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<http://dx.doi.org/10.1111/jfb.13130>

<b>Title</b>	The ART of mating : alternative reproductive tactics and mating success in a nest-guarding fish
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<b>Publication title</b>	Journal of Fish Biology
<b>Publisher</b>	Wiley
<b>Type</b>	Article
<b>USIR URL</b>	This version is available at: <a href="http://usir.salford.ac.uk/id/eprint/39890/">http://usir.salford.ac.uk/id/eprint/39890/</a>
<b>Published Date</b>	2016

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26 Fish use different modalities to access mates for reproduction, often referred to as Alternative  
27 Reproductive Tactics (ARTs). ARTs are an example of coexisting phenotypes, which have to  
28 hold some degree of reproductive success to persist in a population. In the Mediterranean  
29 damselfish (*Chromis chromis*), territorial males colonise nests on rocky reefs, competing for  
30 females, while sneaker males attempt to parasitically spawn in those nests. Here we combine  
31 behavioural observations in the field with molecular analyses, using bi-parentally and  
32 maternally inherited markers, to investigate reproductive success patterns of the two observed  
33 male ARTs in terms of number of eggs sired and number of females contributing to each nest.  
34 Cuckoldry was observed in every nest sampled, with at least two and up to seven sneakers per  
35 nest; however, the nesting male always significantly fathered the large majority of the eggs (on  
36 average 49%) in each clutch. Each sneaker fathered around 7% of the clutch. The average  
37 number of females whose eggs were fertilised by nesting males was 6.76 (ranging 2-13), while  
38 each sneaker on average fertilised the eggs of 1.74 (range 1-8) females. Using this sibship  
39 reconstruction, we investigated some of the factors involved in the regulation of the dynamic  
40 equilibrium of reproductive success between the two ARTs showed by *C. chromis* males. Our  
41 results show that the sneakers' reproductive success was positively linked to egg clutch size;  
42 the density of individuals in the nesting area negatively affected the size of egg clutches; the  
43 rate of defence behaviours performed by nesting males negatively influenced the number of  
44 females contributing to each nest.

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46 Key words: *Chromis chromis*; microsatellites; mtDNA; parentage assignment; sneakers.

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## INTRODUCTION

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Different modalities to access mates for reproduction, also called Alternative Reproductive Tactics (ARTs, Oliveira *et al.*, 2008), are an example of coexisting phenotypes (Taborsky, 1994; Heinze & Keller, 2000), where conspecifics of the same sex in one population invest different amounts of energy and exhibit remarkably different strategies to ensure reproduction (Taborsky *et al.*, 2008). Fish offer a great opportunity to investigate ARTs since they show a huge diversity of reproductive modes (e.g., DeWoody & Avise, 2001, Avise *et al.*, 2002). In particular, males, depending on their mating system, can maximise their fitness through scramble competition, resource defence (e.g., territory, nest, and/or females), reproductive parasitism or even cooperation (Taborsky, 2001). Of these four strategies, resource defence and reproductive parasitism are often coupled as ARTs: territorial males (also called nesting males) conquer and defend a suitable territory and actively attract females, while other males behave as “sneakers”, by parasitically spawning in the nest of a territorial male, without providing any territorial defence or paternal care (Taborsky, 1994; Coleman & Jones, 2011).

While comprehensive knowledge of these mating strategies is available from a behavioural point of view, less is known about the actual relative reproductive success occurring as a consequence of coexisting ARTs (Garant *et al.*, 2001; Reichard *et al.*, 2004; Cogliati *et al.*, 2013). Knowledge of the relative fitness among different tactics can help to clarify if coexisting ARTs represent a plastic/conditional strategy (dependent mainly from environmental conditions, resulting in unequal male fitness) or a genetic polymorphism (maintained by negative frequency dependent selection, resulting in overall similar male fitness) or, as recently proposed, a mixture of these two modalities, called a conditional alternative strategy (Neff & Svensson, 2013; Cogliati *et al.*, 2014). Fitness equilibrium between ARTs can be maintained by a complex interaction of multiple variables, including individual

76 traits, population features and environmental characteristics. According to literature, most of  
77 the species with male nest-defence tactics exhibit a certain level of cuckoldry (i.e., part of their  
78 egg clutch is fertilised by other males; Coleman & Jones, 2011). Yet, the relative number of  
79 eggs fertilised by males other than the nesting one is rather limited (Mackiewicz *et al.*, 2005;  
80 Rios-Cardenas & Webster, 2008; Alonzo & Heckman, 2010). Furthermore, the rate of multiple  
81 maternity for these species is very high, indicating that polygyny is fairly common in these  
82 species, but the average number of dams contributing to a single nest is low (three females on  
83 average; Coleman & Jones, 2011).

84         Members of the family Pomacentridae are known to exhibit ARTs (Gronell, 1989;  
85 Karino & Nakazono, 1993; Tyler, 1995; Barnett & Pankhurst, 1996). The Mediterranean  
86 damselfish, *Chromis chromis* (Linnaeus, 1758), is characterised by male nest-defence. This is  
87 a small (average standard length 6 cm; Bracciali *et al.*, 2014), sexually monomorphic species  
88 commonly distributed in the Mediterranean Sea, extending to the Eastern part of the Atlantic  
89 Ocean. It lives in shoals near rocky reefs or above seagrass meadows at depths between 3 and  
90 30 m (Lythgoe & Lythgoe, 1971; Quignard & Pras, 1986). Throughout the reproductive season  
91 (June-September), colonies go through several reproductive bouts. Some males (nesting males)  
92 colonise nests and guard them until eggs hatch. They attract females to receive egg deposition  
93 by vocalising and performing specific courtship-displays (Picciulin *et al.*, 2002, 2010).  
94 Parasitic spawning by sneaker males is commonly observed in the Mediterranean damselfish;  
95 sneakers do not establish nests, infiltrating a guarded nest, while the nesting male receives egg  
96 deposition by a female. On average, nesting males can receive three intrusions over a 10 min  
97 observation period (Picciulin *et al.*, 2004). However, the success rate of these “attacks” remains  
98 unclear and molecular investigation is needed to unveil the real reproductive success of  
99 sneakers. Additionally, parasitic spawning is known to be occasionally performed even by

100 nesting males (Picciulin *et al.*, 2004). *Chromis chromis* thus offers a model to study the relative  
101 reproductive success attained by nesting males and sneakers.

102 In this study, a combination of bi-parentally and maternally inherited markers was used  
103 to investigate reproductive patterns in *C. chromis*. In particular, the number of breeders  
104 contributing to each nest was examined, with a special focus on the relative reproductive  
105 success of nest-guarding males and sneakers in terms of percentage of egg clutch sired, and the  
106 number of females contributing to each clutch. Additional factors, such as egg clutch size (often  
107 linked to male success), density of the colony (which influences encounter rates between sexes  
108 and male-male competition) and male aggressive behaviours (towards intruders) can also affect  
109 reproductive outcomes. Thus, using sibship reconstruction, the following factors were also  
110 investigated: a) the relationship between egg clutch size and the relative success of nesting  
111 males and sneakers; b) the link between the density of individuals in the breeding colony and  
112 egg clutch size; and c) the relationship between the frequency of nest-defence behaviours  
113 performed by males and the number of females spawning in their nest.

## 114 MATERIALS AND METHODS

### 115 STUDY AREA AND SAMPLE COLLECTION

116 Two damselfish colonies were selected 5-10 m from the coasts of Palermo and Zingaro,  
117 Sicily, Italy (Fig. 1) in May 2011. Twenty-five spherical flowerpots (18 cm diameter; Knapp  
118 & Kovach, 1991) were placed at 7-12 m depth to provide males with artificial nests  
119 (minimising differences in nests' quality and size; Fig. 1). Overall, twelve artificial nests (five  
120 from Palermo and seven from Zingaro) were successfully colonised by *C. chromis*. Colonized  
121 nests were monitored with video cameras during June and August 2011. At the end of the  
122 behavioural observations, artificial nests (which contained all the eggs attached to the pot  
123 surface on a single layer) were collected. Given their fast swimming behaviour, it was not  
124 possible to catch the nesting males. Once on the boat, the relative quantity of eggs present in

125 each nest was estimated (given their small size, it was not possible to count all the eggs). All  
126 egg clutches exhibited a diamond shape; minor and major axes were measured to calculate the  
127 surface area of each egg clutch (cm<sup>2</sup>). All eggs were detached from the flowerpot using a  
128 scalpel and immediately stored in 100% ethanol and subsequently at -20°C.

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#### 130 DNA ISOLATION, PCR AMPLIFICATION, GENOTYPING AND SEQUENCING

131 Single eggs were separated under a stereomicroscope. DNA was isolated from a random  
132 subsample of 48 eggs from each nest (total N = 576) using a modified salt extraction protocol  
133 (Miller *et al.*, 1988) and 376 eggs (30-48 per nest) were successfully amplified at seven  
134 microsatellite loci. Primers from four species of the Pomacentridae family were employed:  
135 2AL2 (*Abudefduf luridus*; Carvalho *et al.*, 2000), Cm\_D006 (*Chromis margaritifer*;  
136 Underwood, 2009), Da360, Da542, Da589, Da590 (*Dascyllus aruanus*; Fauvelot *et al.*, 2009)  
137 and SpTG53 (*Stegastes partitus*; Thiessen & Heath, 2007). Loci were amplified in two  
138 multiplex polymerase chain reactions (PCR). Each reaction was carried out in a total volume  
139 of 12 µl, using 6 µl of QIAGEN® Multiplex PCR kit, 4.4 µl of DNA template and 1.6 µl of  
140 primer mix. Fluorescently-labelled primers (FAM, VIC, NED and PET) were added with the  
141 following concentrations: Cm\_D006 0.17 µM, 2AL2 0.17 µM, SpTG53 0.33 µM (multiplex  
142 1); Da542 0.17 µM, Da589 0.17 µM, Da590 0.17 µM, Da360 0.08 µM (multiplex 2).  
143 Amplification conditions were as follows: 95°C for 15 min; 37 cycles of 94°C for 45 s, 52°C  
144 for 1 min, 72°C for 45 s and a final extension at 72°C for 45 min (multiplex 1); 95°C for 15  
145 min; 37 cycles of 94°C for 45 s, 58°C for 1 min, 72°C for 45 s and a final extension at 72°C for  
146 45min (multiplex 2). PCR reactions were performed using a Biometra T3000 thermocycler.  
147 Allele sizes were determined on an ABI-3130xl Genetic Analyser (Applied Biosystems©) with  
148 an internal size standard (600 LIZ, Applied Biosystems©). Genotypic data were acquired using

149 GeneMapper 4.0 (Applied Biosystems©). Following standard practice, we re-amplified and re-  
150 scored genotypes for 10% (30 eggs) to check for consistency of genotype calling.

151 Furthermore, amplification of 355 bp of the mitochondrial control region (CR) was  
152 carried out on a subsample of eggs from each nest (total N = 222) using the primers CR-A and  
153 CR-E (Lee *et al.*, 1995, Domingues *et al.*, 2005) in a 25 µl reaction volume, according to the  
154 following protocol: NH<sub>4</sub> Buffer X1, dNTPs 800 µM (200 µM each), CR-A 0.3 µM, CR-E 0.3  
155 µM, MgCl<sub>2</sub> 2.5 mM, Taq 2.5 U (BIOLINE), 1 µl template. Amplification conditions were as  
156 follows: 94°C for 5 min; 35 cycles of 94°C for 45 s, 52°C for 45 s, and 72°C for 1 min. Products  
157 were sequenced commercially (Beckman-Coulter Genomics). D-loop sequences were  
158 submitted to GenBank under the accession numbers KX442797-443014.

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## 160 POPULATION GENETIC ANALYSES

161 Expected unbiased ( $H_e$ ) and observed ( $H_o$ ) heterozygosities and average number of  
162 alleles ( $N_A$ ) per nest were calculated using the Microsatellite Toolkit add-in available for  
163 Microsoft Excel (Park, 2001). Allelic richness ( $A_R$ ), departure from Hardy-Weinberg  
164 equilibrium (by calculating  $F_{IS}$  and testing significance through 1320 permutations) and linkage  
165 disequilibrium were estimated using FSTAT 2.9.3.2 (Goudet, 1995).

166 The effective number of breeders ( $N_b$ ) for each nest was initially investigated by  
167 computing the effective population size ( $N_e$ ) for each nest, using the gametic disequilibrium  
168 method implemented in the software LDNe 1.31 (Waples & Do, 2008). Additionally,  
169 probability of individual identity,  $P_{(ID)}$ , was calculated for all seven loci with GIMLET  
170 (Valière, 2002);  $P_{(ID)}$  is defined as the chance that two individuals drawn at random from the  
171 same population will share the same genotypic profile at multiple loci (Waits *et al.*, 2001).  
172 GIMLET allows to calculate both  $P_{(ID)sib}$  (the  $P_{(ID)}$  of a population where siblings are found  
173 and included, Evett & Weir, 1998) and  $P_{(ID)unbiased}$  (the  $P_{(ID)}$  after sample size corrections;



174 Paetkau *et al.*, 1998); the observed  $P_{(ID)}$  lays between  $P_{(ID)sib}$  and  $P_{(ID)unbiased}$  and is estimated by  
175 computing the proportion of all possible pairs of individuals that have identical genotypes.

176 All CR sequences were manipulated on ProSeq 3.0 (Filatov, 2002), and subsequently  
177 aligned with ClustalX 2.1 (Larkin *et al.*, 2007) using the default parameters, whilst resulted  
178 alignments were verified by eye. Summary statistics of haplotypes ( $K$ ), number of polymorphic  
179 sites ( $n$ ), haplotype diversity ( $h$ ), nucleotide diversity ( $\pi$ ) and standard deviations (SD) (Nei,  
180 1987), for the pooled dataset and per nest, were calculated with Arlequin 3.11 (Excoffier *et al.*,  
181 2005). Finally, a median joining network was examined on NETWORK 4.6.1.0 (Bandelt *et al.*,  
182 1999; <http://www.fluxus-engineering.com>).

183

#### 184 RELATEDNESS ANALYSIS AND PARENTAGE ASSIGNMENT

185 Since genetic data from parents were not available, sibship and parentage of offspring  
186 were assigned in Colony 2.0 (Jones & Wang, 2009) using the built-in maximum-likelihood  
187 method for microsatellite data. Half- and full-sib pairs with a probability equal to 1.0 (100%)  
188 were selected and manually grouped under two inferred parents of unknown sex. Mitochondrial  
189 haplotype information was then used to determine the sex of each parent: offspring assigned to  
190 one parent could share the same haplotype (thus the parent was classified as female) or exhibit  
191 more than one haplotype (parent classified as male; Sefc *et al.*, 2008).

192 For those parent-offspring groups with no clear pattern (CR haplotypes were  
193 determined only for 59% of the eggs used to infer sibship), a manual cross-check across the  
194 whole data-set was performed: every offspring was shared by two parents, if one of them was  
195 clearly classified as either sex, the other had to belong to the opposite sex. The male with the  
196 highest number of offspring assigned within a nest was assumed to be the nesting male  
197 (Coleman & Jones, 2011). The number of offspring fertilised by each male in a nest was  
198 recorded and the relative proportion of eggs fathered was estimated. The average of the number

199 of females whose eggs were fertilised by each male was calculated, weighted by the number of  
200 offspring per nest.

201

## 202 BEHAVIOURAL OBSERVATIONS

203 Clear parentage information was successfully obtained for nine out of the 12 nests (N  
204 = 6 for Palermo, N = 3 for Zingaro). For these nests, behavioural data had been obtained from  
205 video cameras installed by a scuba diver in front of each nest, at a distance of 80-120 cm. The  
206 central 75 min of each 90 min video were scored to determine the number of defence-related  
207 behaviours performed (thrusts: focal male moves toward the opponent with a rapid movement  
208 and turns immediately back upon reaching the adversary; chases: focal male does not turn back  
209 after the thrust but instead chases the adversary for several metres; Verginella *et al.*, 1999). The  
210 nine focal males' behaviour was followed for a total of approximately 11 h of observation using  
211 JWatcher 1.0 (Blumstein & Daniel, 2007). The number of defence-related behaviours per hour  
212 were quantified considering only the time spent by focal males in sight of the camera view (i.e.  
213 [defence behaviours \* (total time min) - time spent out of sight min]<sup>-1</sup>) \* 60 min). Damselfish  
214 density (i.e., number of individuals per colony) varied across locations. At the end of randomly  
215 chosen sampling sessions, video files were recorded to estimate density expressed as number  
216 of individuals per breeding colony.

217

## 218 DATA EXPLORATION AND STATISTICAL ANALYSES

219 The clutch size in each nest can be assumed to be a proxy of the success of the nesting  
220 male (Carriço *et al.*, 2014), but this can be hampered by high presence of sneakers (Reichard  
221 *et al.*, 2004) and/or by high density of individuals in the colony (Mück *et al.*, 2013). Thus,  
222 nesting males should increase the number of defence behaviours against sneakers to maximize

223 their fertilization rate. This defence behaviour could become even more predominant as number  
224 of females visiting the nest increases (eggs can be deposited in a nest by a single or multiple  
225 females). Starting with these assumptions and using the available variables estimated for each  
226 nest (number of parents, number of sneakers, number of females, egg clutch size, proportion  
227 of eggs fathered by the nesting male and defence behaviours; Table I), a principal component  
228 analysis (PCA) was run. Although the dataset did not strictly meet all PCA assumptions  
229 (Budaev, 2010), the variables factor map generated was used to visualise and gather a better  
230 understanding of the complex relationships among the variables of this system. Such  
231 relationships were also tested by fitting generalised linear models (GLM) and linear models, to  
232 confirm their significance.

233         The analyses performed were: a) the relationship between the relative reproductive  
234 success of nesting males and size of the egg clutch (GLM with a binomial distribution and a  
235 logit link function: a column-bind matrix was created with the cbind function linking the  
236 number of eggs fertilized by the nesting male with those fertilized by sneakers to consider the  
237 relative frequency of nesting male success using the actual number of eggs and not proportions)  
238 ; b) the relationship between the density of individuals and the size of the clutch (GLM with a  
239 negative-binomial distribution and log link function, due to overdispersion of data; O'Hara &  
240 Kotze, 2010); c) the relationship between the density of individuals and the reproductive  
241 success of the sneakers (number of eggs not fathered by the nesting male) using a linear model.

242         Given that the main source of variability in the number of parents per nest was the  
243 number of females (see results below and Table I), a Pearson correlation was run between the  
244 number of females and the total number of parents per nest. A GLM (Poisson distribution and  
245 log link function) was fitted using the number of females per nest as the response variable and  
246 the number of defence-related behaviour per hour as the explanatory variable.

247 All GLM were tested with and without the study area as a covariate to take into account  
248 general differences between the two areas; the two models for each test were compared and the  
249 best one was chosen according the Akaike information criterion (AIC; Burnham, 2011). Data  
250 exploration and statistical analyses were performed using R statistical software (R 2.13; R  
251 Development Core Team, 2011).

252

253

## RESULTS

254 Overall, 376 offspring were successfully amplified at all seven microsatellite loci (data  
255 is available upon request). Microsatellite markers showed no evidence of linkage-  
256 disequilibrium.  $H_o$ , on average, was not significantly different from  $H_e$  (0.77 and 0.75  
257 respectively). Average number of alleles per nest was 8.08, with nest E showing the lowest and  
258 nest K showing the highest number of alleles and allelic richness respectively (Table II). The  
259 average number of breeders per nest, estimated by  $N_b$ , was 13.97, ranging from 2.3 (nest L) to  
260 29.7 (nest K). Overall  $P_{(ID)sibs}$  and  $P_{(ID)unbiased}$  were respectively  $4.130 \times 10^{-4}$  and  $2.588 \times 10^{-13}$   
261 indicating that two individuals had less than 0.04% probability of sharing the same multilocus  
262 genotypic profile (Table II).

263 In total, 218 mtDNA sequences were generated (Accession numbers: KX442797-  
264 443014), including 49 variable sites and 38 haplotypes. The CR haplotype diversity ranged  
265 from high values (0.931) to null (0.000), whilst values of nucleotide diversity ranged from  
266 0.018 to 0.000 (Table II). The individual haplotype network reflected the high haplotype  
267 diversity across areas (Fig. 2).

268 Of the original 376 eggs, 360 (173 from Palermo and 187 from Zingaro) showed a  
269 probability of 100% to be either full- or half-sib with at least another offspring and thus, they  
270 were used to infer sibship within each damselfish colony. Moreover,  $P_{(ID)sibs}$  and

271  $P_{(ID)}$ unbiased indicated a negligible probability that two individuals could share the same  
272 multilocus genotypic profile.

273 Overall, 201 offspring sequenced for mitochondrial CR (95 from Palermo and 106 from  
274 Zingaro) were used to assign sex to the parents previously inferred by COLONY. It was possible  
275 to extract clear information for nine of the twelve original nests. It was not possible to define a  
276 clear pattern for the remaining three, because either the information provided by mtDNA was  
277 not sufficient to discriminate between males and females (all offspring shared the same  
278 haplotype, nests A and E), or provided unrealistic scenarios (both parents for each given  
279 offspring showed more than one haplotype, e.g. nest B, in contrast with the assumption that  
280 females transmit the same mitotype to all of their offspring). However, even in these instances,  
281 we were able to gauge an idea of the number of parents contributing to each nest.

282 Cuckoldry was observed in every nest sampled. One male fathered most of the eggs in  
283 each nest, on average 49% (range 27-75%) of the egg clutch; we assumed that male to be the  
284 nesting one (Table I). The weighted average number of sneakers per nest was 6.48 (range 2-7);  
285 on average, each sneaker fathered 7% of the egg clutch (Supplementary Table SI). The  
286 weighted average number of females whose eggs were fertilised by nesting males was 6.76  
287 (range 2-13), while each sneaker on average fertilised the eggs of 1.74 (range 1-8) females  
288 (Supplementary Table SI). Female polygamy was also observed: 2 females (out of the 72 that  
289 contributed to our sample) were found to have laid their eggs in two different nests, D and K,  
290 during the same reproductive bout.

291 The first two principal components of the PCA explained more than 85% of the  
292 variance of the dataset (PC1: 58.18%; PC2: 27.32%). From the PCA variables factor plot,  
293 nesting male reproductive success appears to be negatively correlated with the size of the egg  
294 clutch ( $z = -3.309$ ,  $p = 0.0009$ ), which instead is linked to sneakers reproductive success (Fig.  
295 3), as supported by the GLM analysis ( $z = 3.56$ ,  $p = 0.0004$ ). Moreover, the density of

296 individuals in the colony and the clutch sizes show a negative relationship ( $z = -4.445$ ,  $p <$   
297  $0.0001$ ), whereas the density did not affect the sneakers' reproductive success ( $t = -1.696$ ,  $p =$   
298  $0.134$ ). All analyses were repeated excluding nest L, characterized by the smallest egg clutch,  
299 the higher density and the lowest number of sneakers. Without this nest, the relationships  
300 explained by the factor map (PC1+ PC2 = 71%) were maintained but density and number of  
301 sneakers were no longer significant, while the results of all the other tests were the same.

302 In the factor map, the total number of parents per nest was associated with the number  
303 of females and both showed some level of inverse relationship with the number of defence  
304 behaviours performed by nesting males. Pearson correlation between the number of parents  
305 and the number of females per nest was strongly significant ( $r = 0.96$ ;  $p < 0.0001$ ) confirming  
306 that the variability in number of parents among nests was due to the variation in number of  
307 females. The rate of defence behaviours showed to have a significant, negative effect on the  
308 number of females contributing to each nest ( $z = -2.685$ ,  $p = 0.007$ ). No correlation was found  
309 between the number of females and clutch size ( $r = 0.54$ ;  $p = 0.13$ ).

310 The AIC test suggested that adding the study area as a covariate improved only the  
311 model testing for the effect of the size of the egg clutch on sneakers reproductive success, hence  
312 we removed it from the other two models. In any case, there was no significant effect of the  
313 study area in all the models.

314

315

## DISCUSSION

316 In both *C. chromis* colonies from Palermo and Zingaro, cuckoldry was found to be  
317 pervasive in every single nest analysed. This is one of few studies (Munehara & Takenaka,  
318 2000; Alonzo & Heckman, 2010) that report such trends in natural marine fish populations:  
319 despite the very high variability in multiple paternities across species characterised by male

320 nest defence, Coleman & Jones (2011) reported an average of 35% of nests per population  
321 being fertilized by multiple males, while in the current study 100% of the nests were cuckolded.

322         The nesting male was assumed to be the individual that fertilised the majority of eggs  
323 within a nest in agreement with previous studies (Coleman & Jones, 2011). In species for which  
324 parentage studies are available, the proportion of eggs sired by males other than the nesting  
325 one was almost always below 30% (e.g., 12.4% for molly miller, *Scartella cristata*,  
326 Mackiewicz *et al.*, 2005; 15% for pumpkinseed sunfish, *Lepomis gibbosus*, Rios-Cardenas &  
327 Webster, 2008; 28% in the ocellated wrasse, *Symphodus ocellatus*, Alonzo & Heckman, 2010).  
328 However, *C. chromis* sneakers stand out from the “typical” reproductive success patterns  
329 observed in species characterised by male nest defence. On average, 51% of the eggs were  
330 sired by sneakers, with a record value of 73% observed in nest C (where eggs were fertilized  
331 by seven different sneakers). Similar values (an average of 48% of eggs sired by sneakers) have  
332 been found recently in the plainfin midshipman fish, *Porichthys notatus* (Cogliati *et al.*, 2013).  
333 In this species though, nest takeovers have been described, which lower the paternity estimates  
334 for the original nest owner (when takeovers are taken into account, the actual sneaker  
335 contribution to nest decrease to 37%). The high sneaker success in the two population of *C.*  
336 *chromis* under study is not surprising, given the high number of sneakers parasitizing each  
337 nest in the two colonies: with the exception of nest L, the number of sires contributing to each  
338 nest ranged from six to seven (Table I).

339         Multiple maternity is commonly observed in species characterised by male-nest  
340 defence (Coleman & Jones, 2001 and references therein), and in this study the level of  
341 polygyny was particularly high: up to 15 dams per nest were found, double the highest number  
342 reported to date for the molly miller (Mackiewicz *et al.*, 2005). Nesting males are expected to  
343 be polygamous; once the “reproductive resource” (i.e., the nest) is secured, their fitness is  
344 mostly limited by the number of females they manage to mate with. Females, on the other hand,

345 are normally limited by the amount of eggs they can produce and are expected to exert strong  
346 sexual selection by carefully choosing where to lay their eggs. Nests already containing eggs  
347 may be favoured (Pruett-Jones, 1992; Brennan *et al.*, 2008) as males are less likely to desert  
348 full nests (Jennions & Polakow, 2001). In this study, two females were found to lay their eggs  
349 in two different nests during the same reproductive bout. A similar scenario has been rarely  
350 reported (Taborsky *et al.*, 1987; Jones *et al.*, 1998). This strategy might be employed by  
351 females to minimize the risk of predation on their offspring or to select males with different  
352 qualities (Alonzo & Warner, 2000).

353         The size of the egg clutch was positively correlated with the reproductive success of  
354 sneakers. This means that, contrary to expectations (Carrizo *et al.*, 2014), the relative  
355 reproductive success of the nesting male decreases when many eggs are present in his nest.  
356 Indeed, a large clutch of eggs is very likely to be the result of multiple egg deposition events  
357 by one or more females. Considering that the window of opportunity for a sneaker to achieve  
358 parasitic fertilisation is during female spawning (as eggs are fertilised straight away; Picciulin  
359 *et al.*, 2004), more egg deposition events will result in higher chances to sneak. Thus, a larger  
360 clutch size possibly results in higher reproductive success for both types of males but lower  
361 relative reproductive success for the cuckolded nesting males.

362         Colonial nesting with the associated high density of individuals per breeding colony  
363 may favour parasitic spawning (Reichard *et al.*, 2004) and high levels of cuckoldry. Relatively  
364 low reproductive success by nesting males have already been observed in the freshwater fish  
365 *Variabilichromis morii* (Sefc *et al.*, 2008). Literature suggests that density of individuals plays  
366 a major role in parentage patterns due to high encounter rates among conspecifics (Kokko &  
367 Rankin 2006) making it easier for nesting males to attract females. Conversely, high numbers  
368 of individuals are often associated with high numbers of sneakers, augmenting the chances to  
369 be cuckolded (Soucy & Travis, 2003). In *C. chromis* though, clutch size seems to be negatively



370 correlated with the density of individuals in the colonies. Nesting males switch from courtship  
371 to parental care when the trade-off between trying to receive more egg deposition and avoiding  
372 to be cuckolded turns in favour of the latter (Kanoh, 2000). The threshold of this trade-off is  
373 not fixed, and it is likely to shift in response to demographic and environmental variability.  
374 Under high densities, it is possible that males perceive a higher risk of cuckoldry and shift more  
375 quickly to parental care. A similar situation has been reported in *S. ocellatus*, where nesting  
376 males surrounded by many sneakers can give up temporarily courtship activities by preventing  
377 any female to spawn in their nest to avoid competition (Alonzo & Warner, 1999). To do so  
378 effectively, males need to be able to recognise sneakers and distinguish them from females. In  
379 most species a certain dimorphism between the sexes exists (e.g., in colouration and/or size)  
380 and nesting males might be distinguishable from sneakers (Gross & Charnov, 1980; Taborsky  
381 *et al.*, 1987). *Chromis chromis* appears to be morphologically monomorphic, making  
382 individual behaviour (including acoustic behaviour) the most reliable information in  
383 distinguishing males from females. Sneakers do not show significant differences in terms of  
384 size when compared to nesting males (Picciulin *et al.*, 2004). They have however, been  
385 reported to exhibit submissive behaviours, typical of females, when attempting to intrude in  
386 the nests of guarding males (Verginella *et al.*, 1999). This behavioural adaptation, combined  
387 with the lack of evident dimorphism between males exhibiting different ARTs, may explain  
388 the high levels of cuckoldry (especially when densities are not high) and the relatively low  
389 reproductive success of the nesting males observed. Higher density of individuals might inform  
390 nesting males about the risk of cuckoldry.

391 Females must carefully pick their mates to maximise their fitness (Steinwender *et al.*,  
392 2012). Defence related behaviours were found to negatively affect the number of females  
393 laying eggs in the nests. Extremely dominant individuals may not always be the best choice  
394 (Qvarnström & Forsgren, 1998): an excessive amount of energy or time spent in aggressive

395 interactions might cause the nesting male to neglect other important activities related to  
396 parental care, such as egg fanning (Verginella *et al.*, 1999). Moreover, visually conspicuous  
397 behaviours, including defence related ones, might increase the chance to be spotted by  
398 predators (Daly, 1978; Crowley *et al.*, 1991; Dill *et al.*, 1999). For a potential dam an extremely  
399 aggressive male might not be the best carer for her eggs and likely more exposed to predators.  
400 Alternatively, the negative relationship found between the rate of defence behaviours and the  
401 number of dams, could be determined by nesting males decision: when the perceived risk of  
402 cuckoldry is high (i.e., high density of individuals), nesting males switch early to parental care.  
403 This is achieved by actively chasing away any further female approaching the nest (Alonzo &  
404 Warner, 1999).

405 Overall, this study provides new information on the mating system and, more  
406 specifically, the reproductive patterns of the nest-guarding *C. chromis*. Our findings shed new  
407 light on the role and consequences of ARTs in this nesting species: 1) cuckoldry and  
408 promiscuity were found to be widespread; 2) the number of males and females contributing to  
409 the offspring of each nest are the highest reported in literature (Coleman & Jones, 2011); 3) the  
410 reproductive success of nesting males is among the lowest reported for territorial fish (average  
411 49%, lower than 52% recorded by Cogliati *et al.*, 2013 in the plainfin midshipman fish, where  
412 nest takeovers occur; see also reviews by Avise *et al.*, 2002; Coleman & Jones, 2011). The  
413 complex interactions between factors determining the relative success of ARTs require a  
414 multidisciplinary approach ranging from molecular analyses to behavioural observations and  
415 from focal individuals to populations. Knowledge about these interactions becomes extremely  
416 important when predicting fitness patterns in a changing environment that is increasingly  
417 affected by human impacts.

418

419

420 We would like to thank: Jon Yearsley for statistical advice, Natalia Niceta, Jen Coughlan and  
421 Siobhán Bradley, Kelly Hickey, Emma Lawlor, Meabh Nic Mhathúna, Claire Morton and  
422 Martina O'Brien for their help in the field, Stephen Woodward for his help in the lab. We are  
423 grateful to two anonymous reviewers for their constructive comments on a previous version of  
424 this manuscript. This study was supported by the Irish Research Council (IRC) under the  
425 Embark Initiative (RS/2010/2106). Ethical approval was obtained by UCD, University College  
426 Dublin (AREC-P-10-33).

427

### 428 **Supporting Information**

429 Supporting Information may be found in the online version of this paper:

430 **Table SI** Relative proportion of eggs fathered by each male contributing to each nest, and  
431 number of female mates (in brackets); NM: nesting male; SNK: sneaker. Subscripts on the Nest  
432 category define sampling location: Z from Zingaro and P from Palermo.

433

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620 Table I. Variables used to define relationships in alternative reproductive tactics and mating success of *Chromis chromis*. Variables include:  
 621 number of offspring analysed (N), number of parents (N<sub>P</sub>), number of sneakers (N<sub>SNK</sub>), number of males (N<sub>M</sub>), number of females (N<sub>F</sub>), proportion  
 622 of offspring sired by the nesting male (NM<sub>off</sub>), area of the egg clutch expressed in cm<sup>2</sup>, number of defence behaviour per hour and average density  
 623 of individuals at the time of sampling. Subscripts on the Nest category define sampling location; Z from Zingaro and P from Palermo.

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<b>Nest</b>	<b>N</b>	<b>N<sub>P</sub></b>	<b>N<sub>SNK</sub></b>	<b>N<sub>M</sub></b>	<b>N<sub>F</sub></b>	<b>NM<sub>off</sub></b>	<b>Clutch Size</b>	<b>Defence</b>	<b>Density</b>
C <sub>Z</sub>	26	19	7	8	11	0.27	299	31	36.33
D <sub>P</sub>	36	23	7	8	15	0.47	204	7	28.58
F <sub>P</sub>	32	12	6	7	5	0.38	208	43	13.33
G <sub>Z</sub>	31	15	7	8	7	0.35	153	19	69.33
H <sub>Z</sub>	37	18	6	7	11	0.68	117	0	76.05
I <sub>P</sub>	42	13	7	8	5	0.67	171	22	9.33
J <sub>Z</sub>	29	13	7	8	5	0.41	180	22	36.33
K <sub>P</sub>	34	21	6	7	14	0.44	242	7	28.58
L <sub>Z</sub>	8	5	2	3	2	0.75	75	23	175.67

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630 Table II. Summary statistics from microsatellites and mtDNA analyses for each nest: sample size for microsatellite (N, mtDNA in brackets),  
631 unbiased expected heterozygosity ( $H_e$ ), observed heterozygosity ( $H_o$ ), average number of alleles ( $N_A$ ), allelic richness ( $A_R$ ), deviation from Hardy-  
632 Weinberg equilibrium ( $F_{IS}$ , starred if significant), number of breeders ( $N_b$ , 95% C.I. in brackets), number of haplotypes ( $n$ ), haplotype diversity  
633 ( $h$ ), nucleotide diversity ( $\pi$ ). Subscripts on the Nest category define sampling location: Z from Zingaro and P from Palermo.

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Nest	Microsatellites						Mitochondrial			
	N	$H_e$	$H_o$	$N_A$	$A_R$	$F_{IS}$	$N_b$	$n$	$h$	$\pi$
A <sub>Z</sub>	44(20)	0.7026	0.7208	8.57	5.40	-0.026	10.4 (7.8 - 13.5)	1	0	0
B <sub>Z</sub>	14(14)	0.7993	0.7551	7.57	6.88	0.057	17.6 (9.6 - 44.5)	5	0.659	0.00822
C <sub>Z</sub>	27(20)	0.7687	0.6349	7.57	5.94	0.177*	15 (10.3 - 22.9)	3	0.689	0.01047
D <sub>P</sub>	40(20)	0.7633	0.7607	11.14	6.77	0.003	19.6 (15.4 - 25.3)	6	0.721	0.01284
E <sub>P</sub>	29(20)	0.7043	0.7980	4.86	4.15	-0.136*	28.7 (13.8 - 110.6)	1	0	0
F <sub>P</sub>	33(20)	0.7278	0.7403	5.86	5.11	-0.017	7.2 (3.9 - 10.7)	3	0.611	0.00961
G <sub>Z</sub>	32(16)	0.7744	0.7321	8.14	6.13	0.055	3.7 (3.1 - 5.3)	2	0.5	0.00423
H <sub>Z</sub>	38(20)	0.7690	0.8421	9.57	6.54	-0.097*	11.1 (8.6 - 14.1)	5	0.768	0.00722
I <sub>P</sub>	43(20)	0.7612	0.9003	7.43	5.44	-0.185*	7.6 (5.1 - 10.3)	3	0.563	0.01631
J <sub>Z</sub>	29(19)	0.7131	0.7931	7.43	5.35	-0.114*	14.8 (9.8 - 23.6)	1	0	0
K <sub>P</sub>	37(20)	0.7593	0.7799	13.43	7.66	-0.027	29.7 (22.6 - 40.7)	11	0.874	0.01924
L <sub>Z</sub>	10(9)	0.7398	0.8286	5.43	5.43	-0.127*	2.3 (1.6 - 4.8)	2	0.556	0.01095

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636

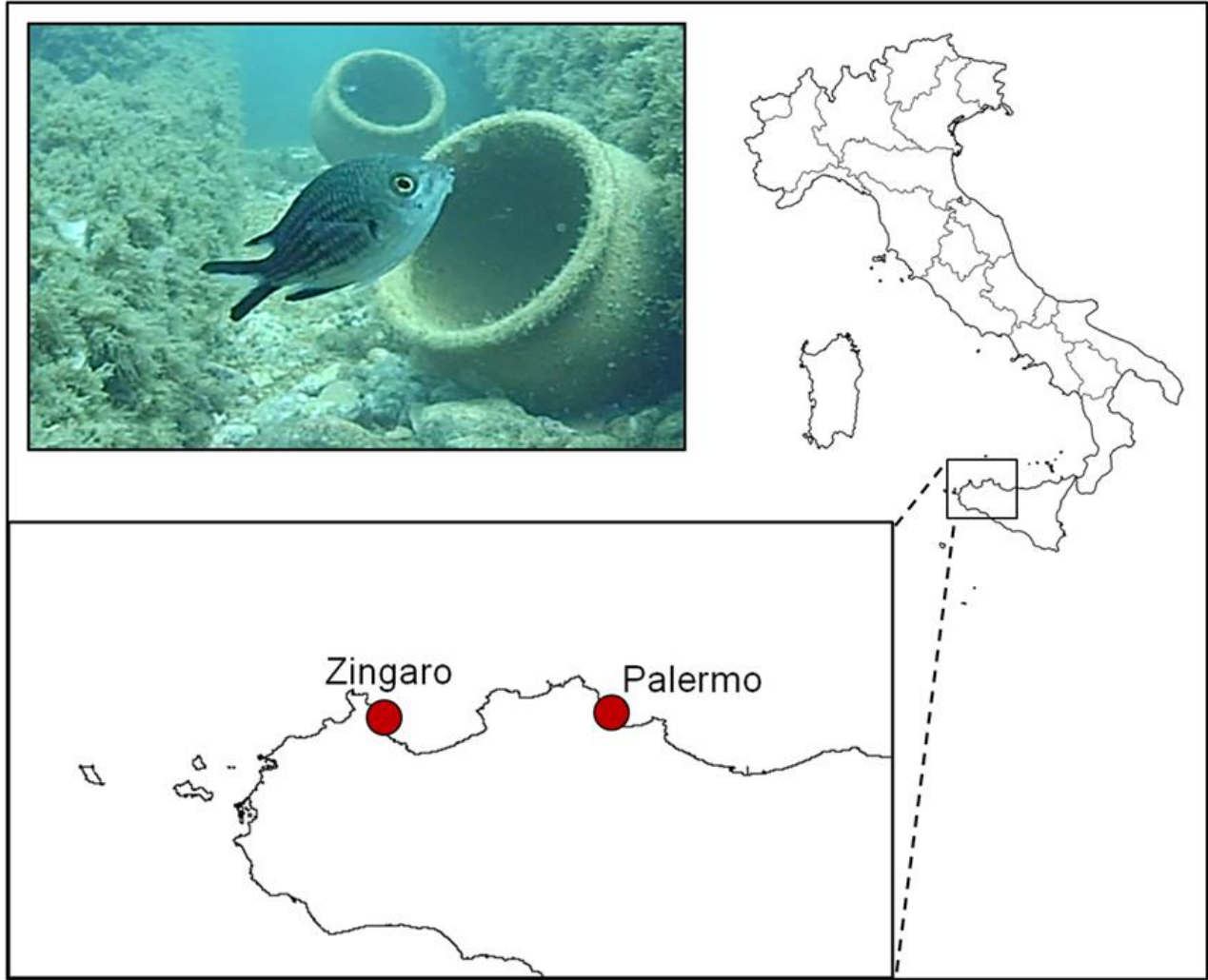
637 FIG. 1. Sampling localities (red circles) for *Chromis chromis* along the Sicilian coast. In the inset, a focal male is guarding an artificial nest

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639 FIG. 2. Median-joining network of mtDNA haplotypes of *Chromis chromis*. The size of each circle corresponds to the relative haplotype  
640 frequencies and black circles represent hypothetical intermediate haplotypes. Single mutational steps are assumed between haplotypes unless  
641 specified. Nests D, E, F, I, K are from Zingaro; nests A, B, C, G, H, J, L are from Palermo.

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643 FIG. 3. PCA variables factor map. Variables showed are: number of parents ( $N_P$ ), number of sneakers ( $N_{SNK}$ ), number of females ( $N_F$ ), proportion  
644 of offspring sired by the nesting male ( $NM_{off}$ ), area of the egg clutch expressed in  $cm^2$ , number of defence behaviour per hour and average density  
645 of individuals at the time of sampling.



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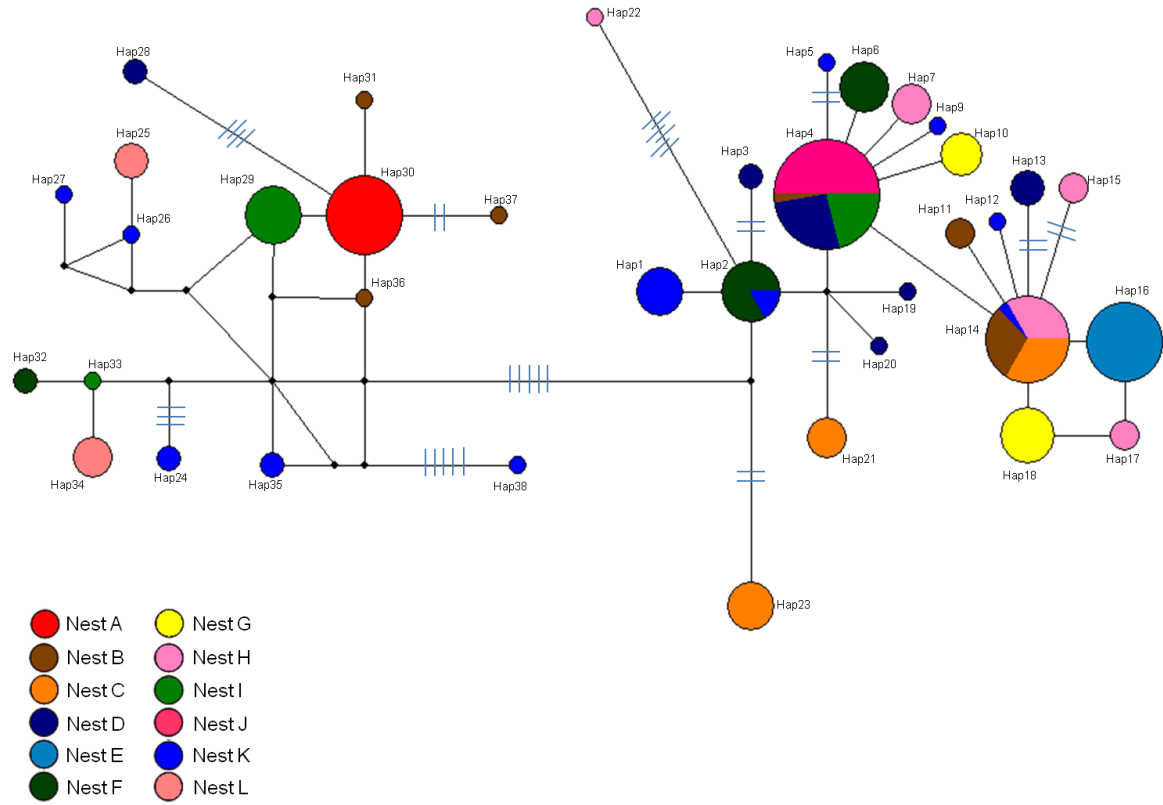
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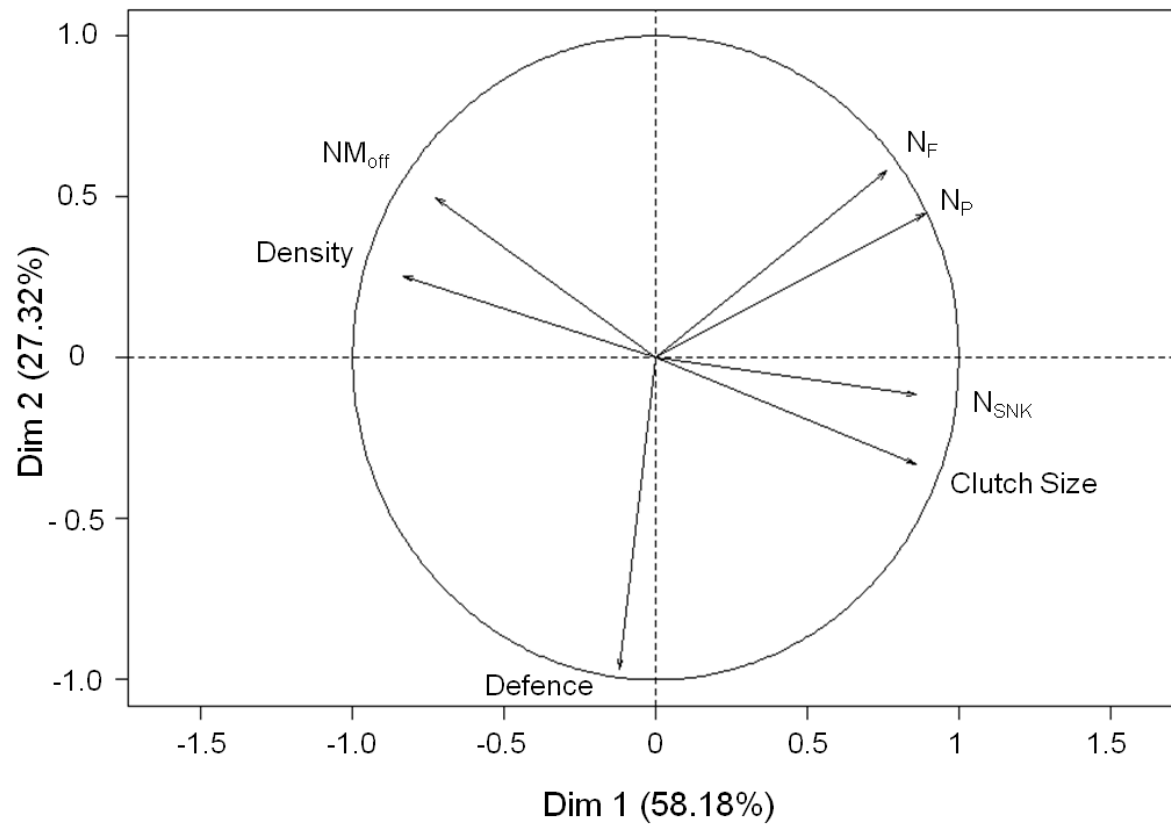


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