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Determinants of Legacy Persistent Organic Pollutant Levels in the European Pond Turtle (*Emys orbicularis*) in the Camargue Wetland, France

Louisiane Burkart, Anthony Olivier, Olivier Lourdais, Marion Vittecoq, Gabriel Blouin-demers, Fabrice Alliot, Clément Le Gac, Nicolas Martin, Aurélie Goutte

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1 Running head: Low levels of POPs in *Emys orbicularis* in the Camargue

2

3 **Determinants of legacy POPs levels in the European**
4 **pond turtle (*Emys orbicularis*) in the Camargue**
5 **wetland, France**

6

7 LOUISIANE BURKART^{1,2}, ANTHONY OLIVIER¹, OLIVIER LOURDAIS^{3,4}, MARION
8 VITTECOQ^{1,5}, GABRIEL BLOUIN-DEMERS⁶, FABRICE ALLIOT², CLÉMENT LE
9 GAC¹, NICOLAS MARTIN¹, AURÉLIE GOUTTE ²

10

11 ¹ La Tour du Valat, Research institute for the conservation of Mediterranean Wetlands,
12 Arles, France

13 ² École Pratique des Hautes Études (EPHE), PSL Research University, UMR 7619
14 Sorbonne University, Paris, France

15 ³ Centre d'études biologiques de Chizé, CNRS, F-79360, Villiers en Bois, France

16 ⁴ School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA

17 ⁵ MIGEVEC Laboratoire maladies infectieuses & vecteurs: écologie, génétique
18 évolution et contrôle, UMR (CNRS/IRD 224/UM), Montpellier, France

19 ⁶ Department of Biology, University of Ottawa, Ottawa, Ontario, Canada

20

21 Correspondence:

22

23 E-mail: aurelie.goutte@ephe.psl.eu

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26 **Highlights**

27

28 • We analyzed POPs levels in 174 European pond turtles in the Camargue
29 wetland.

30 • Only 3 POPs were detected in more than 10% of individual turtles.

31 • Sex, date, and mass did not explain the occurrence and levels of POPs in
32 turtles.

33 • The occurrence and levels of PCB 153 increased with turtle age.

34 • Turtles from the two study sites differed in POP burdens, possibly due to
35 different hydraulic system.

36

37 **Abstract**

38 Many banned persistent organic pollutants (POPs) remain for decades in the aquatic
39 environment and can have harmful effects on long-lived predators because of their
40 high bioaccumulation and biomagnification potentials. We investigated the
41 occurrence and levels of 18 polychlorinated biphenyls (PCBs) and 16 organochlorine
42 pesticides (OCPs) in European pond turtles (n = 174) from April to July 2018 in the
43 Camargue wetland, France. Although the Camargue was highly contaminated in the
44 last decades, plasma occurrence and levels of POPs were very low: we detected only
45 3 of the 34 compounds we analyzed in more than 10% of the turtles. POP burdens
46 did not differ between males and females and was uncorrelated with sampling date
47 and body mass. We observed differences in POP burdens between turtles from the
48 two sampling sites. One possible explanation is the agricultural hydraulic system:
49 plasma occurrence and levels were higher for PCB-52 and HCB in turtles captured in
50 drainage channels, but lower for PCB-153 at the site that receives irrigation. Finally,
51 the occurrence and levels of PCB 153 in turtles increased with age, likely because of
52 bioaccumulation and much higher exposure 20-30 years ago than now.

53

54 Key words: reptiles, PCB, organochlorine pesticides, delta wetlands

55

56

57 **1. Introduction**

58 In Europe, the production and use of persistent organic pollutants (POPs) are
59 prohibited or severely restricted since 2004 through the Stockholm Convention
60 (<http://www.pops.int/>), but POPs still cause environmental concerns.
61 Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) remain in
62 the environment for decades because of their slow biodegradation and improper
63 handling of contaminated wastes. Levels of POPs in animals increase with age
64 (Vives et al., 2005; Binnington & Wania, 2014) and trophic level (Goutte *et al.*, 2020).
65 Exposure to POPs causes a wide range of adverse effects, including neurotoxicity,
66 endocrine disruption, immune dysfunction, reproductive impairment, and
67 developmental abnormalities, which may ultimately compromise survival and
68 reproductive output and lead to population declines of wild vertebrates (Goutte *et*
69 *al.*, 2014, 2015; Salice *et al.*, 2014).

70 Pollution, and especially industrial and agricultural discharge, is one of the
71 major threats to freshwater ecosystems (Holt, 2000; Dudgeon *et al.*, 2006). Wetlands
72 support an extremely rich biodiversity, but are among the most transformed and
73 threatened ecosystems of the world (Revenga *et al.*, 2005), experiencing rates of
74 population declines and species extinctions far higher than those in forests,
75 grasslands, and coastal ecosystems (Dudgeon *et al.*, 2006). Deltas are often exposed to
76 high water pollution from intensive agriculture (Kuenzer & Renaud, 2012) and from
77 discharge of contaminants in upstream waters. The Camargue, in the Rhône River
78 delta, is the largest wetland in France and is of international importance under the
79 Ramsar Convention (<https://www.ramsar.org/>), but it is heavily impacted by

80 human activities (Cheiron *et al.*, 2018). The contamination of water bodies by
81 agricultural, industrial, and urban discharges has been well studied over the past
82 decades (Comoretto *et al.*, 2007). In particular, levels of PCBs in sediments of the
83 Rhône River increase from upstream to downstream, reaching 417 µg/kg dw for the
84 sum of 7 PCB congeners (28, 52, 101, 118, 138, 153, and 180, Mourier *et al.* 2014).
85 Mean flux over the 2011–2016 period averaged 14 kg/year for PCB 180 in suspended
86 particulate matter at the outlet of the Rhône River basin (Poulier *et al.*, 2019).
87 Previous studies conducted in the Camargue confirmed that birds (Berny *et al.*, 2002)
88 and fish (Roche *et al.*, 2002, 2003, 2009a) are exposed to OCPs and PCBs.

89 Freshwater turtles remain poorly studied in ecotoxicology although they can
90 provide useful information on local contamination because of their longevity
91 (Campbell & Campbell, 2002 ; EL Hassani *et al.*, 2019 ; Gaus *et al.*, 2019), high trophic-
92 level, and low dispersal capacity (Ming-ch'eng Adams *et al.*, 2016). Moreover, as
93 ectothermic vertebrates, they have a lower ability to metabolize pollutants than
94 endothermic mammals and birds (de Solla, 2015). The European pond turtle, *Emys*
95 *orbicularis*, a long-lived (> 40-80 years) opportunistic predator (fish, amphibian,
96 crayfish) and scavenger (Ottonello *et al.* 2005; Ficetola & De Bernardi, 2006), is facing
97 significant population declines due to multiple environmental alterations, including
98 water pollution (Cheylan 1998). This species is listed as “near threatened” (NT) on
99 the UICN Red List of threatened species in France (REF). Pollution by trace metal
100 elements has been studied in *E. orbicularis* (Namroodi *et al.*, 2017 ; Guillot *et al.*, 2018 ;
101 Beau *et al.*, 2019), but data are lacking on burdens of POPs.

102 We studied contamination levels in two populations of *E. orbicularis* at the
103 Natural Reserve of the Tour du Valat, in the Camargue, France. Several individuals
104 were of known-age due to capture-mark-recapture (CMR) program, starting more
105 than 20 years ago (Olivier *et al.*, 2010 ; Ficheux *et al.*, 2014 ; Arsovski *et al.*, 2018). The
106 hydraulic system combines irrigation canals originating from the Rhône river and
107 drainage canals, which are exposed to various environmental contaminants from the
108 Rhône river and agricultural plots, especially rice fields (Chauvelon, 1996). In the
109 present study, we assessed recent (2018) levels of OCPs and PCBs in plasma of the
110 European pond turtle (n = 174). We tested the effects of individual traits (mass, sex,
111 and age) on POP burdens. We expected that older and larger individuals should
112 have higher levels of POPs because of bioaccumulation and length of exposure.
113 Moreover, we compared POP burdens of turtles from two populations in different
114 locations in the agricultural hydraulic system (irrigation vs drainage). We expected
115 high levels of OCPs in turtles from the drainage site, as a possible consequence of the
116 remobilization of pesticides from agricultural soils, and high levels of PCBs in turtles
117 from the irrigation channel, as a possible consequence of the historical
118 contamination.

119

120 **2. Material and methods**

121 **2.1. Sampling sites and capture**

122 We conducted the study in the Natural Reserve of the Tour du Valat (43°30' N, 4°40'
123 E, [Fig. 1](#)) in France. We captured European pond turtles (n = 174) in canals and
124 marshes from 24 April to 26 July 2018 by hand or with funnel traps (Olivier *et al.*,

125 2010; Ficheux *et al.*, 2014). We captured turtles at two sites: (1) irrigation canals and
126 their associated marshland (site of Esquineau, n = 126) and (2) drainage canals of the
127 Fumemorte basin and marshes filled with water by these canals (site of Faïsses, n =
128 48). The sex ratio was the same between the two sites (58.3% females at Faïsses and
129 60.3% females at Esquineau, Pearson's Chi-squared test, $p = 0.95$).

130 Individual turtles were identified as part of a long-term CMR program with
131 shallow notches on the marginal and nuchal scales in unique combinations (Olivier,
132 2002 ; Olivier *et al.*, 2010). Individuals were weighed and sexed by visual observation
133 of the secondary sexual characters. The year of birth can be determined if the first
134 capture occurred within the first 5 years of life by counting the number of growth
135 streaks (Castanet, 1988). This long-term survey starts in 1997, although a few tens of
136 individuals were also marked as adults from 1976. In our data set, 66 of the 174
137 individuals were of known-aged (5 to 26 year-old, corresponding to a first capture
138 between 1997 and 2018) and some were first captured as adults in 1976, thus being
139 considered as more than 44 year-old. When turtles were captured after their first 5
140 years of life (N = 108 in the present study), we determined a stage of growth
141 according to growth streaks located on the plastron (Olivier, 2002). By combining
142 estimated age and stage of growth, we then attributed an age class to each turtle: 4 to
143 8 year-old (N = 20), 9 to 13 year-old (N = 46), 14 to 22 year-old (N = 54), and 23 to 44+
144 year-old (N = 54). Turtles were released on the same day at their capture site.

145

146 **2.2. Blood sampling**

147 Plasma is a good matrix to determine individual burdens of POPs because blood
148 sampling is minimally invasive and because circulating levels of POPs in blood are

149 significantly correlated to concentrations of POPs stored in fat (Keller *et al.*, 2004 ;
150 Dabrowska *et al.*, 2006), liver, kidney, and muscle tissues (Van de Merwe *et al.*, 2010).
151 We collected blood samples (2 ml, always less than 1% of the turtle body mass) from
152 the dorsal coccygeal vein (Innis & Knotek, 2020; Keller *et al.*, 2004) with a Terumo
153 Company® syringe (Somerset, USA) pre-impregnated with heparin to prevent blood
154 clotting during collection and equipped with a 25G needle (Franklin Lakes, USA).
155 We then centrifuged the samples to separate the plasma from the red blood cells. We
156 collected blood samples from 174 individuals (126 from Esquineau and 48 from
157 Faïsses), 66 individuals were of known age ranging from 5 to 31 years old (32 females
158 and 19 males from Esquineau; 9 females and 6 males from Faïsses). Samples were
159 stored at -18 °C until analysis in the UMR 7619 METIS of Sorbonne Université,
160 France.

161

162 **2.3. Chemical analyses**

163 We determined the levels of 17 OCPs (p,p'-DDT and metabolites (p,p'-DDE and p,p'-
164 DDD); pentachlorobenzene (PeCB); hexachlorobenzene (HCB);
165 pentachloronitrobenzene (Quintozene); four isomers of hexachlorocyclohexane (α -,
166 β -, γ - (lindane), δ -HCH); aldrin; endrin; isodrin; telodrin (Isonbenzan); heptachlor;
167 heptachlor epoxide; heptachlor endo-epoxide), 7 marker PCBs (M-PCBs, IUPAC
168 numbers # 28, 52, 101, 118, 138, 153 and 180), and 12 dioxin-like PCBs (DL-PCBs,
169 IUPAC numbers # 77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189) in the 174
170 plasma samples.

171 Samples were processed through Solid Phase Extraction (SPE), using hexane:
172 dichloromethane, 9:1, using a validated protocol (Tapie *et al.* 2011) that was adapted

173 for plasma samples (see Supplementary Material for a detailed description of the
174 procedure and method validations). PCBs and OCPs were analyzed using an Agilent
175 7890 A gas chromatograph (GC) coupled to a 7000 B triple quadrupole mass
176 spectrometer system (MS/MS) (Agilent Technologies, Les Ulis, France). Recovery
177 rates (% RR) of compounds were assessed on replicate plasma samples of European
178 pond turtles ($n = 4$) with spiked solutions (100 ng of each compound). The
179 repeatability of the method was assessed in terms of relative standard deviation (%
180 RSD) of the recovery. RR and RSD are provided in Table S2 (Supplementary
181 Material). Recovery rates were not satisfactory ($< 75\%$ or $> 125\%$) for isodrin, endrin,
182 heptachlor epoxid, heptachlor-endo-epoxide, p,p'-DDD, PCB-77, PCB-189 (Table S2)
183 and they were not further considered.

184

185 **2.4. Statistical analyses**

186 We performed statistical analyses with R software version 3.3.2 (R Core Team, 2016).
187 Only POPs detected (i.e. $> \text{LOQ}$) in at least 10% of the samples were included in the
188 statistical analyses. Since age and weight were positively correlated ($t = 3.65$, $df = 64$,
189 $p = 0.0005$) and females were significantly heavier than males ($W = 474$, $p < 0.0001$),
190 body mass, sex and age were tested separately..

191 We used generalized linear models (GLM) with a binomial distribution and a
192 logit link function to test for the effects of sex, site, the interaction sex * site, sampling
193 date, and age (or body mass) on contaminant occurrence. For each model, we used a
194 backward elimination to progressively remove non-significant terms ($p > 0.05$). For
195 all analyses, model specification and validation were based on residual analysis.

196 The effects of sex, sampling site, date, and age (or body mass) on pollutant
197 concentrations were tested by considering left-censored data, i.e. pollutant levels
198 below the LOQ. To do so, group comparisons and linear regressions were performed
199 using Peto-Prentice tests and tobit models respectively, with the function `cendiff` of
200 the NADA package and the function `tobit` of the AER R-package.

201

202 **3. Results**

203

204 **3.1. POP levels and occurrences**

205 In 37 of the 174 samples, all contaminant levels were below the limit of
206 quantification, which represented 10.4% of turtles sampled in Faïsses and 24.4% of
207 turtles sampled in Esquineau. Eleven compounds were never detected in the plasma
208 samples: PeCB, Lindane, Quintozene, Heptachlor, and the dioxin-like PCBs: PCB-
209 118, -105, -114, -126, -156, -157 and -167. The following seven compounds were
210 detected at least in one sample from Esquineau, but not in samples from Faïsses:
211 alpha-HCH, 44'-DDT and PCBs 28, 101, 123, 169 (Table 1). Overall, the contaminant
212 concentrations in the plasma samples were very low (Table 1).

213

214 **3.2. Influence of individual traits, sampling site, and date on POP occurrences and 215 levels**

216 Only three POPs (HCB, PCB-52 and -153) had substantial occurrence, being detected
217 in more than 10% of individuals (Table 1). Plasma occurrences of PCB-52 and HCB
218 were significantly higher in turtles captured at Faïsses compared to Esquineau, while

219 the occurrence of PCB-153 was higher in turtles captured at Esquineau (Tables 1 and
220 2). HCB occurrence slightly increased with sampling date (Table 2). Sex, mass, and
221 age class did not explain POP occurrence in turtles, except for PCB-153 whose
222 detection frequency was higher in older individuals (Table 2).

223 Levels of HCB and PCB-52 were significantly higher in turtles from Faïsses,
224 while PCB-153 levels tended to be higher in turtles from Esquineau (Tables 1 and 3).
225 Moreover, levels of PCB-153 were lower in the youngest turtles (Table 3, Fig. 2). Sex,
226 mass, and date did not explain variation in levels of POPs among turtles (Table 3).

227

228 **4. Discussion**

229

230 The aim of this study was to characterize legacy levels of POPs (OCPs and PCBs) in
231 the plasma of European pond turtles in the Camargue, France, and to determine
232 whether these levels were a function of individual traits and habitat types. Plasma
233 levels of POPs were low and often below limits of quantification (from 0.2 to 3.1
234 ng/mL). We found significant differences in burdens of POPs between turtles from
235 the two sampling sites and these differences could be attributed to the hydraulic
236 system (drainage/irrigation). The occurrence and levels of PCB-153 were higher in
237 older turtles.

238 The Rhône River has been historically contaminated, leading to an
239 accumulation of POPs in sediments downstream (Mourier et al. 2014). Previous
240 studies conducted on several animal taxa in the Camargue confirmed high exposure
241 during the last decades, with high concentrations of PCBs in muscles of eels (*Anguilla*
242 *anguilla*) fished between 1997 and 2000 (Roche et al., 2004), but also in eggs of little

243 egrets (*Egretta garzetta*) collected in 1996 (Berny *et al.*, 2002). We detected low levels of
244 POPs in the pond turtle in Camargue, which could indicate a decline in legacy POP
245 exposure for wild species in the Camargue. Although a longitudinal study of the
246 same species at the same locations would be required to test this hypothesis,
247 decreasing PCB concentrations have been observed in the Rhône River sediments
248 over the last decades (Liber *et al.*, 2019) and water analyses conducted by the Nature
249 Protection National Society (SNPN) have not detected PCBs and OCPs in the
250 Fumemorte canal since 2011 (Cheiron, 2019). Moreover, organochlorine
251 contamination across food webs tended to diminish in the Vaccarès lagoon (Roche *et*
252 *al.*, 2009a), the largest water body of the Camargue Biosphere Reserve. Exotic red
253 swamp crayfish (*Procambarus clarkii*), the main prey of European pond turtles in the
254 Camargue (Ottonello *et al.*, 2005), were not contaminated by POPs in 2019 (i.e. <
255 LOQ, with LOQ ranging from 0.9. to 1.98 ng/g dw, unpublished data).

256 One of the first studies looking at the plasma concentration of POPs in turtles
257 documented high PCBs and OCPs levels in snapping turtles (*Chelydra serpentina*), in
258 Ontario, Canada and sampled in 2001–2004 (Letcher *et al.*, 2015): plasma
259 concentrations of OCPs (sum of 17 contaminants) ranged from 0.2 to 236 ng/g w.w.
260 and the most abundant pesticide was p,p'-DDE (mean \pm SE: 27 ± 6 ng/g w.w.). In our
261 study, plasma concentrations of POPs (sum of 16 contaminants) were much lower,
262 ranging from 0 to 2 ng/g w.w and p,p'-DDE levels didn't exceed 2.3 ng/g ww.
263 Snapping turtles are freshwater turtles with similar feeding habits than European
264 pond turtle: they also consume plant and animal matter, including aquatic
265 invertebrates, fish, frogs, reptiles (Ernst *et al.* 1994). Other studies of turtles

266 documented plasma concentrations of POPs comparable to those in our study; for
267 instance, for PCB 153 and HCB in another freshwater species, the Western pond
268 turtle (*Actinemys marmorata*) in Sequoia National Park, USA, in 2012 (Meyer *et al.*,
269 2016), as well as in marine turtle, such as Loggerhead sea turtles (*Caretta caretta*) in
270 the Eastern Atlantic Ocean in 2011-2012 (Bucchia *et al.*, 2015), and in Green turtles
271 (*Chelonia mydas*) and Hawksbill turtles (*Eretmochelys imbricata*) in Cape Verde
272 between 2009 and 2011 (Camacho *et al.*, 2014).

273 In terms of occurrence of POPs, our results in European pond turtles were low
274 compared to other studies (Bucchia *et al.*, 2015 ; Meyer *et al.*, 2016). The occurrences
275 of dioxin-like PCBs in 8% of European pond turtles, as well as p,p'-DDE in 2% of
276 individuals were much lower than those found in plasma of Loggerhead sea turtles
277 that ranged from 63% in the Atlantic Ocean to 100% in the Adriatic Sea for dioxin-
278 like PCBs and 100 % for p,p'-DDE (Bucchia *et al.*, 2015).

279 Despite low detection frequencies and levels, we found differences in POP
280 burdens between turtles from the two sampling sites. Turtles in Faïsses, a site
281 receiving water from drainage canals, exhibited significantly higher occurrence and
282 levels of HCB compared to turtles from Esquineau, likely because of the
283 remobilisation of trapped OCPs in soils. On the other hand, the occurrence and levels
284 of PCB 153 were higher in turtles from Esquineau, a site with irrigation channels. A
285 previous study has documented higher concentrations of PCBs in the aquatic fauna
286 in irrigation canals compared to the drainage canals downstream of rice fields (Roche
287 *et al.*, 2009b). We also found that concentrations of PCB-52 were higher in turtles at
288 Faïsses, however, which may be due to a lower sedimentation rate of PCB-52

289 associated with its lower molecular weight and a greater solubility due to a lower
290 level of chlorination compared to PCB-153 (Gong *et al.*, 1998 ; Alkhatib & Weigand,
291 2002). The processes of deposition and release of PCBs from the irrigation part to the
292 drainage part of the canals need to be further studied. In addition, other populations
293 of pond turtles in the two types of hydraulic systems should be studied to confirm
294 this pattern.

295 The occurrence and levels of PCB-153 were lower in the youngest individuals
296 (4 to 8 year old), which could be attributed to a shorter exposure period. PCB-153 is a
297 recalcitrant chemical with a high hydrophobicity (high octanol-water partition
298 coefficient), high resistance to metabolic transformation, and slow respiratory
299 elimination through air-exhalation (high octanol-air partition coefficient). PCB-153 is
300 also more prone to bioaccumulation than many other PCB congeners (Kelly *et al.*
301 2007), especially in long-lived air-breathing predatory species (Rowe, 2008). It is also
302 possible that there is a dietary shift during growth in pond turtles leading to the
303 consumption of higher trophic-level prey with age, and these higher trophic level
304 prey would be more PCB contaminated due to biomagnification. This hypothesis,
305 however, is not supported by dietary studies of *E. orbicularis* in Camargue), showing
306 a shift to a more herbivorous diet with age, using prey items identification in faecal
307 samples (Ottonello *et al.*, 2005) or no difference in the proportion of plants,
308 invertebrates and vertebrates, using metabarcoding analyses (Duccotterd *et al.*
309 2020).)

310 We did not detect differences in POP loads between male and female pond
311 turtles, despite males often being more contaminated than females in other turtle

312 species (Guirlet *et al.*, 2010 ; Bangma *et al.*, 2019; Lambiase *et al.*, 2021) due to a
313 transfer of pollutants from the mother to the eggs through vitellogenesis (Moss *et al.*,
314 2009 ; de Solla, 2010).

315

316

317 **Declaration of competing interest**

318 The authors declare that they have no known competing financial interests or
319 personal relationships that could have appeared to influence the work reported in
320 this paper.

321

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331

332 **Author contributions**

333 AO, LB, CLG and NM conducted the field work and collected the blood samples. LB
334 and FA conducted the laboratory work to prepare plasma samples for analysis with
335 gas chromatography and tandem mass spectrometry. LB, OL, AG, AO, GBD, and MV
336 conceived and coordinated the study, participated in data analysis, and in writing
337 the manuscript. All authors gave final approval for publication and agree to be held
338 accountable for the work performed therein.

339

340 **Ethical consideration**

341 This study was performed in accordance with laws relative to capture, transport, and
342 experiments on *E. orbicularis* (DREAL permit # CERFA_13616-01) and all procedures
343 were approved by an independent ethical committee (APAFIS#17899-
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- 524

525 **Figure captions**

526 **Figure 1:** Sampling locations for European pond turtles in the regional nature reserve
527 of Tour du Valat, Camargue, France. The two capture locations are Esquineau (in
528 blue), irrigated with water pumped from the Rhône River, and Faïsses (in orange),
529 consisting of drainage canals of agricultural parcels.

530

531 **Figure 2:** Increase in plasma PCB 153 (ng/mL) levels in European pond turtles with
532 age class (4 to 8, 9 to 13, 14 to 22, 23 to higher than 44 years old). Each point
533 represents an individual. Red and green squares correspond to mean and median
534 levels for age class, respectively. Age class was estimated according to growth
535 streaks located on the plastron (see methods).