ARTICLE IN PRESS

Science of the Total Environment xxx (xxxx) xxx

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Assessing the seasonal and intrinsic variability of neurotoxic and cyto-genotoxic biomarkers in blood of free-living Eleonoras' falcons

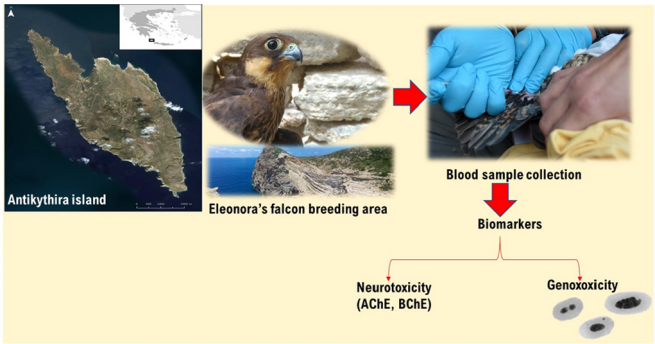
Vasiliki Tsarpali [a], Christina Kassara [a], Christos Barboutis [b], Maria Papadimitraki [a], Maria Kloukinioti [a], Sinos Giokas [a], Stefanos Dailianis [a,*]

[a] Department of Biology, Section of Animal Biology, University of Patras, GR-26500 Patras, Greece
[b] Antikythira Bird Observatory, Hellenic Ornithological Society/BirdLife Greece, Themistokleous str. 80, GR-10681 Athens, Greece

HIGHLIGHTS

- Neural and genetic biomarkers in blood of Eleonorás falcons were investigated.
- Birds are subject to environmental pressure during their pre- and breeding period.
- Cholinesterase activity and nuclear abnormalities were measured in plasma and erythrocytes.
- Birds are likely to be exposed to neurotoxic and cyto-genotoxic potential.
- Birds' reproductive and nutritional status regulate the observed biomarker values.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 July 2019
Received in revised form 4 October 2019
Accepted 19 October 2019
Available online xxxx

Editor: Lotfi Aleya

Keywords:
Avian blood
Biomarkers
Cholinesterase
Cyto-genetic abnormalities
Falco eleonorae
Migratory birds

ABSTRACT

In the present study we investigated seasonal and intrinsic variability of neurotoxic and cyto-genotoxic biomarkers in blood plasma and erythrocytes of free-living Eleonoras' falcons, captured during the pre-breeding (May of 2017 and 2018) and breeding period (September of 2017) on the Antikythira Island (Greece). Specifically, blood samples of captured birds were prepared for the determination of cholinesterase (ChEs, i.e. acetylcholinesterase/AChE and butyrylcholinesterase/ BChE) activity, as well as the formation of nuclear (i.e. the formation of micronuclei into the cells/MN, binucleated cells/BN and others), and cellular/cytoplasmic (i.e. echinocytes/EC, acanthocytes/AC and notched cells/NC) abnormalities in blood plasma and erythrocytes, respectively. Our results indicated that birds sampled in late May had higher ChE and BChE activity levels, as well as higher frequency of total nuclear abnormalities. The latter were also higher in second calendar year (2cy) birds. Cellular/cytoplasmic abnormalities were less frequent in falcons having better body condition, sampled in late May, as well as in light-morph falcons. The observed ChEs activities, as well as nuclear and cellular/cytoplasmic abnormalities revealed that Eleonora's falcons are likely to be exposed to chemical agents with neurotoxic and cyto-genotoxic potential year round, while different aspects of their biology and ecology, such as their reproductive and nutritional status, could mediate their levels. Although we encourage more sampling campaigns to verify the identified seasonal and intrinsic sources of variation in biomarkers tested, the current study enriches the existing knowledge about their usefulness in the environmental monitoring and risk assessment of migratory birds, like Eleonoras' falcon.

* Corresponding author.
E-mail address: sdailianis@upatras.gr (S. Dailianis).

https://doi.org/10.1016/j.scitotenv.2019.135101
0048-9697/

Please cite this article as: V. Tsarpali, C. Kassara, C. Barboutis et al., Assessing the seasonal and intrinsic variability of neurotoxic and cyto-genotoxic biomarkers in blood of free-living Eleonoras' falcons, Science of the Total Environment, https://doi.org/10.1016/j.scitotenv.2019.135101

1. Introduction

Avian species are considered among the most useful bioindicators of environmental quality, thus responding rapidly to environmental contaminants (Grue and Shipley, 1984; García-Fernández et al., 1995; Spahn and Sherry, 1999; Dauwe et al., 2000; Parker and Goldstein, 2000; Wayland et al., 2001; Baesse et al., 2015). Among them, migratory birds are of great concern due to their relatively increased mobility compared to other animal taxa, which in turn increases the risk of exposure to a variety of contaminants and human-derived substances while visiting different geographic areas throughout the year (Bakaloudis, 2012). Specifically, due to their exposure to trace elements/metals (Oliveira et al., 2004) and organic substances (i.e., pesticides and other biocides, veterinary residues and detergents) via the food chain, as well as to air pollutants (gases or particles, etc.) (Esselink et al., 1995; Eeva and Lehikoinen, 1996; Valdes, 2010; Sanderfoot and Holloway, 2017), migratory birds are threatened by serious metabolic disorders (Smith, 1991; Baesse et al., 2015) as well as neurotoxic and genotoxic damages, which in turn may affect various aspects of their fitness, including their breeding performance and survival (Santos et al., 2012, 2017). Based on the latter, the investigation of biomarkers in their tissues can contribute to the identification of early threats during different stages of their annual cycle (Rattner and Fairbrother, 1991).

Biomarkers commonly linked with neurological and genetic disorders, like cholinesterase (ChE) activity and the formation of micronuclei (MN assay) are widely measured in blood plasma and erythrocytes of birds, respectively. In fact, the family of ChEs, mainly formed by acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8), is widely used for assessing the neurotoxic potency of various pollutants, such as organophosphates and carbamate pesticides (Hooper et al., 1989; Thompson, 1991; Rainwater et al., 1995; Goldstein et al., 1999; Parsons et al., 2000; Maul and Farris, 2004; Santos et al., 2012). Those ChE enzymes catalyze the hydrolysis of the neurotransmitter acetylcholine, but differ in substrate specificity, inhibitor susceptibility and tissue distribution (Monteiro et al., 2005; Radic and Taylor, 2006). Specifically, although AChE is mainly found in the brain while BChE is predominant in plasma of avian species (Claudie et al., 2005), several studies showed that AChE might also occur in avian plasma with wide interspecies differences (Claudie et al., 2005; Strum et al., 2008). On the other hand, the detection of MN and other nuclear abnormalities in different cellular types indicates the presence of bioavailable xenobiotics with genotoxic and mutagenic potential (Baesse et al., 2015; Hussain et al., 2012, 2014). The MN assay has been previously performed in erythrocytes from free-living birds (Quirós et al., 2008; Skarphedinsdottira et al., 2010) and/or other captive taxa (Zúñiga-González et al., 2000, 2001; Pinhati et al., 2006) as valuable tool for identifying alterations in DNA integrity caused by mutagenic and clastogenic agents released in the environment (Sutherland et al., 2004).

Given that the investigation of biomarkers could be a promising tool for the establishment of efficient conservation measures (Rattner and Fairbrother, 1991), the major aim of the present study was to determine ChEs (AChE and BChE) activity in blood plasma as well as the formation of nuclear (NA) and cellular/cytoplasmic (CA) abnormalities in erythrocytes of a long-distance migratory top predator, namely Eleonora's falcon *(Falco eleonorae* Géné, 1839). Eleonora's falcon is a trans-equatorial migratory raptor of the Palearctic region that breeds colonially in the Mediterranean Sea, the Canary Islands and the northwestern coast of Africa and migrates to South-East (SE) Africa, mainly to Madagascar, to overwinter (Walter, 1979). Interestingly, Greece hosts over 85% of Eleonora's falcon world population during the breeding period (Dimalexis et al., 2008), while one of the largest colonies worldwide is located on the island of Antikythira in the southern Aegean Sea. Although Eleonorás falcon is classified as "Least Concern" by the IUCN (Birdlife International 2019), it is considered a priority species in Europe, included in Annex I of the Directive 2009/147/EC on the conservation of wild birds and in Annex II of the Bern Convention. In this context, monitoring the health status of Eleonora's falcon is of great importance not only for evaluating the conservation status of the species' populations, but also as a tool for assessing the habitat quality of geographically disjunct areas year-round (Bakaloudis, 2012).

Previous studies measuring pesticide residues and trace elements, in excrements (Bianchi et al., 2003), eggs (Clark and Peakall, 1977; Ristow et al., 1980; Bianchi et al., 2003; Gschweng et al., 2011), tissues of dead falcons and tissues of their prey (Clark and Peakall, 1977) suggested that Elenora's falcons can be exposed to some extent to environmental contaminants during their annual cycle, which is anticipated considering their foraging ecology and distribution pattern year-round. More specifically, during the breeding season the species' diet comprises mainly of migratory, primarily insectivorous, passerines that constitute a locally superabundant food source in the vicinity of its colonies (Walter, 1979). Throughout the rest of the year the species switches to an insectivorous diet (Ristow, 2004); the spatiotemporal patterns of insect availability result in a relatively wider distribution of Eleonora's falcons during the pre-breeding and wintering period that encompasses a variety of habitats, such as forests, cultivated areas, wetlands, both in lowland and mountainous areas (Ristow and Wink, 1995; Ristow, 2010; Mellone et al., 2013a; Kassara et al., 2017 and references therein). Pesticide use in cultivated areas both in Europe and Madagascar could thus lead to secondary poisoning through the consumption of contaminated food, either migratory passerines or insects, during different stages of its annual cycle (Clark and Peakall, 1977; Ristow et al., 1980; Gschweng et al., 2011). Even if in previous studies the measured levels of contaminant load were low and did not seem to incur adverse effects on the individuals' fitness (but see Clark and Peakall, 1977), recorded incidents of Eleonora's falcons from southern Greece, including one falcon found on Antikythira island, exhibiting poisoning symptoms suggested that direct exposure to contaminants can even be lethal and could lead to population decline (Ristow, 2001). Therefore, the investigation of biomarkers in tissues of Eleonora's falcons that do not show clinical symptoms could provide a broader perspective on the exposure of the species to environmental pollutants and complement previous findings.

Moreover, being a well-studied species, Eleonora's falcon is considered appropriate for evaluating seasonal and intrinsic variability in this respect (Gard and Hooper, 1993; Maul and Farris, 2004 and references therein). More specifically, recent studies have provided evidence for morph-, age- and sex-related variability in the species' immune defense, namely antioxidant capacity, incidence of pathogen infection and immune status (Galván et al., 2010; Gangoso et al., 2011, 2015, 2016). Therefore, in the framework of the present study we carried out for the first-time a thorough exploratory analysis to investigate the effect of body condition, age, sex, morph and stage of the annual cycle on the activity levels of ChEs and the frequency of nuclear and cellular abnormalities. Consequently, our study is expected to furnish further insight into the ecophysiology of the species and serve as reference for future work.

2. Materials and methods

2.1 Ethics statement

Trapping, bird ringing and sampling of Eleonora's falcons were carried out under the licenses 6Y0Ξ4653Π8-ΞΓ5 and

Ψ9Θ24653Π8-PT3 issued by the Greek Ministry of Environment and Energy.

2.2. Morphological and sexual characteristics of Falco eleonorae

Eleonora's falcon is a sexually dimorphic raptor; sexes can be easily distinguished by differences in the coloration of the eye ring and cere (Wink et al., 1982). Both sexes can also appear in two distinct morphs, a dark and a light one, that follow a typical simple Mendelian inheritance pattern (Wink et al., 1978). Breeding age is typically reached after 2–3 years (Ristow et al., 1983) (age class 6 in this text). Until then and while at their breeding grounds, sexually immature falcons tend to disperse a lot (Ristow and Wink, 1995; Ristow, 2010) visiting a variety of habitats (see Introduction).

2.3. Sample collection

Eleonorás falcons were captured with mist nets during the pre-breeding period (May of 2017 and 2018) and breeding period (September of 2017). In all cases, trapping efforts took place between 09.00 and 12.00 am (local hour, UTC + 3), thus avoiding possible diurnal variation in ChEs activity, at a natural pond where the falcons usually visit to drink and bath on the island of Antikythira (Fig. 1).

A total of 48 birds were captured during the trapping surveys ($N_{May\ 2017}$ = 12, $N_{May\ 2018}$ = 17, $N_{September\ 2017}$ = 19). All captured individuals were marked with a metal and plastic leg ring according to Bairlein et al. (1995), aged, sexed and classified per morph according to Forsman (1999) and weighed to the nearest 0.1 g, using an electronic balance. Other morphometrical parameters, including wing length, were measured following well-established protocols (Eck et al., 2011). A size-corrected body mass index (body mass/wing length^3 cf. Ekman and Hake, 1990; hereafter, body index) was calculated for every captured bird.

Blood smears were prepared for all captured birds, while plasma samples were available for 42 birds. Specifically, blood samples (ca 300–500 μL per individual, corresponding to <1% of body weight) were drawn from the brachial vein, using a sterile syringe (1 mL; 27′′ needle). A few drops (almost 50–100 μL) were used for preparing blood smears (in duplicate) according to Hussain et al. (2014), in order to determine both nuclear and cellular/cytoplasmic abnormalities. The remaining blood sample (almost 200–400 μL) was transferred to heparinized microcentrifuge tubes (in triplicate per individual) and kept on ice box until further processing later during the day (i.e. within two hours). In particular, the plasma was isolated following centrifugation (4000 × *g*, 10 min, 4 °C) and stored at −70 °C until ChE analysis in the laboratory.

2.4. Cholinesterase (ChE) analysis

Total plasma ChE and acetylcholinesterase (AChE) activities were measured according to a modified Ellman's method (Ellman et al., 1961), using a 96-well microplate spectrophotometer (Gard and Hooper, 1993). All samples were 10-fold diluted and analyzed at 25 °C using 0.05 M Tris buffer (pH = 8), 3.23 × 10^{-4} M 5,50-dithiobis (2-nitrobenzoic acid) (DTNB/Sigma-Aldrich, Germany) and 10^{-3} M acetylthiocholine Iodide (AThCh/Sigma-Aldrich, Germany) as substrate. Samples were also incubated for 5 min in tetra-isopropyl-pyrophosphoramide (iso-OMPA/Sigma-Aldrich, Germany), a selective inhibitor of BChE, before AThCh addition, in order to determine AChE activity. BChE activity was obtained by calculating the difference between total ChE and AChE activities. Horse serum samples were used as standards and blank wells were also used to correct the obtained results. All samples were read every 30 s for 5 min at a wavelength of 412 nm and the results were expressed as nmol min^{-1} mL^{-1}, using a molecular coefficient (ε = 13,600 $M^{-1}cm^{-1}$) (Ellman et al., 1961).

2.5. Determination of nuclear and cellular/cytoplasmic abnormalities in blood cells

Blood smears (N = 2 per individual) were air-dried, fixed in methanol for 3 min and finally stained with 5% v/v Giemsa stain for 5–7 min. Thereafter, all slides were mounted in Canada balsam and analyzed under a light microscope (100 × magnification, using immersion oil).

A total number of 2000 erythrocytes per individual (N = 1000 cells $slide^{-1}$ × 2 slides) bearing flat and intact cytoplasm, without overlapping with other cells and without intracytoplasmic detritus, with bounded nucleus and homogeneous staining throughout the cell and the nucleus, were examined for the presence of both nuclear (NA) and cellular/cytoplasmic (CA) abnormalities, according to well-established criteria (see Fig. 2 and SM Table 1) (for more details see Guilherme et al., 2008; Fenech et al., 2011; Anbumani and Mohankumar, 2012; Harabawy and Mosleh, 2014; Jindal and Verma, 2015; Quero et al., 2016; Taha et al., 2019).

Statistical analysis

The Shapiro-Wilk test was used to assess whether the values of the examined biomarkers followed the normal distribution. With the exemption of ChE and AChE, all biomarkers violated the normality assumption, thus non-parametric tests were adopted to obtain comparable results.

Birds sampled in early and late May were considered separately in the statistical analyses described thereafter, given that in the former case the falcons are more likely to have just arrived at their breeding grounds after a long and energetically demanding migration journey, while the arrival date of the latter remained unknown. More specifically, in our study area the first falcons are already observed in the second half of April, but their numbers build up in May. However, due to their high pre-breeding mobility (Wink et al., 1991; Ristow and Wink, 1995; Ristow, 2010; Mellone et al., 2013a) falcons visiting the natural ponds and trapped in late May could have already completed spring migration 2–3 weeks ago, in which case the measured enzyme activity levels and cellular abnormalities could rather reflect the environmental quality at the pre-breeding areas than that at their wintering and staging areas. Our choice to consider two subperiods in spring was also supported by the results of a preliminary comparison (results not shown here) between the values of the examined biomarkers of the individuals caught in early May with the ones captured in late May, which indicated statistically significant differences in half of the cases.

Next, we explored whether (a) the measured ChEs (AChE, BChE, and their ratio) activity levels and the frequency of nuclear and cellular abnormalities (NA and CA respectively) correlated with the body index of the sample birds and (b) the former also varied with age, sex, morph and/or period of sampling (hereafter, period). To this end, we considered the frequency of CA, namely echinocytes (EC), acanthocytes (AC) and notched cells (NC), the frequency of total NAs (T_{NA}), but also the frequency of micronuclei (MN) separately for reasons of comparison with previous MN studies. More specifically, we estimated the correlation among enzyme activity levels, the frequency of cellular abnormalities and body index using the Spearman correlation coefficient (r_s), while explored differences in enzyme activity levels and the frequency of cellular abnormalities per grouping variable via non-parametric tests,

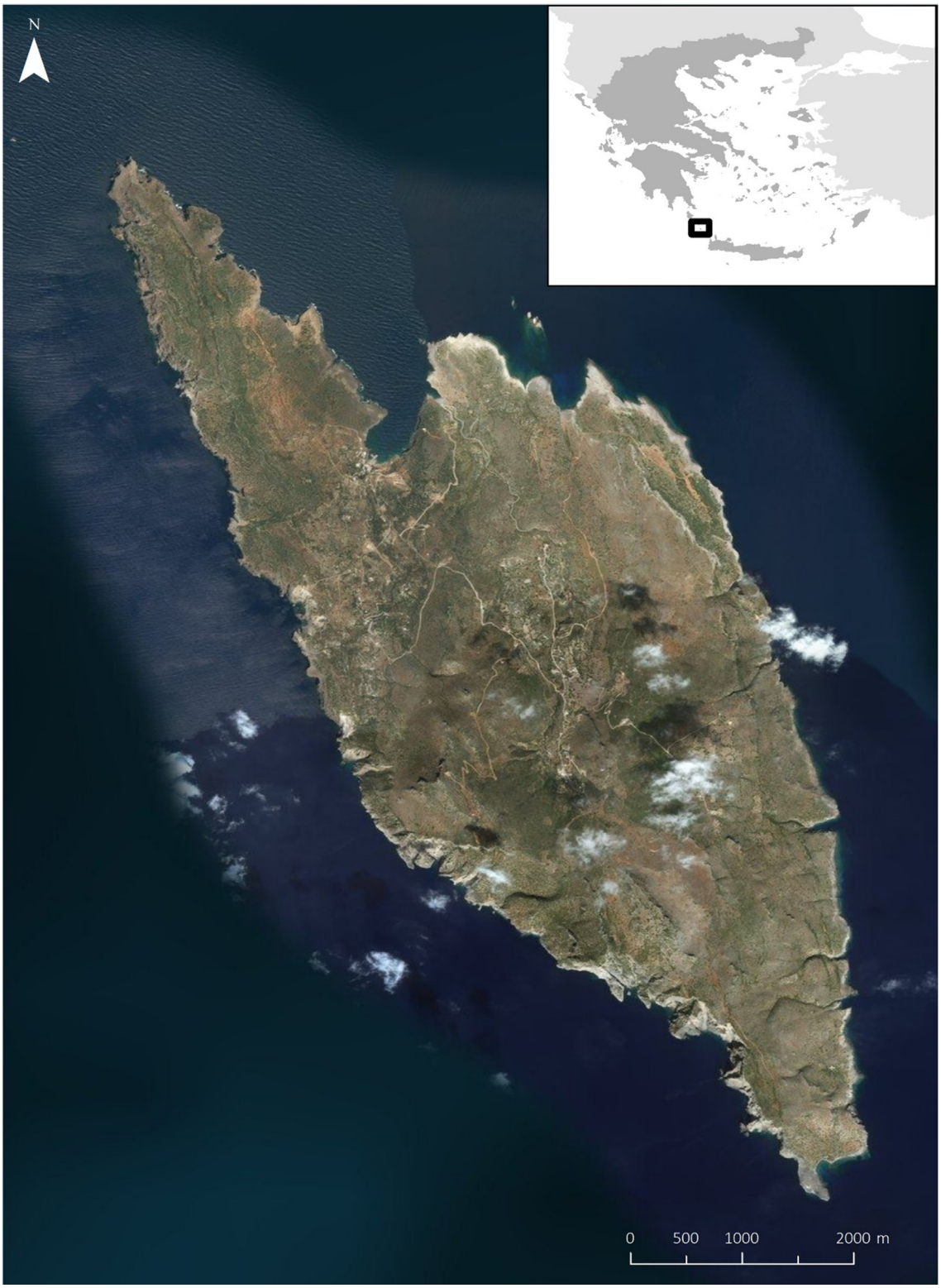


Fig. 1. The location of the study area, namely island of Antikythira, in south Greece Satellite imagery © 2019 Microsoft Corporation Earthstar Geographics SIO.

namely Mann Whitney U-tests (for age, sex and morph) and Kruskal Wallis test (for period). Regarding the latter, post-hoc tests were run to determine the statistical significance of all pairwise comparisons among sampling periods.

Statistical analyses were performed in IBM SPSS statistics version 20 and statistical significance was set at $\alpha = 0.05$.

3. Results

3.1. Enzyme activities in blood plasma

Statistically significant seasonal differences were found regarding the ChE (χ^2 = 12.857, p = 0.002), the BChE (χ^2 = 21.251,

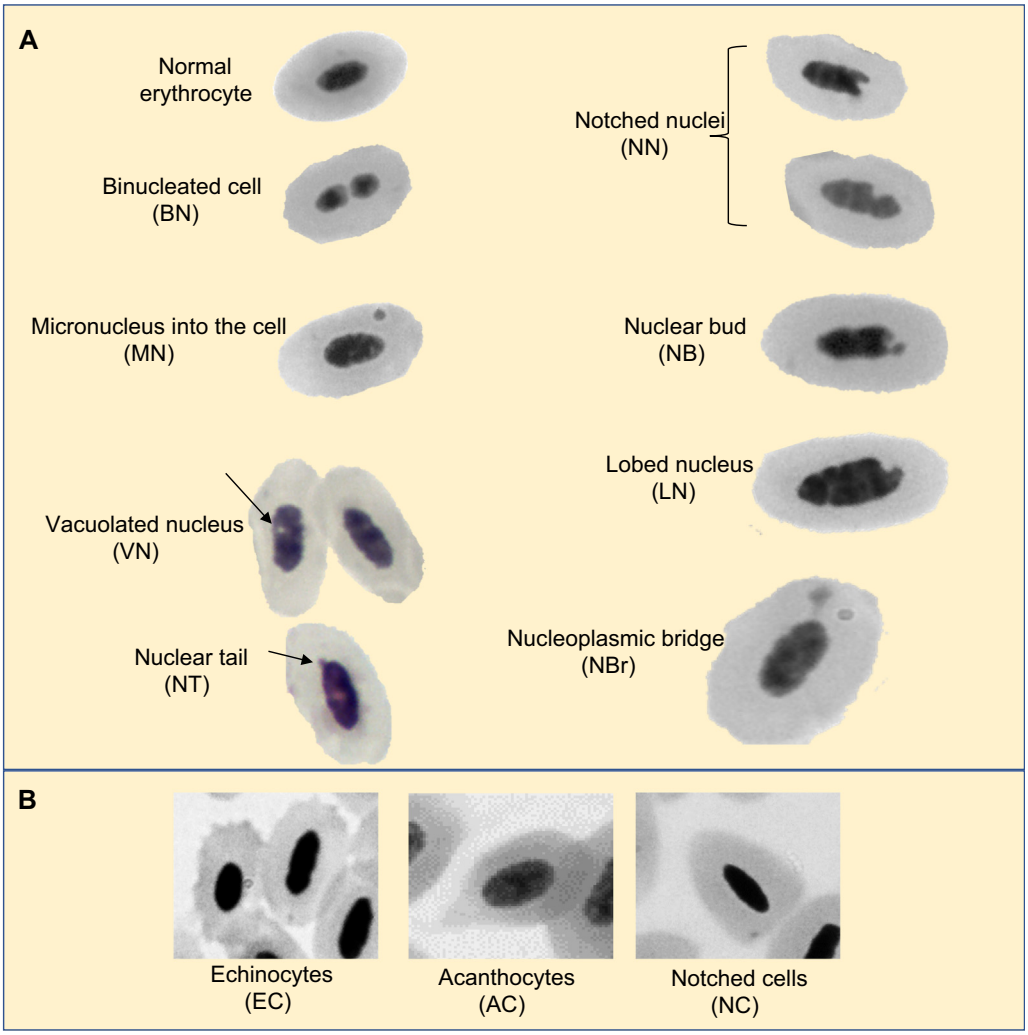


Fig. 2. Representative light microscope pictures of (A) nuclear and (B) cellular/cytoplasmic abnormalities (NAs and CAs respectively) in red blood cells of *Falco eleonorae* (x 400 magnification).

Table 1
Descriptive statistics (mean and standard deviation) of biomarkers measured in blood plasma and erythrocytes of Eleonorás falcons during the sampling campaigns. Sample sizes are given in parentheses.

	Age		**Sex**		**Morph**		**Period**		
	2nd cy	**after 2nd cy**	**male**	**female**	**dark**	**light**	**September**	**late May**	**early May**
ChE	7.02 ± 3.35(13)	5.98 ± 2.54(29)	6.15 ± 3.14(17)	6.40 ± 2.64(25)	5.49 ± 2.63(10)	6.55 ± 2.86(32)	5.94 ± 2.32(17)	8.04 ± 2.71(16)	3.89 ± 1.81(9)
AChE	3.95 ± 2.39(13)	3.89 ± 1.57(29)	3.61 ± 1.47(17)	4.10 ± 2.05(25)	3.81 ± 1.85(10)	3.93 ± 1.85(32)	4.10 ± 1.95(17)	4.18 ± 1.67(16)	3.03 ± 1.79(9)
BChE	3.02 ± 1.92(13)	2.10 ± 1.70(29)	2.58 ± 2.15(17)	2.26 ± 1.56(25)	1.70 ± 1.42(10)	2.60 ± 1.87(32)	1.71 ± 1.21(17)	3.97 ± 1.55(16)	0.85 ± 0.75(9)
AChE / BChE	3.06 ± 5.95(13)	5.40 ± 9.48(28)	5.67 ± 11.63(16)	4.01 ± 5.93(25)	3.92 ± 3.63(9)	4.87 ± 9.48(32)	6.70 ± 11.74(17)	1.36 ± 1.39(16)	6.91 ± 7.34(8)
T_{NA}	6.53 ± 3.53(14)	4.47 ± 2.99(34)	4.30 ± 3.20(20)	5.62 ± 3.25(28)	4.30 ± 3.16(10)	5.27 ± 3.29(38)	7.08 ± 2.56(19)	4.52 ± 3.49(17)	2.67 ± 1.77(12)
MN	1.21 ± 0.84(14)	0.80 ± 0.80(34)	0.78 ± 0.78(20)	1.03 ± 0.86(28)	0.69 ± 0.77(10)	0.98 ± 0.84(38)	0.92 ± 0.82(19)	1.00 ± 0.87(17)	0.81 ± 0.83(12)
BN	2.27 ± 1.41(14)	0.93 ± 1.20(34)	1.61 ± 1.67(20)	1.11 ± 1.15(28)	1.27 ± 1.76(10)	1.34 ± 1.31(38)	0.89 ± 1.51(19)	1.95 ± 1.26(17)	1.11 ± 1.16(12)
LN	0.49 ± 0.82(14)	0.29 ± 0.46(34)	0.31 ± 0.50(20)	0.38 ± 0.65(28)	0.23 ± 0.48(10)	0.38 ± 0.61(38)	0.61 ± 0.66(19)	0.24 ± 0.57(17)	0.10 ± 0.29(12)
NN	1.08 ± 1.55(14)	0.71 ± 1.04(34)	0.50 ± 0.75(20)	1.04 ± 1.42(28)	0.48 ± 0.63(10)	0.90 ± 1.31(38)	1.68 ± 1.46(19)	0.31 ± 0.51(17)	0.15 ± 0.36(12)
NB	0.65 ± 0.79(14)	0.18 ± 0.44(34)	0.19 ± 0.32(20)	0.41 ± 0.73(28)	0.20 ± 0.63(10)	0.35 ± 0.59(38)	0.34 ± 0.60(19)	0.52 ± 0.72(17)	0.00 ± 0.00(12)
NBr	0.32 ± 0.33(14)	0.98 ± 1.26(34)	0.61 ± 1.01(20)	0.91 ± 1.18(28)	0.84 ± 1.05(10)	0.77 ± 1.14(38)	1.47 ± 1.38(19)	0.25 ± 0.34(17)	0.46 ± 0.78(12)
VN	0.15 ± 0.37(14)	0.12 ± 0.41(34)	0.10 ± 0.31(20)	0.14 ± 0.45(28)	0.22 ± 0.63(10)	0.10 ± 0.31(38)	0.18 ± 0.51(19)	0.15 ± 0.39(17)	0.00 ± 0.00(12)
NT	0.36 ± 0.62(14)	0.47 ± 0.82(34)	0.22 ± 0.49(20)	0.59 ± 0.89(28)	0.39 ± 0.66(10)	0.45 ± 0.80(38)	0.97 ± 0.99(19)	0.12 ± 0.17(17)	0.04 ± 0.14(12)
EC	4.51 ± 2.83(14)	4.26 ± 5.55(34)	3.18 ± 3.47(20)	5.16 ± 5.61(28)	5.20 ± 4.78(10)	4.11 ± 4.95(38)	6.63 ± 6.18(19)	2.02 ± 2.26(17)	3.98 ± 3.83(12)
NC	4.31 ± 2.45(14)	5.16 ± 4.81(34)	3.95 ± 3.64(20)	5.60 ± 4.57(28)	7.37 ± 7.11(10)	4.26 ± 2.92(38)	5.79 ± 4.90(19)	3.14 ± 2.66(17)	6.02 ± 4.47(12)
AC	0.33 ± 0.61(14)	0.30 ± 0.67(34)	0.38 ± 0.88(20)	0.25 ± 0.42(28)	0.19 ± 0.25(10)	0.34 ± 0.72(38)	0.24 ± 0.35(19)	0.50 ± 0.99(17)	0.15 ± 0.25(12)

ChE: cholinesterase; AChE: acetylcholinesterase; BChE: butyryl-cholinesterase; T_{NA}: total nuclear abnormalities; MN: micronucleus; BN: binucleated cells; LN: lobed nucleus; NN: notched nucleus; NB: nuclear bud; NBr: nucleoplasmic bridge; VN: vacuolated nucleus; NT: nuclear tails; EC: echinocytes; NC: notched cells; AC: acanthocytes; cy: second calendar year.

Please cite this article as: V. Tsarpali, C. Kassara, C. Barboutis et al., Assessing the seasonal and intrinsic variability of neurotoxic and cyto-genotoxic biomarkers in blood of free-living Eleonoras' falcons, Science of the Total Environment, https://doi.org/10.1016/j.scitotenv.2019.135101

Table 2
Univariate test results for the measured biomarkers and the explanatory variables considered in this study. The value of the statistic and the associated p-value are given. Statistically significant differences are highlighted in bold. For variable abbreviations see Table 1.

	Mann Whitney tests Age	Sex	Morph	Kruskal Wallis tests Period
ChE	144.0, p = 0.226	194.0, p = 0.635	123.5, p = 0.281	**12.9, p = 0.002**
AChE	186.5, p = 0.957	187.0, p = 0.513	155.5, p = 0.894	2.5, p = 0.287
BChE	136.0, p = 0.153	205.0, p = 0.848	112.5, p = 0.161	**21.2, p = 0.000**
AChE/BChE	121.0, p = 0.087	183.5, p = 0.659	107.5, p = 0.250	**12.7, p = 0.002**
T_{NA}	165.0, p = 0.097	215.0, p = 0.173	163.0, p = 0.493	**16.5, p = 0.000**
MN	**148.0, p = 0.039**	226.0, p = 0.253	142.5, p = 0.222	0.7, p = 0.689
EC	178.5, p = 0.177	219.0, p = 0.202	164.5, p = 0.517	**11.7, p = 0.003**
NC	237.0, p = 0.982	200.5, p = 0.096	143.5, p = 0.237	5.8, p = 0.056
AC	237.0, p = 0.979	279.5, p = 0.990	189.5, p = 0.988	0.3, p = 0.860

ChE: cholinesterase; AChE: acetylcholinesterase; BChE: butyryl-cholinesterase; T_{NA}: total nuclear abnormalities; MN: micronucleus; EC: echinocytes; NC: notched cells; AC: acanthocytes.

p = 0.000) and the ratio of AChE and BChE activity levels (χ^2 = 12.748, p = 0.002). More specifically, BChE and the ratio of AChE and BChE activity levels were higher in birds sampled in September compared to those sampled in late May (Tables 1 and 2, Fig. 3). In addition, ChE and BChE activity levels were higher in birds sampled in late May compared to those sampled in early May, while the opposite pattern was observed for the AChE/ BChE ratio activity levels (Tables 1 and 2, Fig. 3). Apart from the above-mentioned seasonal differences, the measured enzyme levels did not vary by the examined intrinsic factors (Table 2).

3.2. Nuclear and morphological/cytoplasmic abnormalities in erythrocytes

MN frequency was significantly higher in younger birds (Mann Whitney U = 148, p = 0.039; Table 1). Statistically significant differences were also found among periods regarding the frequency of T_{NA} (χ^2 = 16.547, p = 0.000) and EC (χ^2 = 11.710, p = 0.003) and only marginally for NC (χ^2 = 5.750, p = 0.056). More specifically, T_{NA} were more frequent in birds sampled in September compared to both early and late May (Tables 1 and 2, Fig. 4). Similarly, EC were more frequent in birds sampled in September compared to those sampled in late May (Tables 1 and 2, Fig. 4). No other statistically significant differences were identified based on the univariate tests (Table 2).

3.3. Correlations among parameters

ChE levels were positively correlated with AChE (r_s = 0.798) and BChE (r_s = 0.748) levels (Table 3). The ratio AChE/BChE was negatively correlated with the frequency of MN (r_s = −0.313). The latter was also positively correlated with the frequency of T_{NA} (r_s = 0.466). The frequency of EC was negatively correlated with ChE (r_s = −0.369), AChE (r_s = −0.309) and BChE levels (r_s = −0.309), while positively correlated with the frequency of T_{NA} (r_s = 0.472), MN (r_s = 0.515) and NC (r_s = 0.645). Moreover, neither the measured enzyme activity levels nor the frequency of EC, AC, NC and MN were related to the body condition of the sampled birds (Table 3).

4. Discussion

Given that migratory birds' health status could indicate the quality of their habitat, including their breeding and visiting geographic areas (Bakaloudis, 2012), in the present study was investigated for the first time biomarkers commonly used for the assessment of both neural (i.e. ChEs, like AChE and BChE activity) and cellular/genetic damages in blood plasma and erythrocytes of free-living (wild) Eleonora's falcons. Moreover, since the investi-

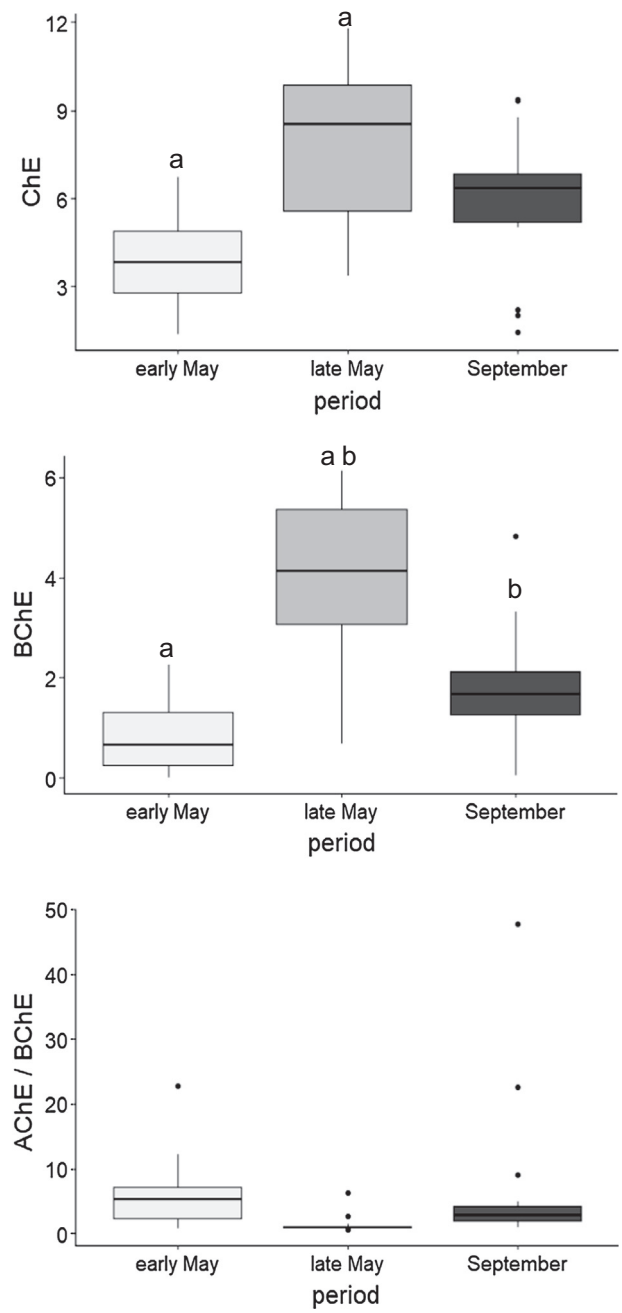


Fig. 3. Boxplots of enzyme activity levels (ChE, BChE, and AChE/BChE, expressed as nmol min^{-1} mL^{-1}) per sampling period. Statistically significant differences according to post-hoc tests are denoted by Latin letters.

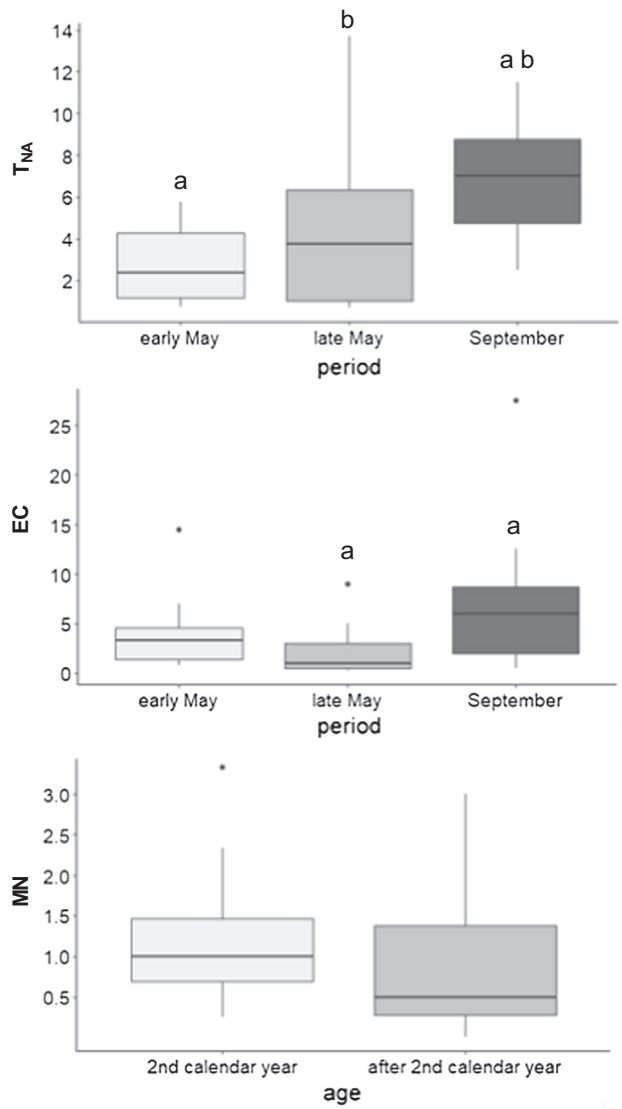


Fig. 4. Boxplots of the frequency (expressed as ‰) of total nuclear abnormalities (T_{NA}) and of echinocytes (EC) per sampling period, as well as of micronuclei (MN) with age class. In the case of sampling period, statistically significant differences according to post-hoc tests are denoted by Latin letters.

gation of sources of variation in biomarkers is highly recommended (Maul and Farris, 2004), a thorough exploratory analysis was performed for evaluating the effect of intrinsic factors, such as the body condition, age, sex, morph and stage of the annual cycle on the obtained biomarkers values. The latter is of great concern, thus enriching the existing knowledge about the usefulness of biomarkers in the environmental monitoring and risk assessment of migratory birds, like Eleonoras' falcon.

4.1. ChEs levels in blood plasma of Eleonora's falcon

Although AChE levels are considered to be lower than BChE in avian species (Maul and Farris, 2004; Santos et al., 2012), the higher AChE levels measured in blood plasma of captured Eleonoras' falcon are in accordance with those reported in other species of the family Falconidae (Roy et al., 2005). Similarly, the total ChE activity measured in blood plasma of Eleonoras' falcon seemed to be lower than those measured in other species, including Falconidae (Lanzarot et al., 2001; Maul and Farris, 2004), a fact that could be due to several factors, previously mentioned to mediate the obtained ChE activity, such as age (Grue et al., 1981; Gard and Hooper, 1993; Wolf and Kendall, 1998), species (Greig-Smith, 1991; Wolf and Kendall, 1998), reproductive condition (Fairbrother et al., 1990), nutritional status and temperature (Rattner, 1982), as well as sample handling and treatment (Fairbrother et al., 1991).

Although ChEs levels are sex-dependent in avian species, like the Japanese quail (*Coturnix japonica*) (Ludke et al., 1975; Hill, 1989), the northern bobwhite (*Colinus virginianus*) (Hill and Murray, 1987), and the American kestrel (*Falco sparverius*) (Rattner and Franson, 1984), the analyses did not reveal any variability in plasma ChE activity between males and females, which is in accordance with relevant previous studies in avian species (Niethammer and Baskett, 1983; Grue and Shipley, 1984; Hill and Murray, 1987; Robinson et al., 1988). On the other hand, birds sampled in early May had lower ChEs levels than those captured in late May and September, thus indicating seasonal variations in ChEs activity. These results are in accordance with Fildes et al. (2009), regarding ChEs activity in King Quails (*Excalfactoria chinensis*), Budgerigars (*Melopsittacus undulatus*), White-plumed Honeyeaters (*Lichenostomas penicillatus*), Yellowthroated Miners (*Manorina flavigula*), Willie Wagtails (*Rhipidura leucophrys*), Australian Reed-Warblers (*Acrocephalus australis*), Brown Songlarks (*Cincloramphus cruralis*), Double-barred Finches (*Taeniopygia bichenovii*) and Australasian Pipits (*Anthus novaeseelandiae*). However, the observed differences in ChEs values in our study are mostly due to the enhancement of BChE levels, as well as of AChE/BChE ratios, in blood plasma of birds captured in early May rather than a significant increase of AChE activity, a fact that could question the significance of the obtained seasonal ChEs differences. In fact, seasonal alterations of BChE activity, which is considered as the predominant ChE enzyme in most species, except in Falconidae, could differentially contribute to the obtained total ChEs levels (Wolf and Kendall, 1998; Roy et al., 2005), as indicated by

Table 3
Spearman correlation coefficient between all pairs of examined variables. Statistically significant values are highlighted in bold.

	body index	ChE	AChE	BChE	AChE / BChE	T_{NA}	MN	EC	NC	AC
body index	1									
ChE	−0.043	1								
AChE	0.028	**0.798**	1							
BChE	−0.152	**0.748**	0.267	1						
AChE/BChE	0.171	−0.295	0.221	**−0.812**	1					
T_{NA}	0.079	0.094	0.054	0.047	−0.042	1				
MN	−0.024	0.021	−0.162	0.184	**−0.313**	**0.466**	1			
EC	0.035	**−0.369**	**−0.309**	**−0.309**	0.040	**0.472**	**0.515**	1		
NC	0.242	**−0.361**	−0.276	−0.219	−0.071	0.264	0.280	**0.645**	1	
AC	0.013	−0.189	−0.213	−0.090	−0.009	0.178	−0.169	0.137	0.150	1

ChE: cholinesterase; AChE: acetylcholinesterase; BChE: butyryl-cholinesterase; T_{NA}: total nuclear abnormalities; MN: micronucleus; EC: echinocytes; NC: notched cells; AC: acanthocytes.

Please cite this article as: V. Tsarpali, C. Kassara, C. Barboutis et al., Assessing the seasonal and intrinsic variability of neurotoxic and cyto-genotoxic biomarkers in blood of free-living Eleonoras' falcons, Science of the Total Environment, https://doi.org/10.1016/j.scitotenv.2019.135101

the results of the current study. Since little is known about the circadian rhythms and the effect of seasonality on ChE activity (Garcia-Rodriguez et al., 1987; Rattner and Fairbrother, 1991), the captured birds' reproductive and nutritional status could merely contribute to the obtained seasonality in ChEs, AChE and BChE levels. Specifically, the first incoming birds (early May) are characterized by low energy reserves that could contribute towards reduced ChEs activity (Rattner and Fairbrother, 1991; Gard and Hooper, 1993). Eleonora's falcons cover more than 7000 km between their wintering and breeding grounds. Their migration represents a typical loop migration system (Mellone et al., 2013b), with spring routes being located east to the autumn ones, however both involving the crossing of arid environments, namely the Sahara and Arabian desert, respectively (Gschweng et al., 2008; López-López et al., 2010; Kassara et al., 2012; Mellone et al., 2013b). Eleonora's falcons have adopted a mixed stop-over/fly-and-forage strategy during migration (Mellone et al., 2013b). Especially during spring migration, they pause their north-bound journey for approximately a week hunting over diffuse staging areas located in the Horn of Africa (Gschweng et al., 2008; López-López et al., 2010; Kassara et al., 2012; Mellone et al. 2013b) where local weather conditions favor increased food (i.e. insect) availability during this time of the year (Pearson and Lack, 1992). Once they reach their breeding grounds in mid-April and until the onset of another energetically-demanding period, namely the breeding period, in mid-July, sexually mature Eleonora's falcons spend two months replenishing their fuel reserves by consuming mainly insects in a wide variety of habitats lying several hundred kilometers away from their colonies (Ristow and Wink, 1995; Ristow, 2010; Mellone et al., 2013a). Thus, during this prolonged pre-breeding period ChEs levels and especially BChE activity could elevate (late May), followed by a slight decrease during the breeding period (September) when their energetic demands increase again (Hill and Murray, 1987). Still, differences in the migration phenology among the captured birds, including timing of arrival and selection of migratory routes and staging areas, could also contribute to the observed variability between the two trapping periods in spring. Thus, further data on the migration phenology of the species are required for elucidating the latter. A larger sample size would be also desired in the future to account for any differences in the quality of the individuals and thus their resistance to contaminant exposure.

4.2. Cellular and nuclear abnormalities in erythrocytes of Eleonora's falco

Given that the quantification of MN is considered a reliable tool for evaluating the genotoxic effects in challenged organisms (Sutherland et al., 2004), including free-living birds (Quirós, et al., 2008; Skarphedinsdottira et al., 2010), the present study showed that Eleonora's falcon is subject to environmental pressure. Moreover, apart from MN and other NAs, the present study showed for the first time the presence of CAs (in terms of EC, AC and NC) in peripheral blood erythrocytes of Eleonora's falcon, as well as their significant relationship with either nuclear abnormalities or ChEs activity, thus elucidating the complementarity between those biomarkers in neurotoxicity and cyto-genotoxicity assessment, as recently reported (reviewed from Jindal and Verma, 2015).

According to the results, the MN frequency in peripheral blood erythrocytes per individual (0.92‰) was close to those reported for other species (for more details see Zúñiga-González et al., 2000; Zúñiga-González et al., 2001; Pinhati et al., 2006; Baesse et al., 2015 and references therein), while binucleated (BN) erythrocytes (1.32‰), erythrocytes with nuclear buds (NB) and nucleoplasmic bridges (NBr) (0.81 and 0.79‰, respectively) were also detected (see also SM Table 3). Although the mechanism being responsible for the formation of these types of NAs remains to be unveiled, there is evidence that NBu, originating from interstitial or terminal acentric fragments during nuclear division or in S-phase as a stage in the extrusion of extra DNA, could give rise to MN formation (Lindberg et al., 2007), while BN cells that are considered as genotoxic analog of MN (Serrano-Garcia and Montero-Montoya, 2001), could be attributed to failure of tubuline polymerization and difficulty of formation of mitotic fuse caused by aneugenic action of toxicants (de Campos Ventura et al., 2008), thus resulting in genetic imbalance and carcinogenesis (Tsangaris et al., 2011).

Bearing in mind that MN frequencies higher than 0.35‰ could reveal the exposure of organisms to genotoxic agents (Zúñiga-González et al. 2001; Hussain et al., 2012; Quero et al., 2016), the obtained results clearly indicates that MN and other NAs in erythrocytes of Eleonoras' falcon could be formatted by spontaneous chromosomal changes, as well as by clastogenic and aneugenic agents, being present in food, water and air (Brown et al., 1997; Saleh and Sarhan, 2007). Interestingly, the high frequencies of EC and NC (4.34 and 4.91‰, respectively) and to a smaller extent AC (0.31‰), as well as the statistically significant correlations between T_{NAs} and MN, clearly indicates the presence of agents with cyto-genotoxic potential in the habitats where Eleonora's falcons occur throughout the year. The latter could also explain differences in MN and NAs frequencies observed in erythrocytes of incoming birds (early and possibly late May) and breeding birds (September). The latter could be attributed to the reasons contributing to seasonal variability in the nutritional status and energetic requirements of the falcons as explained above.

Regarding MN and NAs, there is evidence that different parameters could mediate their frequencies, including (a) the nature of toxic agents each species are subjected to, (b) the period of the year, and (c) the chemical kinetics of the toxins in combination with the speed of the hemopoietic cycle (Okonkwo et al., 2011; Osman et al., 2011; Kumar, 2012). According to the latter, following the long, north-bound journey towards their breeding grounds, once there the falcons intensify their hunting activity to replenish their energy reserves, thus increasing the risk of accumulating genotoxic contaminants (Grisolia et al., 2009; Braham et al., 2017), whose fate and effects could be merely depended on birds' metabolic/detoxification efficiency. In this context, erythrocytes' malformation could be due to the increasing amount of contaminants, being capable of enhancing ROS generation within cells, thus leading to lipid peroxidation and disturbance of cellular membrane integrity, changes in protein conformation and ATP depletion (Sorg, 2004; Ahmad et al., 2006; Jindal and Verma, 2015). Previous studies assessing heavy metal and pesticide residuals in egg-shells of breeding Eleonora's falcons originating from colonies across the breeding range of the species suggest a rather low contaminant load during the breeding period that does not seem to affect egg fertility despite regional differences in the reported values (Clark and Peakall, 1977; Ristow et al., 1980; Bianchi et al., 2003; Gschweng et al., 2011). However, exposure to contaminants might in some cases lead even to death, as was the case of the high frequency of Eleonora's falcon found poisoned in Crete, most of which near a breeding colony in the north-eastern part of the island (Ristow, 2001). These incidents were linked to the observed population decline in the same colony at that time, namely late 90 s – early 2000 (Ristow, 2001). Although the evidence provided was mostly circumstantial and the exact cause of death was not determined in all cases, secondary poisoning via the consumption of water contaminated with insecticides is the most likely explanation that can either incur direct mortality or reduce the falcons' fertility (Ristow, 2001). Thus, at least within the species' breeding grounds habitat quality could affect the observed levels of nuclear abnormalities.

On the other hand, MN frequency in erythrocytes of younger birds was higher than those observed in older ones, a fact that could be due to differences among the clearance efficiency of their reticuloendothelial system, since the ability to eliminate old erythrocytes (lifespan almost 100–120 days) or those containing NAs, improves with age (Ramírez-Munõz et al., 1999; Zúñiga-González et al., 2000). Previous immune assays and parasitological studies on samples obtained from Eleonora's falcons during the breeding season pertained to sexually mature falcons, thus neither seasonal nor age-related patterns have been investigated to date, except for the prevalence of parasites (Martínez-Abraín and Urios, 2002; Gangoso et al., 2019). However, these studies have indicated morph-related variability in the antioxidant capacity (Galván et al., 2010), inflammatory cell-mediated response (Gangoso et al., 2011, 2015) and the immune response of nestlings (Gangoso et al., 2015), as well as in the parasite prevalence of adults (Gangoso et al., 2016), according to which light morph individuals are better armored against pathogens than dark morph ones. However, given that no other age- or morph-related variability was observed in the remaining variables that we examined, the observed differences should be further investigated considering a larger sample size. The latter remains to be elucidated in the current case, since the number of captured individuals was small and probably not statistically robust for verifying the hypothesis.

5. Conclusion

In the present study were investigated for the first-time biomarkers commonly linked with neurological (i.e. ChEs, like AChE and BChE) and cyto-genetic (i.e. cellular and nuclear abnormalities) disorders in peripheral blood (plasma and erythrocytes) of free-living migratory species, like Eleonora's falcon. The current findings revealed that those biomarkers could be complimentarily used in monitoring campaigns for evaluating the conservation status of the species' populations and the quality of their breeding and non-breeding areas. According to the observed ChEs levels, NAs (in terms of MN and other NA) and CAs (in terms of EC, AC and NC) frequencies measured in birds captured during the pre-breeding and breeding period, it is likely that Eleonora's falcons are exposed to chemical agents with neurotoxic and cyto-genotoxic potential in the habitats they occur that could affect their health status overtime. Moreover, the reproductive and nutritional status of migratory birds could mediate the obtained ChEs, nuclear and/or cellular/cytoplasmic values, thus leading to season-related variability in the obtained results. However, the absence of any trait-related differences in most of the tested biomarkers with sex, age classes, morphs and the body condition of captured birds, probably due to the small sample size, reinforces the need for more extensive sampling campaigns (i.e. larger sample size), in order to increase the statistical robustness and the knowledge about any potential trait-related sources of, as well as annual variation in biomarkers tested. Last but not least, the identification of areas that serve as foraging grounds for the species during different periods of its annual cycle could contribute to the unveiling of both the source and location of exposure to genotoxic agents, and thus provide valuable information for the conservation of Eleonora's falcon.

Acknowledgements

The present work was conducted in the framework of the project "LIFE13 NAT/GR/000909 Conservation measures to assist the adaptation of *Falco eleonorae** to climate change" with the financial support of the European Union LIFE Instrument and the Green Fund. The authors declare that there are no conflicts of interest. All persons gave their informed consent prior to their inclusion in the study, read and approved the paper and materially participated in the research and/or article preparation. This is contribution No 25 from Antikythira Bird Observatory – Hellenic Ornithological Society. Further data associated with the current article can be found within the supplementary file.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.135101.

References

Ahmad, I., Maria, V.L., Oliveira, M., Pacheco, M., Santos, M.A., 2006. Oxidative stress and genotoxic effects in gill and kidney of *Anguilla anguilla* L. exposed to chromium with or without pre-exposure to beta-naphthoflavone. Mutat. Res. 608, 16–28.

Anbumani, S., Mohankumar, M.N., 2012. Gamma radiation induced micronuclei and erythrocyte cellular abnormalities in the fish *Catla catla*. Aquat. Toxicol. 122–123, 125–132.

Baesse, C.Q., de Magalhães, Carneiro, Tolentino, V., da Silva, A.M., de Andrade Silva, A., Ferreira, G.A., Paniago, L.P.M., Nepomuceno, J.C., de Melo, C., 2015. Micronucleus as biomarker of genotoxicity in birds from Brazilian Cerrado. Ecotoxicol. Environ. Saf. 115, 223–228.

Bakaloudis, D.E., 2012. A preliminary overview of monitoring for raptors in Greece. Acrocephalus 33, 293–295.

Bairlein, F., Jenni, L., Kaiser, A., Karlsson, L., Noordwijk, A., Peach, W., Pilastro, A., Spina, F., Walinder, G., 1995. European-African songbird migration network: manual of field methods. Wilhelmshaven, Germany.

Bianchi, N., Leonzio, C., Casini, S., Fossi, M.C., Focardi, S., 2003. Valutazione dello stato di contaminazione nel *Falco eleonorae* attraverso l' utilizzo di metodologie non distruttive. Università di Siena, Italy, Dipartamento di Scienze Ambientali.

BirdLife International, 2019. Species factsheet: *Falco eleonorae* Downloaded from http://www.birdlife.org, .

Braham, R.P., Blazer, V.S., Shaw, C.H., Mazik, P.M., 2017. Micronuclei and other erythrocyte nuclear abnormalities in fishes from the Great Lakes basin. USA. Environ. Mol. Mutagen. 58, 570–581.

Brown, R.E., Brain, J.D., Wang, N., 1997. The avian respiratory system: a unique model for studies of respiratory toxicosis and for monitoring air quality. Environ. Health Perspect. 105, 2.

Clark, A.L., Peakall, D.B., 1977. Organochloride residues in Eleonora's falcon *Falco eleonorae*, its eggs and its prey. Ibis 119, 353–358.

Claudie, R., Grolleau, G., Chamoulaud, S., Rivere, J.L., 2005. Plasma B-esterase activities in European raptors. J. Wildl. Dis. 41, 184–208.

Dauwe, T., Bervoets, L., Blust, R., Pinxten, R., Eens, M., 2000. Can excrement and feathers of nestling songbirds be used as biomonitors for heavy metal pollution? Arch. Environ. Contam. Toxicol. 39, 541–546.

de Campos Ventura, B., de Franceschi de Angelis, D., Marin-Morales, M.A., 2008. Mutagenic and genotoxic effects of the atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. Pestic. Biochem. Phys. 90, 42–51.

Dimalexis, A., Xirouchakis, S., Portolou, D., Latsoudis, P., Karris, G., Fric, J., Georgiakakis, P., Barboutis, C., Bourdakis, S., Ivovic, M., Kominos, T., Kakalis, E., 2008. The status of Eleonora's Falcon (*Falco eleonorae*) in Greece. J. Ornithol. 149, 23–30.

Directive 2009/147/EC of the European Parliament and of the Council of 30 November 2009 on the conservation of wild birds OJ L 20, 26.1.2010.

Eck, S., van den Elzen, R., Fiebig, J., Fiedler, W., Heynen, I., Nicolai, B., Töpfer, T., Winkler, R., Woog, F., 2011. Measuring birds. Vögel vermessen. Deutsche Ornithologen-Gesellschaft, Wilhelmshaven.

Eeva, T., Lehikoinen, E., 1996. Growth and mortality of nestling great tits (*Parus major*) and piedflycatchers (*Ficedula hypoleuca*) in a heavy metal pollution gradient. Oecologia 108, 631–639.

Ekman, J.B., Hake, M.K., 1990. Monitoring starvation risk: adjustments of body reserves in greenfinches (*Carduelis chloris* L.) during periods of unpredictable foraging success. Behav. Ecol. 1, 62–67.

Ellman, G.L., Courtney, K.D., Andres Jr, V., Featherstone, R.M., 1961. A new rapid and colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Esselink, H., Vandergeld, F.M., Jager, L.P., Posthuma-Trumpie, G.A., Zoun, P.E.F., Baars, A.J., 1995. Biomonitoring heavy metals using the barn owl (*Tyto albaguttatus*): sources of variation especially relating to body condition. Arch. Environ. Contam. Toxicol. 28, 471–486.

Fairbrother, A., Craig, M.A., Walker, K., O'Loughlin, D., 1990. Changes in mallard *Anas platyrhynchos* serum chemistry due to age, sex, and reproductive condition. J. Wildl. Dis. 26, 67–77.

Fairbrother, A., Marden, B.T., Bennett, J.K., Hooper, M.J., 1991. Methods used in determination of cholinesterase activity. In: Mineau, P. (Ed.), Cholinesterase-inhibiting insecticides. Elsevier Science, Amsterdam, pp. 35–71.

Fenech, M., Kirsch-Volders, M., Natarajan, A.T., Surralles, J., Crott, J.W., Parry, J., Norppa, H., Eastmond, D.A., Tucker, J.D., Thomas, P., 2011. Molecular

mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis 26, 125–132.
Fildes, K.J., Szabo, J.K., Astheimer, L., Hooper, M., Butterner, W.A., 2009. Plasma cholinesterase characteristics in native Australian birds: significance for monitoring avian species for pesticide exposure. Journal of Birdlife Australia 109, 41–47.
Forsman, D., 1999. The raptors of Europe and the Middle East: a handbook of field identification. London, T & AD Poyser.
Galván, I., Gangoso, L., Grande, J.M., Negro, J.J., Rodríguez, A., Figuerola, J., Alonso-Alvarez, C., 2010. Antioxidant machinery differs between melanic and light nestlings of two polymorphic raptors. PLoS ONE 5, e13369.
Gangoso, L., Grande, J.M., Ducrest, A.-L., Figuerola, J., Bortolotti, G.R., Andrıs, J.A., Roulin, A., 2011. MC1R-dependent, melanin based colour polymorphism is associated with cell-mediated response in the Eleonora's falcon. J. Evol. Biol. 24, 2055–2063.
Gangoso, L., Roulin, A., Ducrest, A.L., Grande, J.M., Figuerola, J., 2015. Morph-specific genetic and environmental variation in innate and acquired immune response in a color polymorphic raptor. Oecologia 178, 1113–1123.
Gangoso, L., Gutiérrez-López, R., Martínez-de la Puente, J., Figuerola, J., 2016. Genetic colour polymorphism is associated with avian malarial infections. Biol. Lett. 12, 20160839.
Gangoso, L., Gutiérrez-López, R., Martínez-de la Puente, J., Figuerola, J., 2019. Louse of flies of Eleonora's falcons that also feed on their prey are evolutionary dead-end hosts for blood parasites. Mol. Ecol. 28, 1812–1825.
Gard, N.W., Hooper, M.J., 1993. Age-dependent changes in plasma and brain cholinesterase activities of Eastern bluebirds and European starlings. Wildl. Dis. 29, 1–7.
García-Fernández, A.J., Sánchez-García, J.A., Jimenez-Montalban, P., Luna, A., 1995. Lead and cadmium in wild birds in southeastern Spain. Environ. Toxicol. Chem. 14, 2049–2058.
Garcia-Rodriguez, T., Ferrer, M., Recio, R., Castroviejo, J., 1987. Circadian rhythms of determined blood chemistry values in buzzards and eagle owl. Comp. Biochem. Physiol. Part 88A, 663–669.
Goldstein, M.I., Lacher, T.E., Zaccagnini, M.E., Parker, M.L., Hooper, M.J., 1999. Monitoring and assessment of Swainson's hawks in Argentina following restrictions on monocrotophos use, 1996–1997. Ecotoxicology 8, 215–224.
Greig-Smith, P.W., 1991. Use of cholinesterase measurements in surveillance of wildlife poisoning in farmland. In: Mineau, P. (Ed.), Cholinesterase inhibiting insecticides, their impact on wildlife and the environment. Elsevier Science, Amsterdam, pp. 127–150.
Grisolia, C.K., Rivero, C.L.G., Starling, F.L.R.M., da Silva, I.C.R., Barbosa, A.C., Dorea, J. G., 2009. Profile of micronucleus frequencies and DNA damage in different species of fish in a eutrophic tropical lake. Gen. Molecul. Biol. 32, 138–143.
Grue, C.E., Powell, G.V.N., Gladson, N.L., 1981. Brain cholinesterase (ChE) activity in nestling starlings: Implications for monitoring exposure of nestling songbirds to ChE inhibitors. Bull. Environ. Contam. Toxicol. 26, 544–547.
Grue, C.E., Shipley, B.K., 1984. Sensitivity of nestling and adult starlings to dicrotophos, and organophosphate pesticide. Environ. Res. 35, 454–465.
Gschweng, M., Kalko, E.K.V., Querner, U., Fielder, W., Berthold, P., 2008. All across Africa: highly individual migration routes of Eleonora's falcon. Proc. R. Soc. London Ser. B 275, 2887–2897.
Gschweng, M., Tataruch, F., Fröhlich, O., Kalko, E.K.V., 2011. Dead embryos despite low contaminant loads in eggs of Eleonora's falcon. ISRN Zool. https://doi.org/10.5402/2011/510202.
Guilherme, S., Valega, M., Pereira, M.E., Santos, M.A., Pacheco, M., 2008. Erythrocytic nuclear abnormalities in wild and caged fish (*Liza aurata*) along an environmental mercury contamination gradient. Ecotoxicol. Environ. Saf. 70, 411–421.
Harabawy, A.S.A., Mosleh, Y.Y.I., 2014. The role of vitamins A, C, E and selenium as antioxidants against genotoxicity and cytotoxicity of cadmium, copper, lead and zinc on erythrocytes of Nile tilapia, *Oreochromis niloticus*. Ecotoxicol. Environ. Saf. 104, 28–35.
Hill, E.F., 1989. Sex and storage affect cholinesterase activity in blood plasma of Japanese quail. J. Wildl. Dis. 25, 580–585.
Hill, E.F., Murray, H.C., 1987. Seasonal variation in diagnostic enzymes and biochemical constituents of captive northern bobwhites and passerines. Comp. Biochem. Physiol. 87B, 933–940.
Hooper, M.J., Detrich, P.J., Weisskopf, C.P., Wilson, B.W., 1989. Organophosphorus insecticide exposure in hawks inhabiting orchards during winter dormant-spraying. Bull. Environ. Contam. Toxicol. 42, 651–659.
Hussain, R., Khan, R., Mahmood, F., Rehan, S., Ali, F., 2014. Clinico-hematological and tissue changes induced by butachlor in male Japanese quail (*Coturnix japonica*). Pestic. Biochem. Physiol. 109, 58–63.
Hussain, R., Mahmood, F., Khan, A., Javed, M.T., Rehan, S., Mehdi, T., 2012. Cellular and biochemical effects induced by atrazine on blood of male Japanese quail (*Coturnix japonica*). Pestic. Biochem. Physiol. 103, 38–42.
Jindal, R., Verma, S., 2015. In vivo genotoxicity and cytotoxicity assessment of cadmium chloride in peripheral erythrocytes of Labeorohita (Hamilton). Ecotoxicol. Environ. Saf. 118, 1–10.
Kassara, C., Fric, J., Gschweng, M., Sfenthourakis, S., 2012. Complementing the puzzle of Eleonora's Falcon (*Falco eleonorae*) migration: new evidence from an eastern colony in the Aegean Sea. J. Ornithol. 153, 839–848.
Kassara, C., Gangoso, L., Mellone, U., Piasevoli, G., Hadjikyriakou, T.G., Tsiopelas, N., Giokas, S., López-López, P., Urios, V., Figuerola, J., Silva, R., Bouten, W., Kirschel, A.N.G., Virani, M.Z., Fiedler, W., Berthold, P., Gschweng, M., 2017. Current and future suitability of wintering grounds for a long-distance migratory raptor. Sci. Rep. 7, 8798.
Kumar, S.P., 2012. Micronucleus assay: a sensitive indicator for aquatic pollution. Int. J. Res. Biosci. 1 (2), 32–37.
Lanzarot, M.D.P., Montesinos, A., San Andrés, M.I., Rodríguez, C., Barahona, M.V., 2001. Hematological, protein electrophoresis and cholinesterase values of free-living nestling peregrine falcons in Spain. J. Wildl. Dis. 37 (1), 172–177.
Lindberg, H.K., Wang, X., Jarventaus, H., Falck, G.C.-M., Norppa, H., Fenech, M., 2007. Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutat. Res. 617, 33–45.
López-López, P., Limiñana, R., Mellone, U., Urios, V., 2010. From the Mediterranean Sea to Madagascar: are there ecological barriers for the long-distance migrant Eleonora's falcon? Landscape Ecol. 25, 803–813.
Ludke, J.L., Hill, E.F., Dieter, M.P., 1975. Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. Arch. Environ. Contam. Toxicol. 3, 1–21.
Maul, J.D., Farris, J.L., 2004. The effect of sex on avian plasma cholinesterase enzyme activity: a potential source of variation in an avian biomarker endpoint. Arch. Environ. Contam. Toxicol. 47, 253–258.
Martínez-Abraín, A., Urios, G., 2002. Absence of blood parasites in nestlings of the Eleonora's falcon (*Falco eleonorae*). J. Raptor Res. 36 (2), 139–141.
Mellone, U., López-López, P., Limiñana, R., Urios, V., 2013a. Summer pre-breeding movements of Eleonora's Falcon *Falco eleonorae* revealed by satellite telemetry: implications for conservation. Bird Conserv. Int. 23, 487–494.
Mellone, U., López-López, P., Limiñana, R., Piasevoli, G., Urios, V., 2013b. The trans-equatorial loop migration system of Eleonora's falcon: differences in migration patterns between age classes, regions and seasons. J. Avian Biol. 44, 417–426.
Monteiro, M., Quintaneiro, C., Morgado, F., Soares, A.M.V.M., Guilhermino, L., 2005. Characterization of the cholinesterases present in head tissues of the estuarine fish *Pomatoschistus microps*: Application to biomonitoring. Ecotoxicol. Environ. Safe. 62, 341–347.
Niethammer, K.R., Baskett, T.S., 1983. Cholinesterase inhibition of birds inhabiting wheat fields treated with methyl parathion and toxaphene. Arch. Environ. Contam. Toxicol. 12, 471–475.
Okonkwo, J.C., Obiakor, M.O., Nnabude, P.C., 2011. Micronuclei profile: an index of chromosomal aberrations in freshwater fishes (*Synodontis clarias* and *Tilapia nilotica*). Online J. Anim. Feed Res. 1, 40–45.
Oliveira, F.N.S., Freire, F.C.O., Aquino, A.R.L., 2004. Bioindicadores de Impacto Ambiental em Sistemas Agrícolas Orgânicos. Embrapa Agroind, Trop., p. 24.
Osman, A.G.M., Abd-El-Baset, M., El Reheem, A., Moustafa, M.A., Mahmoud, U.M., Abuel-Fadl, K.Y., Kloas, W., 2011. In situ evaluation of the genotoxic potential of the river Nile: I. micronucleus and nuclear lesion tests of erythrocytes of oreochromis niloticus niloticus (Linnaeus, 1758) and clarias gariepinus (Burchell, 1822). Toxicol. Environ. Chem. 93 (5), 1002–1017.
Parker, M.L., Goldstein, M.I., 2000. Differential toxicities of organophosphate and carbamate insecticides in the nestling European starling (*Sturnus vulgaris*). Arch. Environ. Contam. Toxicol. 39 (2), 233–242.
Parsons, K.C., Matz, A.C., Hooper, M.J., Porkras, M.A., 2000. Monitoring wading bird exposure to agricultural chemicals using serum cholinesterase activity. Environ. Toxicol. Chem. 19, 1317–1323.
Pearson, D.J., Lack, P.C., 1992. Migration patterns and habitat use by passerine and near-passerine migrant birds in eastern Africa. Ibis 134, 89–98.
Pinhati, V.R., Allgayer, M.C., Breyer, A.S., Pereira, R.A., Silva, J., 2006. Determinação de danos basais no DNA de araras canindé (*Ara ararauna*) através do Teste de Micronúcleos: uma ferramenta na avaliação da saúde animal e seu uso no biomonitoramento da poluição ambiental. Acta Sci. Vet. 34 (3), 313–317.
Quero, A.A.M., Ferré, D.M., Zarco, A., Cuervo, P.F., Gorta, N.B.M., 2016. Erythrocyte micronucleus cytome assay of 17 wild bird species from the central Monte desert. Argentina. Environ. Sci. Pollut. Res. 23, 25224–25231.
Quirós, L., Ruiz, X., Sanpera, C., Jover, L., Piña, B., 2008. Analysis of micronucleated erythrocytes in heron nestlings from reference and impacted sites in the Ebro basin (NE Spain). Environ. Pollut. 155, 81–87.
Radic, Z., Taylor, P., 2006. Structure and Function of Cholinesterases. In: Gupta, R.C. (Ed.), Toxicology of Organophosphate and Carbamate Pesticides. Elsevier Academic Press, Waltham, pp. 161–186.
Ramírez-Munõz, M.P., Zúñiga, G., Torres-Bugarín, O., Portilla, E., García-Martínez, D., Ramos-Mora, A., Cantú, J.M., Sánchez-Corona, J., 1999. Evaluation of micronucleus test in peripheral blood erythrocytes by means of the splenectomized model. Lab. Anim. Sci. 49, 418–420.
Rainwater, T.R., Leopold, V.A., Hooper, M.J., Kendall, R.J., 1995. Avian exposure to organophosphorus and carbamate pesticides on a coastal South Carolina golf course. Environ. Toxicol. Chem. 14, 2155–2161.
Rattner, B.A., 1982. Diagnosis of anticholinesterase poisoning in birds: effects of environmental temperature and underfeeding on cholinesterase activity. Environ. Toxicol. Chem. 1, 329–335.
Rattner, B.A., Fairbrother, A., 1991. Biological variability and the influence of stress on cholinesterase activity. In: Mineau, P. (Ed.), Cholinesterase-inhibiting Insecticides. Elseviers, Amsterdam, pp. 89–107.
Rattner, B.A., Franson, J.C., 1984. Methyl parathion and fenvalerate toxicity in American kestrels: Acute physiological responses and effects of cold. Can. J. Physiol. Pharmacol. 62, 787–792.
Ristow, D., 2001. Poison is causing the sudden population decline of Eleonora's Falcon. Int. Hawkwatcher 3, 10–17.
Ristow D., 2004. On the insect diet of Eleonora's Falcon Falco eleonorae and its importance for coloniality, in: Chancellor, R.D., Meyburg, B.-U. (Eds.), Raptors Worldwide, WWGBP/MME, pp. 705–712.

Ristow D., 2010. Up-date on breeding status and review on Eleonora's Falcon Falco eleonorae when away from the breeding sites. II-Merill 32, 1-5.

Ristow, D., Conrad, B., Wink, C., Wink, D., 1980. Pesticide residues of failed eggs of Eleonora's falcon *Falco eleonorae* from an Aegean colony. Ibis 122 (1), 74–76.

Ristow, D., Wink, M., 1995. Distribution of non-breeding Eleonora's falcon Falco eleonorae. II-Merill 28, 1-10.

Ristow, D., Wink, M., Wink, C., Friemann, H., 1983. Biologie des Eleonorenfalken (Falco eleonorae): 14. Das Brutreifealter der Weibchen. Journal für Ornithologie 124, 291–293.

Robinson, S.C., Kendall, R.J., Robinson, R., Driver, C.J., Lacher Jr., T.E., 1988. Effects of agricultural spraying of methyl parathion on cholinesterase activity and reproductive success in wild starlings (*Sturnus vulgaris*). Environ. Toxicol. Chem. 7, 343–349.

Roy, C., Grolleau, G., Chamoulaud, S., Rivière, J.L., 2005. Plasma b-esterase activities in european raptors. J. Wildl. Dis. 43, 184–208.

Saleh, K., Sarhan, M.A.A., 2007. Clstogenic analysis of chicken farms using micronucleus test in peripheral blood. J. Appl. Sci. Res. 3, 1646–1649.

Sanderfoot, O.V., Holloway, T., 2017. Air pollution impacts on avian species via inhalation exposure and associated outcomes. Environ. Res. Lett. 12, 083002.

Santos, C.S.A., Brandao, R., Monteiro, M.S., Bastos, A.C., Soares, A.M.V.M., Loureiro, S., 2017. Assessment of DNA damage in Ardeacinerea and Ciconiaciconia: A 5-year study in Portuguese birds retrieved for rehabilitation. Ecotoxicol. Environ. Safe. 136, 104–110.

Santos, C.S.A., Monteiro, M.S., Soares, A.M.V.M., Loureiro, S., 2012. Characterization of cholinesterases in plasma of three portuguese native bird species: Application to Biomonitoring. PloS One 7, e33975.

Serrano-Garcia, L., Montero-Montoya, R., 2001. Micronuclei and chromatid buds are the result of related genotoxic events. Environ. Mol. Mutagen. 38, 38–45.

Skarphedinsdottira, H., Hallgrimssonb, G.T., Hanssona, T., Hägerrotha, P.A., Liewenborga, B., Tjärnlunda, U., Åkermana, G., Barsiene, J., Balka, L., 2010. Genotoxicity in herring gulls (*Larus argentatus*) in Sweden and Iceland. Mutat. Res. 702, 24–31.

Smith, A.G., 1991. Chlorinated hydrocarbon insecticides. In: Hayes, W.J., Laws, E.R. (Eds.), Handbook of Pesticide Toxicology. Academic Press, California, pp. 731–916.

Sorg, S., 2004. Oxidative stress: a theoretical model or a biological reality? C. R. Biol. 327, 649–662.

Spahn, S.A., Sherry, T.W., 1999. Cadmium and lead exposure associated with reduced growth rates, poorer fledging success of little blue heron chicks (*Egretta caerulea*) in south Louisiana wetlands. Arch. Environ. Contam. Toxicol. 37, 337–384.

Strum, K.M., Alfaro, M., Haase, B., Hooper, M.J., Johnson, K.A., et al., 2008. Plasma cholinesterases for monitoring pesticide exposure in Nearctic-Neotropical migratory shorebirds. Ornitol. Neotrop. 19, 641–651.

Sutherland, W.J., Newton, I., Green, R.E., 2004. Bird Ecology and Conservation: A Handbook of Techniques. Techniques in Ecology and Conservation Series. Oxford University Press, p. 153.

Taha, H., Abdelnour, S., Alagwany, M., 2019. Growth performance, biochemical, cytological and molecular aspects of rabbits exposed to lead toxicity. J. Anim. Physiol. a Anim. Nutr. 103, 747–755.

Thompson, H.M., 1991. Serum "B" esterases as indicators of exposure to pesticides. In: Mineau, P. (Ed.), Cholinesterase inhibiting insecticides: Their Impact on Wildlife and the Environment. Elsevier, Amsterdam, Netherlands, pp. 109–126.

Tsangaris, C., Vergolyas, M., Fountoulaki, E., Goncharuk, V.V., 2011. Genotoxicity and oxidative stress biomarkers in *Carassius gibelio* as endpoints for toxicity testing of Ukrainian polluted river waters. Ecotoxicol. Environ. Saf. 74, 2240–2244.

Valdes, S.A.C., 2010. Avaliação da exposição a agrotóxicos em aves silvestres de vida livre. In: Von Matter, S., Straube, F.C., Accordi, I., Piacentini, V., Candido, J.F. (Eds.), Ornitologia e conservação: ciência aplicada, técnicas de pesquisa e levantamento. Technical Books Editora, Rio de Janeiro, pp. 429–439.

Walter, 1979. Eleonora's Falcon: adaptations to prey and habitat in a social raptor. University of Chicago Press, Chicago and London.

Wayland, M., García-Fernández, A.J., Neugebauer, E., Gilchrist, H.G., 2001. Concentrations of cadmium, mercury and selenium in blood, liver and kidney of common eider ducks from the Canadian Arctic. Environ. Monit. Assess. 71, 255–267.

Wink, M., Wink, C., Ristow, D., 1978. Biologie des Eleonoren falken (*Falco eleonorae*). 2. Zur Verebrung der Gefiederpahsen (hell-dunkel). Journal für Ornithologie 119, 421–428.

Wink, M., Wink, C., Ristow, D., 1982. Biologie des Eleonoren falken (*Falco eleonorae*). 12. Biometrie des Sexualdimorphismus adulter und flugger Falken. Vogelwarte 103, 225–229.

Wink, M., Biebach, H., Feldmann, F., Scharlau, W., Swatschek, I., Wink, C., Ristow, D., 1991. Contribution to the breeding biology of Eleonora's Falcon (*Falco eleonorae*). In: Proceedings of The Hawk and Owl Trust Conference, University of, pp. 59–72.

Wolf, M.F., Kendall, R.J., 1998. Age-dependent toxicity of Diazinon and Terbufos in European starlings (*Sturnus vulgaris*) and red-winged blackbirds (*Agelaius phoeniceus*). Environ. Toxicol. Chem. 17, 1300–1312.

Zúñiga-González, G., Torres-Bugarín, O., Luna-Aguirre, J., González-Rodríguez, A., Zamora-Perez, A., Gómez-MEDA, B.C., Ventura-Aguilar, A.J., Ramos-Ibarra, M.L., Ramos-Mora, A., Ortíz, G.G., Gallegos-Arreola, M.P., 2000. Spontaneous micronuclei in peripheral blood erythrocytes from 54 animal species (mammals, reptiles and birds): part two. Mutat. Res. 467, 99–103.

Zúñiga-González, G., Torres-Bugarín, O., Zamora-Perez, A., Gómez-Meda, B.C., Ramos Ibarra, M.L., Martínez-González, S., González-Rodríguez, A., Luna-Aguirre, J., Ramos-Mora, A., Ontiveros-Lira, D., Gallegos-Arreola, M.P., 2001. Differences in the number of micronucleated erythrocytes among young and adult animals including humans: Spontaneous micronuclei in 43 species. Mutat. Res. 494, 161–167.

Please cite this article as: V. Tsarpali, C. Kassara, C. Barboutis et al., Assessing the seasonal and intrinsic variability of neurotoxic and cyto-genotoxic biomarkers in blood of free-living Eleonoras' falcons, Science of the Total Environment, https://doi.org/10.1016/j.scitotenv.2019.135101