

1 **Red operculum spots, body size, maturation and evidence for a satellite**
2 **male phenotype in non-native European populations of pumpkinseed**
3 ***Lepomis gibbosus***

4
5 Grzegorz Zięba^{1,2} | Carl Smith^{2,3,4} | Michael G. Fox⁵ | Stan Yavno^{5,6} | Eva Záhorská⁷ |
6 Mirosław Przybylski² | Gérard Masson⁸ | Julien Cucherousset⁹ | Hugo Verreycken¹⁰ |
7 Hein H. van Kleef¹¹ | Gordon H. Copp^{1,5,12}

8
9 ¹Salmon and Freshwater Team, Centre for Environment, Fisheries and Aquaculture Science,
10 Pakefield Road, Lowestoft, NR33 0HT, UK

11 ²Department of Ecology and Vertebrate Zoology, University of Łódź, Poland

12 ³Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno, Czech
13 Republic

14 ⁴School of Biology and Bell-Pettigrew Museum of Natural History, University of St Andrews, UK

15 ⁵Environmental and Life Sciences Graduate Program, Trent University, Peterborough, Ontario,
16 Canada

17 ⁶Department of Zoology, Tel Aviv University and Inter-University Institute for Marine Sciences,
18 Eilat, Israel

19 ⁷Comenius University, Faculty of Natural Sciences, Department of Ecology, Bratislava, Slovakia

20 ⁸Laboratoire Interdisciplinaire des Environnements Continentaux, Université de Lorraine, Metz,
21 France

22 ⁹Laboratoire Evolution and Diversité Biologique (EDB UMR 5174), Université Paul Sabatier,
23 CNRS, ENFA, UPS, Toulouse, France

24 ¹⁰Research Institute for Nature and Forest, Brussels, Belgium

25 ¹¹Bargerveen Foundation, Department of Animal Ecology and Ecophysiology & Department of
26 Environmental Science, Faculty of Science, Radboud University Nijmegen, Nijmegen, The
27 Netherlands

28 ¹²Centre for Conservation and Environmental Change, School of Conservation Sciences,
29 Bournemouth University, Dorset, UK

30

31 Correspondence

32 Gordon H. Copp

33 gordon.copp@cefas.co.uk; +44 01502 52 7751

34

35

36 **Running title:** Operculum spot of non-native pumpkinseed

37

38 **Abstract**

39 Carotenoid-based pigmentation is a striking feature of many taxa, yet the function, if any,
40 of colour traits is often unclear. Pumpkinseed *Lepomis gibbosus*, a widely introduced
41 freshwater sunfish that exhibits alternative male mating strategies, express a striking, red
42 operculum spot. To investigate the potential function of this red spot as a signal in this
43 species' mating system, we determined the presence and measured the size of red
44 operculum spots in fish collected from 12 populations in five European countries in which
45 pumpkinseed is an established non-native species. We subsequently related the
46 presence and size of the red spot to body size and mating strategy, based on an analysis
47 of relative gonad size (gonado-somatic index, GSI), using a mixed modelling approach.
48 The study demonstrated that the presence of a red operculum spot in pumpkinseed is
49 associated with sexual maturation, with GSI frequency distributions suggesting that
50 cuckolders in some non-native populations comprised both sneaker and satellite males,
51 the latter not having previously been reported for this species. Further, the size of red spot
52 correlated strongly with body size in parental and cuckolder males, though there was no
53 difference in the presence or size of the red operculum spot between male mating
54 strategies. The function of a red operculum spot in females is not clear but may be partly
55 mediated by pleiotropic genetic mechanisms. Red operculum spots appear to function as
56 signals of male maturation and body size in pumpkinseed, irrespective of mating strategy.

57

58 **Keywords** alternative mating strategy · carotenoid · GLMM · sexual selection · visual
59 signal · Centrarchidae

60 1 | INTRODUCTION

61 Visual signals play a key role in the environmental biology of teleost fishes (Marshall,
62 2000; Wootton, 1998). Pigmentation is an important component of visual signals in fishes,
63 both within and among species. The expression of colour, which often comprises multi-
64 component signals, involves pigment-based and structural colours (Price, Weadick, Shim,
65 & Rodd, 2008; Wootton & Smith, 2015). Carotenoid-based colouration, which typically
66 appears as yellow, orange or red, has been of particular interest because these are costly
67 to produce and thereby serve as a reliable indication of individual condition and foraging
68 ability (Olson & Owens, 1998), with a potential role in mating system evolution (Wootton
69 & Smith, 2015). Different carotenoid pigments are responsible for conveying different
70 information to potential mates or rivals (McLennan, 2007). Colour traits can function in
71 sexual selection; selection that acts on differences in fitness among individuals that arise
72 due to the number and identity of their mates. Because sexual selection tends to act more
73 strongly on males than females (Andersson, 1994), it is males that usually express the
74 brightest and most elaborate visual traits, including nuptial colouration. Though a broad
75 simplification, there is a tendency in many taxa for a preference to mate with more
76 colourful and ornamented individuals (Andersson, 1994; Price et al., 2008).
77 Understanding the adaptive value of mate choice (*sensu* Kokko, Brooks, Jennions &
78 Morley, 2003) based on visual signals is a controversial field of research, but may arise
79 through fitness benefits associated with mate choice, or an arbitrary mechanism
80 (Andersson, 1994; Wootton & Smith, 2015).

81 Pigmentation in North American sunfishes (Centrarchidae), in common with most other
82 teleosts, is more extensive in males than in females (Scott & Crossman, 1973). In the
83 pumpkinseed *Lepomis gibbosus* males have a striking, red operculum spot that is
84 associated with male aggression (Stacey & Chiszar, 1978). In aquarium studies, breeding
85 males were shown to attack fish models that intruded into their nesting area, and models
86 with red colour on the operculum spot or iris received more attacks and aggressive
87 displays than those without colour (Stacey & Chiszar, 1978). It is recognised that
88 dominance relationships among sunfishes are related to colouration, with dominant,
89 brightly-coloured parental (territorial) pumpkinseed males building and defending nests,
90 and performing courtship behaviour to attract females to their nest for spawning. Male
91 sunfishes also adopt alternative mating strategies (*sensu* Wootton, 1984), whereby sub-
92 dominant 'cuckolder' males attempt to intrude into the nest of a parental male that is in
93 the act of spawning (Gross, 1979, 1982). Cuckolder males are early-maturing, do not

94 perform courtship and avoid fitness costs associated with parental care (Smith & Wootton,
95 1995). Small cuckolders, typically possessing disproportionately large testes, are referred
96 to as ‘sneakers’ due to their rapid entry into the nest of a spawning territorial male to
97 release sperm at the moment of spawning. Large cuckolders, which have testes of
98 intermediate size relative to parental and sneaker males, are termed ‘satellites’ and
99 participate in spawning by mimicking female characteristics and entering the nest of a
100 territorial male as a potential mate (Gross, 1982; Garner & Neff, 2013). Despite aggressive
101 defence of their nest by parental males, cuckolder males frequently intrude successfully
102 during spawning events (Rios-Cardenas, 2003). In the close congener, bluegill *Lepomis*
103 *macrochirus*, a model taxon for research on alternative reproductive strategies (Taborsky,
104 2008), Gross (1982) characterised two cuckolder phenotypes, based on a population in
105 Lake Opinicon, Canada. Sneaker bluegill are small and have relatively large testes relative
106 to parental males, with a mean gonado-somatic index (GSI) of 4.6 (contrasting with
107 parental males with a mean GSI of 1.1). In contrast, satellite male bluegill are larger than
108 sneakers, with GSI values (mean 3.3) that are intermediate between those of parental
109 males and sneakers. In applying these criteria to pumpkinseed from the same lake, Gross
110 (1982) identified the sneaker phenotype, but not that of the satellite. In a subsequent
111 paternity study, Neff and Clare (2008) noted that the satellite strategy had yet to be
112 reported for pumpkinseed. In a subsequent genetic study of bluegill–pumpkinseed
113 hybridisation, Garner and Neff (2013) did not distinguish between satellites and sneakers,
114 referring to them collectively as cuckolders. Thus, there are no reports that the satellite
115 phenotype (*sensu* Gross, 1982) is expressed in pumpkinseed and hereafter we use the
116 term ‘cuckolder’ to refer to the alternative male mating strategy in pumpkinseed to the
117 parental strategy.

118 The approach, onset and end of the reproductive period in fishes can often be identified
119 by variation in GSI, the relationship between gonad weight and somatic weight (Wootton,
120 1998). Male mating polymorphisms, particularly the cuckolder strategy, are associated
121 with sperm competition, which occurs when the spermatozoa of different males compete
122 for the same eggs (Parker, 1970). Males expressing alternative mating strategies tend to
123 experience a higher “risk” of sperm competition; i.e. an elevated probability that their
124 sperm will compete with the sperm of other males, because they usually mate in the
125 presence of at least one competitor, typically the territorial male and often other cuckolder
126 males (Wedell, Gage, & Parker, 2002; Wootton & Smith, 2015). An outcome is that
127 cuckolders are predicted to show adaptations for sperm competition, including relatively

128 (or even absolutely) larger testis size, larger ejaculates, and faster swimming
129 spermatozoa (Montgomerie & Fitzpatrick, 2009). Consequently, individual GSI can be
130 used to identify male mating strategy.

131 Pumpkinseed have been widely established outside their native North American range.
132 Most introductions have been in Europe, where the species was introduced in the late 19th
133 and early 20th centuries, establishing populations in at least 28 countries (Copp & Fox,
134 2007). Most of the research undertaken on pumpkinseed in Europe has focused on female
135 growth and life-history traits (reviewed in Fox & Copp, 2014), with relatively few studies
136 on males (e.g. Almeida, Tobes, Miranda, & Copp, 2012; Valente et al., 2016). In its native
137 range, Neff & Clare (2008) observed temporal variability in the patterns of pumpkinseed
138 paternity and suggested that these reflect temporal variation in mating opportunities,
139 parental male defence ability, or cuckolder densities. Given this temporal variability,
140 spatial variations are also feasible, possibly extending to visual signals, such as the
141 operculum spot, which is assumed to be important for males for attracting mates (Stacey
142 & Chiszar, 1978).

143 The aim of the present study was to determine whether the presence and size of the
144 red operculum spot serves as a signal of sex and/or mating strategy in pumpkinseed. To
145 do this we used estimates of GSI to categorise males as nesters or cuckolders and fitted
146 models to test whether spot presence and size varied between the sexes and between
147 males adopting different reproductive strategies. We predicted that parental males, which
148 experience intrasexual contests and intersexual mate choice, would be more likely than
149 cuckolder males and females to express a red operculum spot and that these males would
150 also express larger red spots.

151

152 **2 | Methods**

153 Previously unpublished data for 1118 pumpkinseed were obtained from established non-
154 native populations in Belgium, England, France, the Netherlands and Poland (Table 1),
155 with all data elements being available for 924 specimens of which 465 were males, 387
156 were females, and 266 were juveniles (Table 2). The fish were collected by a variety of
157 sampling gear (Table 1), depending upon location characteristics and gear availability.
158 Note that sampling of the two Netherlands populations was incorrectly reported as by
159 funnel trap in Cucherousset et al. (2009). Where possible, the sampling aimed to obtain \approx
160 100 specimens for growth and life-history trait analysis (e.g. Villeneuve, Copp, Fox, &
161 Stakénas, 2005; Cucherousset et al., 2009) and so was not quantitative. In the three

162 English sites, sampling was by catch-per-unit-effort (CPUE) with minnow traps (see Fox,
163 Villeneuve & Copp, 2011). The data on female growth and life-history traits, but not
164 operculum spot occurrence or size, have been previously published for all these
165 populations (Villeneuve et al., 2005; Cucherousset et al., 2009; Fox et al., 2011; Valente
166 et al., 2016), except the two populations from northwest Poland (Table 1): i) a former clay
167 pit (Brodowski Pond) of ≈ 0.9 ha (maximum depth of 7 m) situated in Szczecin with a water
168 temperature that ranges from 4 °C under thick ice cover in winter to 25 °C in summer; and
169 ii) an artificial channel (4 km long, mean depth of 4.5 m), situated on the lower River Oder
170 near the city of Gryfino that receives heated water effluent from a power plant, resulting
171 in daily water temperature fluctuations of 5–15 °C, with maximum temperatures of 30 °C
172 in summer and 15 °C in winter. There is currently no published study on the North
173 American source populations of pumpkinseed in Europe.

174 For all populations, pumpkinseed were collected at the beginning of the spawning
175 season (between May and June), immediately killed with a lethal dose of anaesthetic
176 following licenced procedures in the country of collection and cooled to freezing for
177 subsequent processing in the laboratory. After thawing, specimens were photographed
178 (left side only) using a Nikon CoolPix 5000 camera for morphological analysis. The
179 presence of operculum spots (denoted as present or absent) was recorded, including the
180 rare occurrence of deviations from red (i.e. yellow or orange, or reddish shades thereof).
181 Note that the reflectance spectra, hue or intensity of operculum spots were not measured,
182 nor was the spectral sensitivity of pumpkinseed in the red region of the light spectrum
183 known. The presence of an operculum spot was recorded for both sides of each specimen
184 assessed, but only the left side of the fish (the standard for fish measurements) was used
185 for quantifying the size of the red operculum spot, which was estimated as the surface
186 area of the spot (ImageJ, 1.48v). Fish were measured for standard length (SL, nearest 1
187 mm) and mass (nearest 1 g) and dissected to determine sex and gonad mass (in mg)
188 when present. GSI was calculated as: $100 \times \text{gonad mass} \div \text{total body mass}$ (Wootton &
189 Smith, 2015).

190

191 **2.1 | Statistical analysis**

192 To categorise males as parentals or cuckolders, a frequency distributions of male GSI
193 was plotted by country. The resulting multimodal GSI frequency distributions were
194 decomposed to normal distributions using the Bhattacharya method (Bhattacharya, 1967),
195 with a meaningful separation of normal distributions identified with a separation index (SI)

196 > 2 (Gayaniilo, Soriano, & Pauly, 1989). GSI values below the threshold value were
 197 categorised as parental and those equal to or above the threshold value were categorised
 198 as cuckolders. Data were pooled by country, rather than population, to maximise the
 199 precision with which threshold values could be identified.

200 To test whether parental males were more likely to express a red operculum spot than
 201 other males and females, the presence of a red spot in pumpkinseed was modelled using
 202 a Bernoulli Generalised Linear Mixed Model (GLMM). Fish SL and mass were collinear,
 203 and mass was subsequently dropped from the analysis. Only a single fish that was
 204 classified as a juvenile expressed a red spot, and juveniles were subsequently dropped
 205 from the analysis. The distribution for the model response variable, link function and
 206 predictor function were specified as:

$$\begin{aligned}
 207 \quad & Spot_{ij} \sim \text{Binomial}(\pi_{ij}, n_{ij}) \\
 208 \quad & E(Spot_{ij}) \sim n_{ij} \times \pi_{ij} \quad \text{and} \quad \text{var}(Spot_{ij}) = n_{ij} \times \pi_{ij} \times (1 - \pi_{ij}) \\
 209 \quad & \eta_{ij} = \beta_1 + \beta_2 \times SL_{ij} + \beta_3 \times \text{strategy}_{ij} + \text{population}_i \\
 210 \quad & \text{logit}(\pi_{ij}) = \eta_{ij} \\
 211 \quad & \text{population}_j \sim N(0, \sigma^2_{\text{population}})
 \end{aligned}$$

212 Where $Spot_{ij}$ is the probability of fish i in population j having a red operculum spot in n_{ij}
 213 independent individuals, which was assumed to follow a binomial distribution with an
 214 expected probability (E) of expressing a spot of mean $\pi_i N_i$ and variance $\pi_i \times (1 - \pi_i)$, with a
 215 logit link function. The logit function ensures the fitted probability of a red spot falls
 216 between 0 and 1. The variable strategy_{ij} is a categorical covariate with three levels,
 217 corresponding with fish mating strategy; female, territorial or cuckolder. The model also
 218 contained a linear effect for fish SL (SL_{ij}). Exploratory analyses demonstrated differences
 219 among populations in the size and expression of red operculum spots. To accommodate
 220 this effect in the model, the random intercept population_j was included to introduce a
 221 correlation structure between observations for different fish from the same population, with
 222 variance $\sigma_{\text{population}}$ distributed normally and equal to 0.

223 To test whether parental males expressed larger red operculum spots than other males
 224 and females, the size of red spots was modelled on a zero-truncated subset of the data
 225 using a Gaussian GLMM, specified as:

$$\begin{aligned}
 226 \quad & \log_{10}Spotsize_{ij} \sim N(\mu_{ij}, \sigma^2) \\
 227 \quad & E(\log_{10}Spotsize_{ij}) = \mu_{ij} \quad \text{and} \quad \text{var}(\log_{10}Spotsize_{ij}) = \sigma^2 \\
 228 \quad & \mu_{ij} = \beta_1 + \beta_2 \times \log_{10}SL_{ij} \times \beta_3 \times \text{strategy}_{ij} + \text{population}_i \\
 229 \quad & \text{population}_j \sim N(0, \sigma^2_{\text{population}})
 \end{aligned}$$

230 Where $\log_{10}Spotsize_{ij}$ is the \log_{10} size of red spot on fish i in population j and $\log_{10}SL_{ij}$ is
231 the \log_{10} standard length of fish i in population j . Expected $\log_{10}Spotsize_{ij}$ is μ_{ij} with
232 variance σ^2 . Analysis was conducted using the *lme4* library (Bates, Maechler, Bolker, &
233 Walker, 2014) in R (R Development Core Team, 2017). Model residuals were examined
234 to ensure assumptions were met and that effects were adequately accounted for by the
235 model.

236

237 **3 | RESULTS**

238 Male GSI distributions were multimodal within countries (Figure 1). With the exception of
239 data from the Netherlands, the overlap of normally-distributed GSI groups was low; SI was
240 always >2 and thereby provided meaningful separation of modal groups, even for the
241 Netherlands specimens (separation index = 2.68). Decomposition of GSI distributions
242 showed the threshold GSI value that distinguished parental and cuckolder males varied
243 among countries, from 2.3 (Poland) to 4.2 (England) (Table 2, Figure1). In Belgian, French
244 and Polish populations, separation of male strategies was ambiguous, with Bhattacharya
245 decomposition revealing two thresholds within these data (Figure 1). In each case, the
246 higher threshold was used as the cut-off between male strategies.

247 The probability of expressing a red operculum spot was predicted by male SL, with a
248 significantly greater likelihood of a red spot in larger males (Table 3; Figure 2). The
249 probability of a red operculum spot was also predicted by sex, with both cuckolder and
250 territorial males significantly more likely to express a red spot than females (Table 3;
251 Figure 2). Among pumpkinseed that expressed a red spot, there was a significantly
252 stronger interaction between SL and spot size in males than in females (Table 4; Figure
253 3), though there was no difference between male strategies classified by GSI (Figure 3).
254 Two specimens were observed to possess a yellow operculum spot, a mature female from
255 Schoapedobbe Pond and a male from Brière Marsh (Table 1).

256

257 **4 | DISCUSSION**

258 Our analysis of introduced pumpkinseed populations across Europe demonstrated that
259 the expression of a red operculum spot was more likely in mature males than females,
260 (Table 3), whereas only a single sexually immature juvenile expressed a red spot. Further,
261 a significantly stronger interaction was observed (Table 4) between red spot size and body
262 size in parental and cuckolder males than in females. Taken together, these findings
263 indicate that in pumpkinseed, the presence and size of a red operculum spot signals

264 sexual maturity and correlates strongly with body size in males. This signal, however,
265 does not distinguish between the male mating strategies of parental and cuckold.

266 Colour signals can have multiple functions, potentially conveying information to rivals,
267 mates or both. In pumpkinseed, the function of the red operculum spot is equivocal. The
268 present findings are consistent with previous studies in demonstrating that a red spot has
269 a potential role in male-male interactions by eliciting an aggressive response by males
270 (Stacey & Chiszar, 1978). Notably, during aggressive intra-sexual contests, male
271 sunfishes flare their opercula (Colgan & Gross, 1977), displaying the red spot to a rival
272 facing them head-on. Our results support a role for the red operculum spot in male
273 contests, with the expression of a red spot primarily associated with sexually mature males
274 and correlating with body size. However, this finding contradicts our prediction that
275 parental males, which experience intrasexual contests and intersexual mate choice,
276 should be more likely than cuckold males to express a red operculum spot.

277 The expression of a red carotenoid-based colour signal may convey information about
278 male condition, which could be functional in the context of male-male contests.
279 Carotenoids are acquired solely in the diet by vertebrates and these compounds have a
280 number of critical physiological functions, making carotenoid-based signals a potentially
281 reliable indication of individual condition and fighting ability (Olson & Owens, 1998).
282 Whether additional information is conveyed by the size, reflectance spectra and intensity
283 of the red colour spot, or whether the red operculum spot comprises one component of a
284 multimodal signal in pumpkinseed, remains to be investigated. Experimental studies
285 would be particularly effective in differentiating these aspects of signal evolution in
286 pumpkinseed.

287 Surprisingly, we detected no significant difference between parental and cuckold
288 males, either in the probability of expressing a red spot, or in the strength of the
289 relationship between red spot size and male size. Alternative mating strategies in fish,
290 which are exclusively shown by males in teleost fishes, are reflected most strikingly in
291 reproductive behaviour, but also in external appearance and physiology. In contrast to
292 Gross' (1982) interpretation of his sunfish model to a single native population of
293 pumpkinseed, our analysis of non-native populations in Europe suggests that, while a
294 clear distinction can be seen between the parental and cuckold male reproductive
295 strategies in relative testes size, the GSI value threshold that distinguishes parental and
296 cuckold males varies among populations, ranging from 2.3 (Poland) to 4.2 (England).

297 A possible alternative explanation for the observed range of GSI values may relate to
298 variation in pumpkinseed population density, which has consequences for the
299 reproductive success of cuckold males. In water bodies where the populations occurred
300 at high density, traits associated with sperm competition, including large testes size, are
301 favoured, thereby potentially selecting for relatively larger gonads (high GSI) in both
302 parental and cuckold males, with a consequent upward shift in the threshold between
303 them. In the three ponds in England for which quantitative density data were available
304 (Fox et al., 2011), pumpkinseed was virtually the only species present in the two ponds
305 where the species have been observed in high density and males in these ponds had
306 higher mean and maximum male GSI values than observed in the low-density pond. In
307 water bodies where populations occur at low densities, where the risk of sperm
308 competition for parental males is low, relative testes size is predicted to be smaller and
309 the GSI threshold between parental and cuckold males lower (Wootton & Smith 2015).

310 A caveat to these conclusions was the finding that many females also expressed red
311 spots in their opercula, albeit with lower probability than males (Figure 2). The size of red
312 operculum spots in females also conveyed less information about size than in males
313 (Figure 3). If red operculum spots function primarily in male-male contests, then it is
314 unclear why females would display this trait. For example, the red operculum spot may
315 have multiple functions, potentially signalling male size and fighting ability, but also
316 undergoing intersexual selection in the context of mate choice. If the red operculum spot
317 is selected through intersexual selection, then it is possible that females express the trait
318 for the same reason as males. Female ornamentation is surprisingly common in teleosts,
319 and in the context of the pumpkinseed mating system, in which males are the sole
320 providers of parental care, the evolution of male mate choice and female ornamentation
321 is possible (Wootton & Smith 2015).

322 The expression of red operculum spots in males is adaptive solely through intra-sexual
323 selection, whereas in females the expression of red spots may be through a genetic
324 correlated response. In threespine stickleback (*Gasterosteus aculeatus*), red throat
325 colouration is known to function in intra- and intersexual selection on males and is a
326 reliable indicator of body condition and parasite resistance (Milinski & Bakker, 1990;
327 Barber, Arnott, Braithwaite, Andrew, & Huntingford, 2001). The red ornament, which has
328 a strong genetic component, is also found in females and its expression in females is
329 consistent with pleiotropy (Yong, Peichel & McKinnon, 2016). Pleiotropy may also mediate
330 the expression of a red operculum spot in female pumpkinseed, and possibly also in

331 cuckold males, though this conclusion relies on there being a shared genetic basis to
332 red spots in males and females and no adaptive value in expressing a red spot in females
333 and cuckolders, and these assumptions remain to be tested.

334 The presence of two male GSI thresholds in pumpkinseed populations in Belgium,
335 France and Poland provides evidence that, contrary to Gross, (1982), both the satellite
336 and sneaker strategies may be present in some non-native European pumpkinseed
337 populations (Figure 1). Alternatively, these findings may simply reflect the greater
338 heterogeneity in age at maturity within some European populations (Copp & Fox, 2007;
339 Fox & Copp, 2014), which is a possible outcome of multiple sources of introduction or
340 adaptation to conditions outside the natural range of the species. Behavioural studies,
341 complemented with morphological analyses, are needed to clarify the existence of a
342 satellite strategy in European pumpkinseed populations. Similarly, variation in predation
343 among populations has the capacity to modify the selective landscape for red operculum
344 spots, since sexual selection for visual signals can be limited through natural selection by
345 predators in teleosts (Endler, 1980; Wootton & Smith, 2015). Although outside the scope
346 of the present study, the effect of selective predation on pumpkinseed in response to the
347 expression of the red operculum spot is not known and warrants further research.

348 In conclusion, the present study demonstrates that the presence of a red operculum
349 spot in pumpkinseed is associated with sexual maturation, primarily in males. The size of
350 red spot correlates with body size in parental and cuckold males, although there was no
351 difference in the presence or relative size of the red operculum spot between male mating
352 strategies. The observed GSI distributions suggest the possible existence of the satellite
353 mating strategy in some European populations, but alternatively this may be due to
354 variations in pumpkinseed population density or to selective predation. The function, if
355 any, of a red operculum spot in females is not clear but is consistent with pleiotropy.

356

357 **ACKNOWLEDGEMENTS**

358 This study, which derives from work initiated as part of a N.A.T.O. Collaborative Linkage
359 Grant (LST-CLG No. 979499), supported by consecutive research contracts (SF0238 and
360 SF0248) from the UK Department of Environment, Food and Rural Affairs (to GHC), which
361 were complemented by a Marie Curie post-doctoral fellowship (PIEF-GA-2008-219707)
362 and a subsequent research grant from the National Science Centre, Poland (decision No
363 DEC-2011/01/D/NZ8/01807) to GZ. We thank two anonymous reviewers for their
364 constructive and perceptive comments.

365

366 **Author contribution**

367 The study was conceived by GHC and MGF. Specimens were collected and processed
368 by GHC, GZ, MGF, SY, EZ, GM, JC, HV and HHvK. Data were analysed by GHC, SY,
369 GZ, MP and CS. The initial draft of the manuscript was prepared by SY, EZ, GZ and GHC,
370 then extensively revised by GZ, CS and MP, with co-author contributions.

371

372

373 **REFERENCES**

374 Almeida, D., Tobes, I., Miranda R., & Copp G. H. (2012). Cuckoldry features of introduced
375 pumpkinseed sunfish (*Lepomis gibbosus*) in contrasting environmental conditions in
376 southern Europe. *Canadian Journal of Zoology*, 90, 1051–1057.
377 <https://doi.org/10.1139/z2012-073>

378 Andersson, M. (1994). *Sexual Selection*. Princeton University Press, Princeton.

379 Barber, I., Arnott, S. A., Braithwaite, V. A., Andrew, J., & Huntingford, F. A. (2001). Indirect
380 fitness consequences of mate choice in sticklebacks: offspring of brighter males grow
381 slowly but resist parasitic infections. *Proceedings of the Royal Society of London Series*
382 *B-Biological Sciences*, 268(1462), 71–76. <https://doi.org/10.1098/rspb.2000.1331>

383 Bates, D., Maechler, M., Bolker, B., & Walker, S. (2014). lme4: Linear mixed-effects
384 models using Eigen and S4. R package version 1(7), 1–23.

385 Bhattacharya, C. G. (1967). A simple method of resolution of a distribution into Gaussian
386 components. *Biometrics*, 23, 115–135. <https://doi.org/10.2307/2528285>

387 Colgan, P. W., & Gross M. R. (1977). Dynamics of aggression in male pumpkinseed
388 sunfish (*Lepomis gibbosus*) over the reproductive phase. *Zeitschrift für*
389 *Tierpsychologie*, 43, 139–151. <https://doi.org/10.1111/j.1439-0310.1977.tb00064.x>

390 Copp, G. H., & Fox, M. G. (2007). Growth and life history traits of introduced pumpkinseed
391 (*Lepomis gibbosus*) in Europe, and the relevance to its potential invasiveness. In F.
392 Gherardi (Ed.), *Biological Invaders in Inland Waters: Profiles, Distribution, and Threats*,
393 (pp. 289–306). Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-6029-8_15

394 Cucherousset, J., Copp, G. H., Fox, M. G., Sterud, E., Van Kleef, H. H., Verreycken, H.,
395 & Záhorská, E. (2009). Life-history traits and potential invasiveness of introduced
396 pumpkinseed *Lepomis gibbosus* populations in northwestern Europe. *Biological*
397 *Invasions*, 11, 2171–2180. <https://doi.org/10.1007/s10530-009-9493-5>

398 Endler, J. A. (1980). Natural selection on color pattern in *Poecilia reticulata*. *Evolution*, 34,
399 76–91.

400 Fox, M. G., & Copp, G. H. (2014). Old world versus new world: life-history alterations in a
401 successful invader introduced across Europe. *Oecologia*, 174, 435–446.
402 <https://doi.org/10.1007/s00442-013-2776-7>

403 Fox, M. G., Villeneuve, F., & Copp, G. H. (2011). Seasonal reproductive allocation, local-
404 scale variation and environmental influences on life history traits of introduced
405 pumpkinseed (*Lepomis gibbosus*) in Southern England. *Fundamental and Applied*
406 *Limnology*, 178, 231–243. <https://doi.org/10.1127/1863-9135/2011/0178-0231>

407 Garner, S. R., & Neff, B. D. (2013). Alternative male reproductive tactics drive
408 asymmetrical hybridization between sunfishes (*Lepomis* spp.). *Biology Letters*, 9(6),
409 20130658. <https://doi.org/10.1098/rsbl.2013.0658>

410 Gayanilo, F. C., Soriano, M., & Pauly, D. (1989). A draft guide to the Complete ELEFAN.
411 International Center for Living Aquatic Resources Management, Manila.
412 (www.worldfishcenter.org/content/draft-guide-compleat-elefan).

413 Gross, M. R. (1979). Cuckoldry in sunfishes (*Lepomis*: Centrarchidae). *Canadian Journal*
414 *of Zoology*, 57, 1507–1509. <https://doi.org/10.1139/z79-197>

415 Gross, M. R. (1982). Sneakers, satellites and parentals: polymorphic mating strategies in
416 North American sunfishes. *Zeitschrift für Tierpsychologie*, 60, 1–26.
417 <https://doi.org/10.1111/j.1439-0310.1982.tb01073.x>

418 Kokko H., Brooks, R., Jennions, M. D., & Morley, J. (2003) The evolution of mate choices
419 and mating biases. *Proceedings of the Royal Society of London Series B-Biological*
420 *Sciences*, 270(1515), 653–664. <https://doi.org/10.1098/rspb.2002.2235>

421 Marshall, N. J. (2000). The visual ecology of reef fish colours. In Y. Espmark, T. Amundsen
422 & G. Rosenqvist (Eds.), *Signaling and Signal Design in Animal Communication*, (pp.
423 83–120). Tapir Academic Press, Trondheim.

424 McLennan D. A. (2007) The umwelt of the three-spined stickleback. In S. Ostlund-Nilsson,
425 I. Mayer & F. Huntingford F (Eds.), *Biology of the Three-Spined Stickleback* (pp. 179–
426 224). CRC Press, Boca Raton.

427 Milinski, M., & Bakker, T. C. (1990). Female sticklebacks use male coloration in mate
428 choice and hence avoid parasitized males. *Nature*, 344(6264), 330–333.
429 <https://doi.org/10.1038/344330a0>

- 430 Montgomerie, R., & Fitzpatrick, J. L. (2009). Testes, sperm, and sperm competition. In B.
431 G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Fishes (Agnathans and*
432 *Bony Fishes)* (pp. 1–53). Science Publishers, Enfield (NH).
- 433 Morrell, L. J., Backwell, P. R., & Metcalfe, N. B. (2005). Fighting in fiddler crabs *Uca*
434 *mjoebergi*: what determines duration? *Animal Behaviour*, *70*, 653–662.
435 <https://doi.org/10.1016/j.anbehav.2004.11.014>
- 436 Neff, B. D., & Clare, E. L. (2008). Temporal variation in cuckoldry and paternity in two
437 sunfish species (*Lepomis* spp.) with alternative reproductive tactics. *Canadian Journal*
438 *of Zoology*, *86*, 92–98. <https://doi.org/10.1139/Z07-121>
- 439 Olson, V. A., & Owens, I. P. (1998). Costly sexual signals: are carotenoids rare, risky or
440 required? *Trends in Ecology & Evolution*, *13*, 510–514. <https://doi.org/10.1016/S0169->
441 [5347\(98\)01484-0](https://doi.org/10.1016/S0169-5347(98)01484-0)
- 442 Parker, G. A. (1970). Sperm competition and its evolutionary consequences in insects.
443 *Biological Reviews*, *45*, 525–567. <https://doi.org/10.1111/j.1469-185X.1970.tb01176.x>
- 444 Price, A. C., Weadick, C. J., Shim, J., & Rodd, F. H. (2008). Pigments, patterns, and fish
445 behavior. *Zebrafish*, *5*, 297–307. <https://doi.org/10.1089/zeb.2008.0551>
- 446 Rios-Cardenas, O. (2003). The use of Molecular Genetic Analyses to Examine Mating
447 Patterns a Paternal Care in the Pumpkinseed Sunfish (PhD dissertation). State
448 University of New York at Buffalo.
- 449 Scott, W. B., & Crossman, E. J. (1973). *Freshwater Fishes of Canada*. Fisheries Research
450 Board of Canada, Ottawa.
- 451 Smith, C., & Wootton, R. J. (1995). The costs of parental care in teleost fishes. *Reviews*
452 *in Fish Biology and Fisheries*, *5*, 7–22.
- 453 Stacey, P. B., & Chiszar, D. (1978). Body color pattern and the aggressive behavior of
454 male pumpkinseed sunfish (*Lepomis gibbosus*) during the reproductive season.
455 *Behaviour*, *64*, 271–297. <https://doi.org/10.1163/156853978X00062>
- 456 Taborsky, M. (2008). Alternative reproductive tactics in fish. In R. F. Oliveira, M. Taborsky
457 & H. J. Brockmann (Eds.), *Alternative Reproductive Tactics: An Integrative Approach*
458 (pp. 251–299). Cambridge University Press, Cambridge.
- 459 Valente, E., Masson, G., Maul, A., Fox, M. G., Meyer, A., & Pihan J. C. (2016). Seasonal
460 gonadal development and age-related maturity patterns of introduced pumpkinseed
461 (*Lepomis gibbosus* Linnaeus, 1758) in a heated thermal reservoir and an adjacent river
462 reach. *Journal of Thermal Biology*, *58*, 60–71.
463 <https://doi.org/10.1016/j.jtherbio.2016.03.004>

- 464 Villeneuve, F., Copp, G. H., Fox, M. G., & Stakėnas, S. (2005). Interpopulation variation
465 in the growth and life history traits of the introduced sunfish, pumpkinseed *Lepomis*
466 *gibbosus*, in Southern England. *Journal of Applied Ichthyology*, 21, 275–281.
467 [https://doi: 10.1111/j.1439-0426.2005.00690.x](https://doi:10.1111/j.1439-0426.2005.00690.x)
- 468 Wedell, N., Gage, M. J. G., & Parker, G. A. (2002). Sperm competition, male prudence
469 and sperm-limited females. *Trends in Ecology and Evolution*, 17, 313-320.
470 [https://doi.org/10.1016/S0169-5347\(02\)02533-8](https://doi.org/10.1016/S0169-5347(02)02533-8)
- 471 Wootton R. J. (1984) Introduction: tactics and strategies in fish reproduction. In G. W.
472 Potts & R. J. Wootton (Eds.), *Fish Reproduction: Strategies and Tactics* (pp. 1–12).
473 Academic Press, London.
- 474 Wootton, R. J. (1998). *Ecology of Teleost Fishes*, 2nd edn. Elsevier, Dordrecht.
- 475 Wootton, R. J., & Smith, C. (2015). *Reproductive Biology of Teleost Fishes*. Wiley-
476 Blackwell, Oxford.
- 477 Yong, L., Peichel, C. L., & McKinnon, J. S. (2016). Genetic architecture of conspicuous
478 red ornaments in female threespine stickleback. *G3-Genes Genomes Genetics*, 6(3),
479 579–588. <https://doi.org/1534/g3.115.024505>

480 **TABLE 1.** Country, population, location (WGS84 latitude and longitude coordinates) and capture method for male pumpkinseed
 481 *Lepomis gibbosus*, including number of specimens (*n*), mean and SD of standard length (mm) and the mean, minimum (min.) and
 482 maximum (max.) of the gonado-somatic index (GSI) for each population by water body.
 483

484	Country	Population	Latitude	Longitude	Method [†]	n	SL (SD)	GSI mean	min.	max.
486	Belgium	Webbekomsbroek Pond	50.975833N	5.076111E	ELF	76	61.2 (15.7)	2.4	0.2	5.3
487	France	Briere Marsh	47.366111N	2.312500W	ELF	63	90.9 (16.4)	1.9	0.5	4.6
488		Grand-Lieu Lake	47.083333N	1.650000W	ELF	51	69.4 (21.2)	3.1	0.2	8.3
489	Netherlands	Schoapedobbe Pond	52.951944N	6.258333E	NET	30	124.2 (16.6)	2.0	0.7	5.6
490		Meeuwven Pond	51.391111N	5.499722E	NET	58	67.3 (3.8)	3.5	1.3	10.7
491	Poland	Brodowski Pond	53.450342N	14.565856E	ELF	24	77.1 (11.1)	2.0	0.2	5.2
492		Power Plant discharge channel	53.213983N	14.468975E	ELF	25	77.1 (18.4)	1.8	0.1	5.7
493	England	Cottesmore Pond (Lower)	51.089167N	0.219444W	TRC	31	64.5 (12.5)	2.3	0.4	9.5
494		Cottesmore Pond (Upper)	51.088056N	0.219444W	TRC	82	64.9 (12.3)	2.6	0.8	8.3
495		Cottesmore Pond (School)	51.087222N	0.219444W	TRC	56	64.4 (16.2)	1.8	0.2	2.9
496		Batts Stream at Cackle Street	51.018611N	0.074444W	ELF	28	75.2 (12.4)	1.7	0.7	6.4
497		Tanyard Specimen Lake 3	51.019843N	0.012377W	ANG	39	92.7 (16.4)	2.4	0.9	12.5

498 [†]ANG = angling; ELF = continuous electrofishing; NET = small seine netting; TRC = minnow traps, catch-per-unit-effort (see Fox et al. 2011)

499 **TABLE 2.** Mean (SD) pumpkinseed body mass, standard length (SL) and gonado-somatic
500 index (GSI; n/a = not available) among countries of origin and reproductive status. Male
501 reproductive strategy (either cuckolder or parental) was identified by separation of GSI
502 frequency distributions by Bhattacharya's (1967) method.

Country of origin	Reproductive status	n	Mass (g)	SL (mm)	GSI
Belgium	Female	61	8.33 (9.89)	61.3 (16.0)	n/a
	Juvenile	10	3.85 (1.63)	52.5 (6.5)	n/a
	Cuckolder	21	4.81 (2.59)	55.9 (7.3)	3.60 (0.86)
	Parental	24	14.86 (10.22)	75.2 (16.4)	1.26 (0.59)
France	Female	66	17.81 (11.68)	81.1 (18.5)	n/a
	Juvenile	16	1.28 (0.75)	38.9 (6.1)	n/a
	Cuckolder	15	5.03 (2.95)	55.6 (8.9)	4.90 (1.11)
	Parental	91	22.58 (14.21)	88.2 (17.1)	2.00 (0.83)
Netherlands	Female	62	23.77 (23.99)	86.7 (30.2)	n/a
	Juvenile	1	7.00	66.6	n/a
	Cuckolder	33	7.25 (1.16)	66.3 (4.5)	4.25 (1.32)
	Parental	51	36.35 (26.58)	101.3 (30.4)	2.10 (0.63)
Poland	Female	18	20.25 (11.76)	76.4 (14.5)	n/a
	Juvenile	0	n/a	n/a	n/a
	Cuckolder	10	11.01 (7.24)	64.2 (10.3)	4.19 (1.05)
	Parental	39	23.79 (12.42)	80.4 (14.4)	1.26 (0.62)
England	Female	180	14.46 (14.64)	76.5 (19.9)	n/a
	Juvenile	45	2.56 (3.14)	46.2 (13.4)	n/a
	Cuckolder	16	5.69 (7.08)	59.4 (14.4)	6.20 (2.23)
	Parental	165	12.29 (10.04)	76.1 (16.2)	n/a

503

504

505 **TABLE 3.** Summary of Bernoulli GLMM to model the probability of pumpkinseed
506 expressing a red operculum spot. Fish from different populations were fitted as random
507 intercepts, with standard deviation of 1.47. $N_{\text{obs}} = 897$.

Model parameter	Estimate	SE	<i>P</i>
Intercept _(female)	-8.02	0.80	<0.001
SL [†]	0.08	0.01	<0.001
Strategy _(cuckolder)	2.30	0.34	<0.001
Strategy _(territorial)	2.35	0.23	<0.001

508 † SL = standard length

509

510

511

512 **TABLE 4.** Summary of a Gaussian GLMM to model the size of pumpkinseed red
 513 operculum spots. Fish from different populations were fitted as random intercepts, with
 514 standard deviation of 0.24. $N_{\text{obs}} = 344$.

Model parameter	Estimate	SE	<i>P</i>
Intercept _(female)	-7.43	0.99	<0.001
Log ₁₀ SL [†]	1.82	0.22	<0.001
Strategy _(cuckolder)	-5.31	2.77	0.055
Strategy _(territorial)	-4.38	1.06	<0.001
Log ₁₀ SL x Strategy _(cuckolder)	1.40	0.66	0.033
Log ₁₀ SL x Strategy _(territorial)	1.18	0.24	<0.001

515 † SL = standard length

516
 517

518 **Figure legends**

519 **FIGURE 1.** Observed gonado-somatic index (GSI): frequency plots for pumpkinseed
520 among country of origin with Gaussian distribution curves and separation indices (SI),
521 generated using Bhattacharyya's (1967) method. Threshold GSI values for separating
522 frequency distributions for male mating strategies are indicated with arrows.

523 **FIGURE 2.** Mean fitted probability (solid line) of pumpkinseed expressing a red operculum
524 spot as a function of standard length (SL in mm) with 95% confidence intervals (shaded
525 area) for females, cuckolder males and parental males. Data were modelled with a
526 Bernoulli GLMM. Fish from different populations were fitted as random intercepts in the
527 model.

528 **FIGURE 3.** Mean fitted Log_{10} area (mm^2) of red operculum spot (solid line) of
529 pumpkinseed as a function of log_{10} standard length (SL in mm) with 95% confidence
530 intervals (shaded area) for females, cuckolder males and parental males fitted with a
531 Gaussian GLMM. Fish from different populations were fitted as random intercepts in the
532 model.