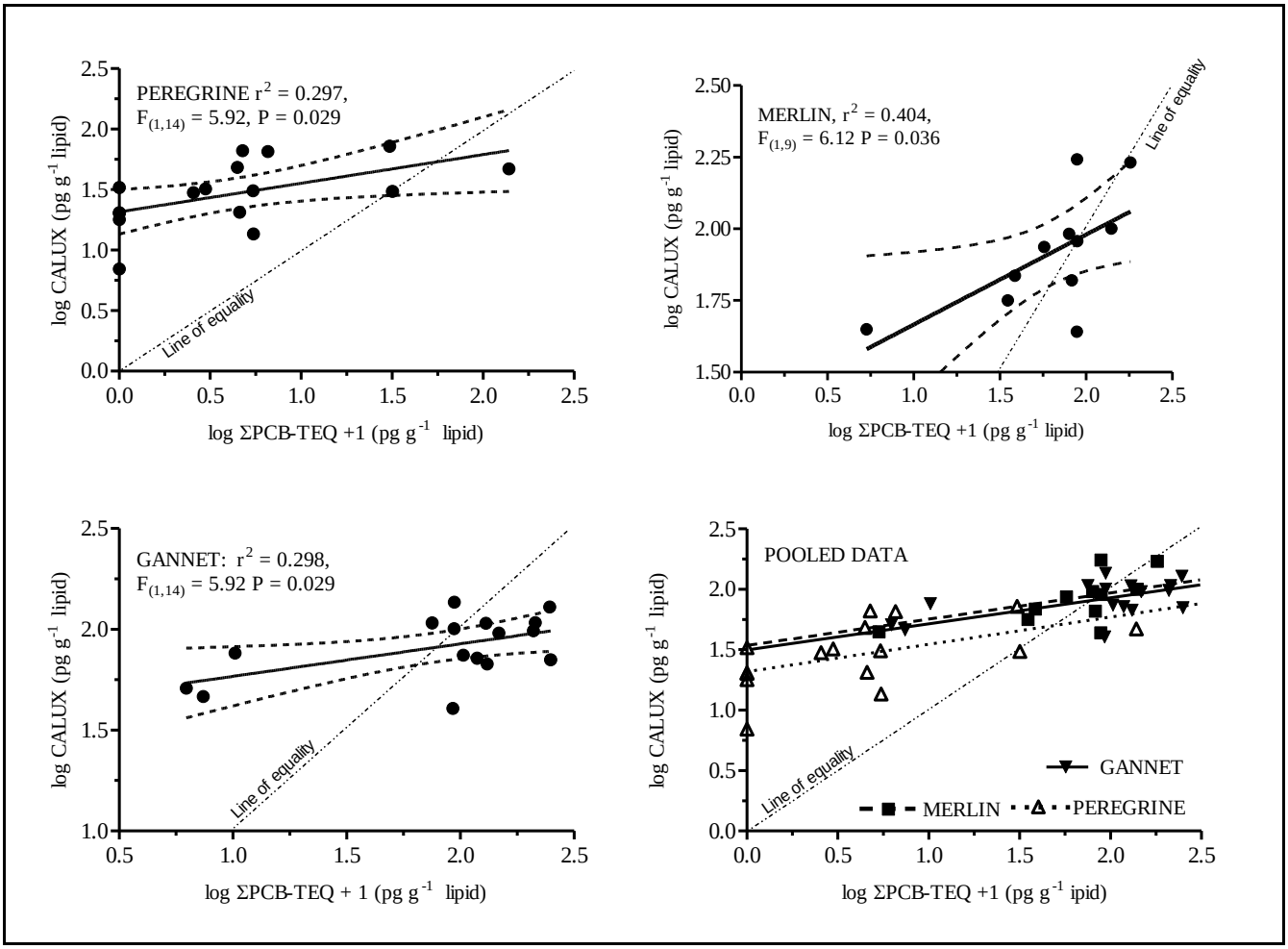

% of samples with detected concentrations
gannet eggs
118 105 114 156 167 157 123 189 77 81 126 169
sparrowhawk livers
114 118 77 105 167 189 81 123 126 156 157 169
merlin eggs
118 105 167 189 114 156 157 123 77 81 126 169
kestrel livers
118 156 105 167 189 77 81 114 123 126 157 169
peregrine eggs
118 156 105 167 157 123 189 77 81 114 126 169
heron livers
118 105 156 167 157 123 189 77 81 114 126 169
0 20 40 60 80 100

Figure 2

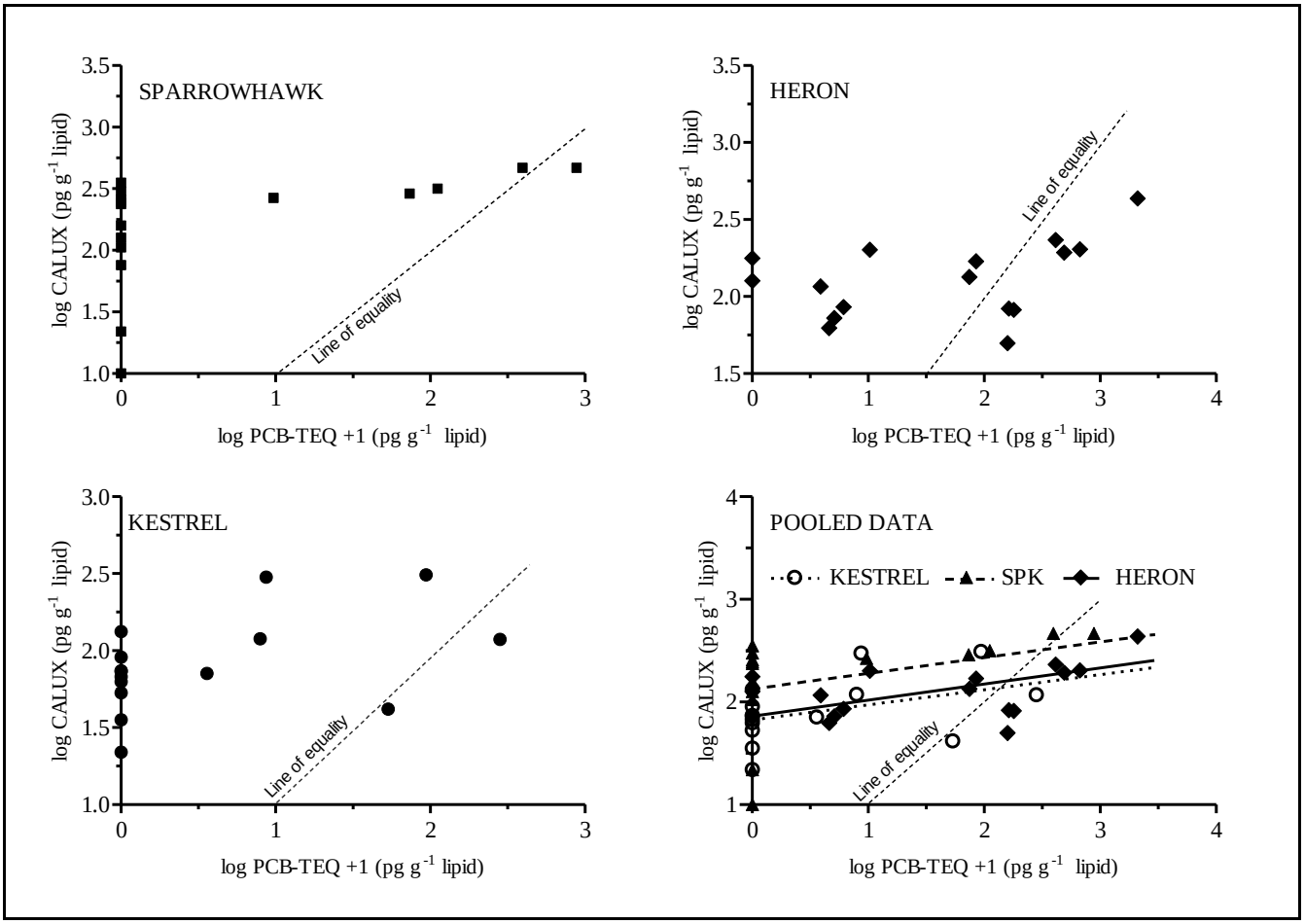

PCB-TEQ
CALUX-TEQ
Median (± IQR) TEQ (pg g-1 lipid)
200
150
100
50
0
***
gannet
merlin
peregrine
400
300
200
100
0
***
***
kestrel
sprwhwk
heron

560 Figure 3
561
562



563

564 Figure 4
565



566
567
568
569
570
571
572
573

Supporting information S1

Introduction

The DR-Calux assay, for detecting dioxin (TCDD) and dioxin-like contaminants in environmental matrices, is based on rat-hepatoma cells (H4IIE) transfected with a stable plasmide which carries the luciferase gene of fireflies (Photinus pyralis) as a reporter gene. In the presence of dioxin-like compounds these transfected cells produce the enzym luciferase. The luciferase thus formed reacts with added luciferine under production of light. The quantity of light produced can be measured in a luminometer. The luciferase production is used to calculate a TEQ- value (TCDD-equivalent) with the aid of an TCDD-standard curve. The TEQ-value is based on the total amount of dioxin-like compounds, including furanes, PCB's, PBB's, PBDE's, etc.

This method was developed at the Toxicology Section of Wageningen University and Research Centre, and validated for samples of whole blood, blood plasma, tissues (liver, muscle) and sediments

Sample preparation

Samples were initially resolved in 100 µl of a 1:1 mixture of acetone and hexane in each vial. The contents was mixed well for 1 minute. After adding 15 µl DMSO for all egg-samples, 20 µl DMSO for all liver samples (except XE010-1 and XE013-1, these were dissolved in 15 µl) and 100 µl DMSO for spiked samples, the acetone/hexane was evaporated under gentle flow of nitrogen gas.

Calux-assay

The assay used in-vitro cultivated H4IIE-luc cells, plated on 96-well Packard ViewPlates. Cells were grown on the plates for 24 hours until a confluent layer was reached. Sample dilutions were prepared in culture medium. Cells were exposed to the sample dilutions for 24 hours (0.4 % of DMSO). After cell lysis, according to assay protocol, the cells were stored at –80 ^{0}C until measurement.

Luminescence measurement

Bio-luminescence was measured using a Luminoskan RS from Thermo Life Science, equipped with two internal injectors to inject the luciferine containing FlashMix, and the NaOH for signal quenching.

Calculations

All samples were corrected for the solvent DMSO signal. A standard graph (Figure S1) was use to interpolate the TEQ's (pM) from the measured Relative Light Units (RLU's). Each plate contained a 0 and a 10 pM calibration sample, which was used for plate-to-plate correction. The difference between the calculated EC50 from the standard graph and the validated EC50 concentration for TCDD (=10 pM) was used to correct data.

Samples were measured in two different dilutions, in calculating the final results always the dilution with RLU between the RLU's of 1 pM and 10 pM TCDD was used. When samples were measured on more than one plate, the average of the results was used for further calculation.

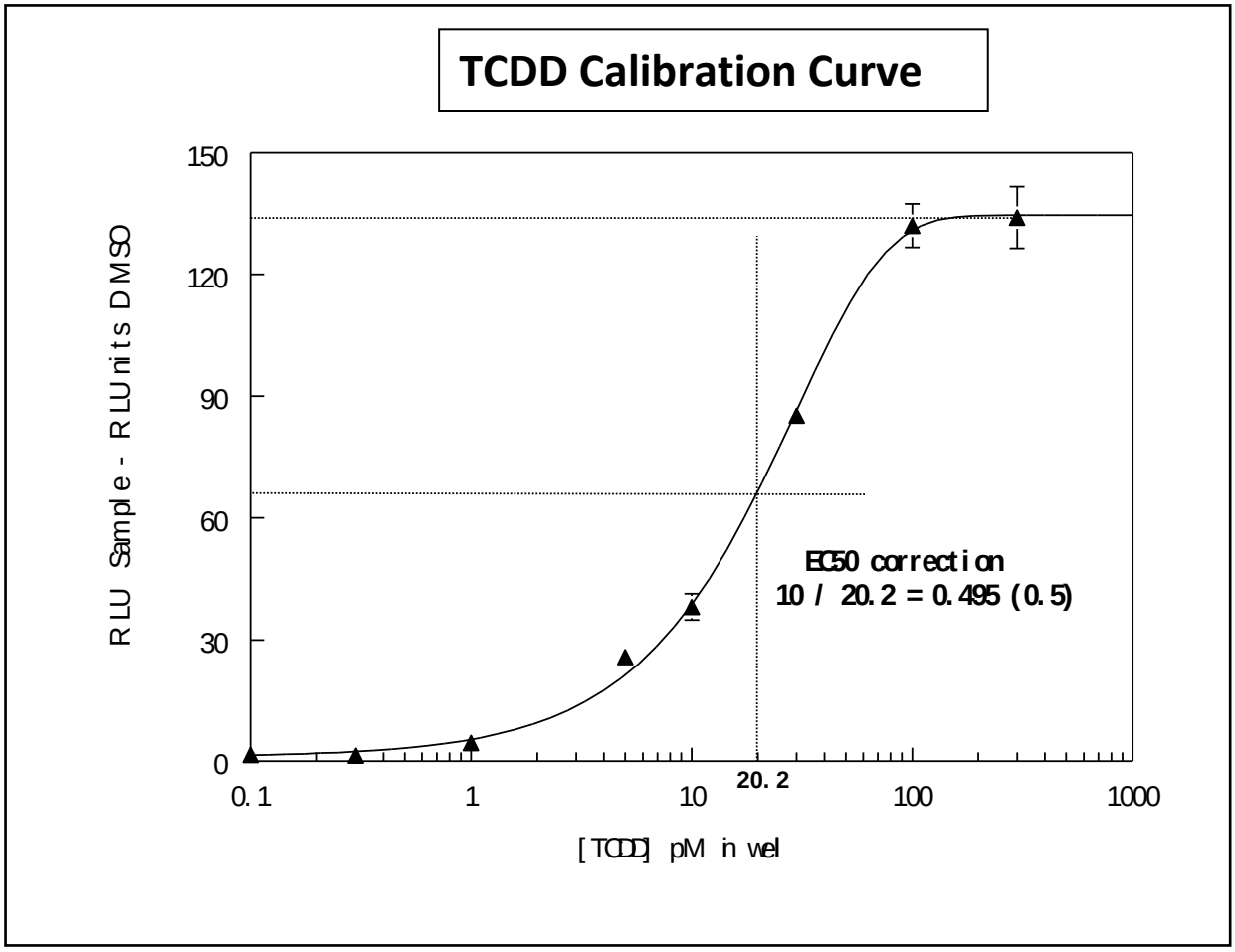


Figure S1. Dose response curve used in CALUX assay to estimate TCDD equivalents in samples