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# Conspicuous carotenoids-based pelvic spine ornament in three-spine stickleback populations ~~- occurrence and inheritance~~

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Reports on reddish carotenoids-based ornaments in female three spine sticklebacks (*Gasterosteus aculeatus*) are few, despite the large interest in the species' behaviour, ornamentation, morphology and evolution. We sampled sticklebacks from 17 sites in north-western Europe in this first extensive study on the occurrence of carotenoids-based female pelvic spines and throat ornaments. The field results showed that females, and males, with reddish spines were found in all 17 populations. Specimens of both sexes with conspicuous red spines were found in several of the sites. The pelvic spines of males were more intensely red compared to the females' spines, and large specimens were more red than small ones. Fish infected with the tapeworm (*Schistocephalus solidus*) had drabber spines than uninfected fish. Both sexes had red spines both during and after the spawning period, but the intensity of the red colour was more exaggerated during the spawning period. As opposed to pelvic spines, no sign of red colour at the throat was observed in any female from any of the 17 populations. ~~A rearing experiment was carried out to estimate a potential genetic component of the pelvic spine ornament by artificial crossing and rearing of 15 family groups during a 12 months period. The results indicated that the genetic component of the red colour at the spines was low or close to zero.~~ The potential adaptive function ~~of the reddish pelvic spines in sticklebacks~~ remains largely unexplained.

of this coloration

How was samplings sites chosen?

Although reddish pelvic spins seem common in populations of Sticklebacks

# Conspicuous carotenoids-based pelvic spines ornament in three-spine stickleback populations - occurrence and inheritance

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## Introduction

Sexual selection has dominated the study of behavioural ecology the last 25 years (Andersson, 1994; Milinski, 2014; Simmons, 2014). Although the main focus has been on female choice and males' elaborate ornaments, the evolution of female ornaments has received attention as well (Amundsen, 2000; Clutton-Brock, 2009; Kraaijeveld, Kraaijeveld-Smit & Komdeur, 2007). Female ornaments come in different varieties and may be female-specific or ~~mutual~~ for both sexes, relatively static over longer time-periods or highly dynamic, for example ~~to~~ signal fertility or ovulation (e.g. McLennan, 1994, 1995; Rowland, Baube & Horan, 1991; Amundsen & Forsgren, 2001). Authors have hypothesized that ornaments signal genetic quality of females (e.g. Zahavi, 1975), or direct benefits such as non-genetic maternal resources (e.g. Blount, Houston & Møller, 2000; Massaro, Davis & Darby, 2003; Gladbach et al., 2010). However, the resources allocated to ornamentation may lead to reduced resources available for offspring and thus constrain female fitness (Fitzpatrick, Berglund & Rosenqvist, 1995; Price, 1996; LeBas, Hockham & Ritchie, 2003; Chenoweth, Doughty & Kokko, 2006) ~~and~~ giving dishonest female signals (Funk & Tallamy, 2000; Bonduriansky, 2001).

Several hypotheses have been proposed to explain the evolution of female ornaments in ~~mutually~~ ornamented species. The "direct selection hypothesis" suggests that female ornaments are under direct sexual selection by males or selection ~~via~~ reproductive competition among females (Amundsen, 2000; Kraaijeveld et al., 2007). Thus, according to this hypothesis, female ornaments are honest signals of some aspects of individual quality. The alternative "genetic correlation hypothesis", predicts that female ornamentation is a genetically correlated response to selection for male ornamentation and this received some support already from Darwin (1871). Later, Lande (1980) suggested that female ornamentation in ~~mutually~~ ornamented species may be just a temporal stage in the evolution of male ornaments. The evolutionary explanations to females' ornaments remain controversial (Nordeide et al., 2013).

The three spine stickleback (*Gasterosteus aculeatus*) has been studied for decades to address diverse topics within ecology, morphology, evolutionary biology, ~~including evolution of anti-predator adaptations, and sexual selection~~ (reviews by Wootton, 1976, 1984; Bell & Foster, 1994; Östlund-Nilsson, Mayer & Huntingford, 2007). Pelvic spines are part of the defence armour protecting three spine sticklebacks from predators (Moodie, 1972), ~~which has~~ been studied in numerous populations in North America (e.g. Moodie, 1972; Hagen & Gilbertson, 1972; Rowland, 1994), and Europe (Klepaker & Østbye, 2008; Gross, 1978). Male sticklebacks develop the nuptial blue eyes and yellow - reddish carotenoid-based throat (reviewed by Rowland, 1994). For simplicity, we refer to the yellow - reddish carotenoids-based ornaments as "red" in the rest of this paper. Red serves as a strong signal eliciting territorial aggression (ter Pelkwijk & Tinbergen, 1937; Tinbergen, 1948) or a dual effect of aggression and fear in male three spine sticklebacks (Rowland, 1994), in addition to being an important mate choice cue for females (Milinski & Bakker, 1990 reviewed by Rowland, 1994). Sticklebacks' eyes have four cone pigments with visual peak absorption maximums around 360 nm (ultra-violet sensitive), 445 nm (short-wavelength sensitive), 530 nm (middle-wavelength sensitive) and 605 nm (long-wavelength sensitive) (Rowe et al., 2004, see also Lythgoe, 1979). ~~These absorption maximums are similar to those of humans except humans lack sensitivity in the ultra-violet (Rowe et al., 2004).~~ Male three spine sticklebacks ~~courted~~ females more when illuminated by full-spectrum light including ultra-violet, compared to females presented in light lacking ultra-violet light (Rick & Bakker, 2008a). Especially the long wavelengths ("red" light) and the short wavelengths (ultra-violet) seem to be important when female courted male three spine sticklebacks (Rick & Bakker, 2008b).

Despite the extensive scientific literature, studies on female three spine sticklebacks with ornaments, especially red ornaments, are few. In a general overview of fishes in Maine (U.S.), several species of Gasterosteidae, including three spine sticklebacks, were ~~mentioned briefly~~ described to have red colours (Bigelow & Schroeder, 1953). Three spine sticklebacks (sex not specified) ~~were described as~~

73 "...the fin membrans often are red", whereas female three spine sticklebacks ~~were described as~~ "...the
74 whole body except the top of the back may then be reddish..." during the spawning season (Bigelow &
75 Schroeder, 1953). In an overview of fishes in western Europe, red colour in female three spine
76 sticklebacks was not mentioned whereas males were described as having red throat during the
77 spawning period (Pethon, 1985). More recent and detailed studies reported red pelvic spines in both
78 sexes of a population of the brook stickleback (*Culaea inconstans*) from Washington (Hodgson, Black
79 & Hull, 2013 see also McLennan, 1995). Some gravid female three spine sticklebacks from a
80 population in Long Islands have vertical barring on the upper half of the body (Rowland et al., 1991;
81 Rowland, 1994). Red colour was observed at the throat of female three spine sticklebacks from
82 California (Pescadero Creek, von Hippel, 1999), as well as at the throat and at the membrane of the
83 pelvic spines of females from two stream-resident three-spine stickleback populations from British
84 Columbia (Little Campbell River, McKinnon et al., 2000). Moreover, female three spine sticklebacks
85 from another site in California (Matadero Creek) were reported to have red ornaments both at their
86 throat and their pelvic spines (Yong et al., 2013). McKinnon et al. (2000) ~~refer~~ to personal
87 communications with colleagues who have observed red ornaments to "... occur at least occasionally
88 in other populations" of three spine sticklebacks. Only one population from Europe has been reported
89 to have red ornamented females ~~as far as we know~~ (Lake Nedre Vollvatn, Nordeide, 2002; Nordeide,
90 Rudolfsen & Egeland, 2006). ~~These~~ females were reported to have a red membrane attached to the
91 pelvic spines but not red throat, whereas the males had both red pelvic spines and red throat. Extensive
92 studies are absent on the prevalence of pelvic spine ornaments in male and female sticklebacks, and on
93 carotenoids-based throat ornament in female stickleback populations. The few published studies have
94 reported males to have more exaggerated red ornaments compared to females, and body length to be
95 associated with the elaboration of the ornament (McKinnon et al., 2000; Yong et al., 2013). The
96 difference in the intensity of the ornament between ovulating and non-ovulating females seems to be

97 minor (McKinnon et al., 2000; Yong et al., 2013). Ambiguous results were reported on the relationship
98 between the elaboration of red ornaments and body condition of sticklebacks (Hodgson et al., 2013;
99 Yong et al., 2013), whereas red ornaments were negatively affected by the parasitic cestoda
100 *Schistocephalus solidus* (Milinski & Bakker, 1990; Barber 2007; Candolin & Voigt, 2001; Folstad et
101 al., 1994).

102 A large environmental component is expected in carotenoids-based ornaments, since animals
103 cannot synthesize carotenoids and must acquire them through the feed (Goodwin, 1984). Empirical
104 estimates of the relative roles of genes and environment on ornaments are contradictory although the
105 genetic contribution is often low (Pagani-Núñez et al., 2014; Evans & Sheldon, 2012; Hadfield &
106 Owens, 2006; Hadfield et al., 2006; Hill, 1993a). On the other hand, some studies have shown a
107 genetic component in carotenoids-based characters, like the red ornamented throat in male three spine
108 sticklebacks (Bakker, 1993), flesh colour in Chinook salmon (*Oncorhynchus tshawytscha*) ($h^2 > 0.71$)
109 (Withler, 1986) and in Arctic charr (*Salvelinus alpinus*) ($h^2 = 0.26 \pm$ S.E. 0.16) (Elvingson & Nilsson,
110 1994)).

111 The aim of this study was to give the first extensive overview of prevalence of red pelvic spine
112 ornaments of three spine sticklebacks, from north-west European populations. Additionally we aimed
113 to test for potential effects of sex, body size, parasitism and season on the elaboration of the ornament.
114 ~~Finally, we report from an experiment where wild sticklebacks from one of the populations were~~
115 ~~crossed and their offspring reared, in order to estimate a potential genetic component of red pelvic~~
116 ~~spine ornaments in sticklebacks.~~
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120

## Materials and methods

### Field study

Three-spine sticklebacks were sampled at 17 sites in north-west Europe from 22 May to 20 August 2012, to estimate (*i*) the occurrence of red pelvic spines, (*ii*) how the intensity of red varied between stickleback populations, and (*iii*) whether intensity of red was affected by sex, parasitism and body size. All samples were from landlocked freshwater populations except two (no 13 and 14) which were brackish. Ten of the populations were from North Norway, one (no 7) was from mid-Norway, and the remaining six populations (no 1-6) were from the southern parts of Norway (Fig. 1, Table 1). The majority of the samples were collected in May - July, whereas two (no 7 and 16) were sampled in August (Table 1). An additional sample was included from one (Lake Pallvatn, Table 1) of these 17 sites, to examine potential change in intensity of red at the pelvic spines within the spawning season compared to 3 - 4 months later.

All fish were caught by traps with no bait. The majority of the samples were collected using traps made by cutting 1.5 l transparent soda bottles into two parts, turning the upper part (about 1/3 of the bottle) upside down, and assembling the two parts by twine. Fish from lakes 3 – 6 were caught by passive traps made of plexiglas (Breder, 1960), and by minnow-traps made of small-meshed nets of nylon. The traps fished during a period of 20-24 hours. The sticklebacks were killed by an overdose of tricaine methanesulfonate (MS222) immediately after the traps were emptied. After the required exposure time (approximately 1-2 min), the dead sticklebacks were quickly rinsed in freshwater to remove any anaesthetic residue and placed on ice in a dark container. The sticklebacks were kept in a freezer until transported to the University of Nordland in Bodø, where they were kept in complete darkness in a - 40° C freezer until they were photographed (see below). This was to ensure that the carotenoids on the pelvic spines did not oxidize due to light exposure.

145

146

147 Rearing experiment

148 The rearing experiment lasted from June 2008 to June 2009. Parents were caught 3 – 16 June
149 2008 in Lake Nedre Vollvatn (Table 1, Fig. 1). These fish were kept in the holding tanks for 4 – 7 days
150 before sacrificed by an overdose MS222. To fertilize eggs of parents and rear their sibling groups of
151 offspring we used the method described by Barber & Arnott (2000), with some modifications. The
152 female's (mother's) abdomen was gently squeezed and eggs and ovarian fluid were collected in a Petri
153 dish. The male's (father's) gonad was first removed then cut into small pieces in the Petri dish before
154 the eggs and semen were physically mixed and left for fertilization the next 5 min. In the hatchery, the
155 offspring in each sibling group, produced from each of the fertilizations trials, were reared separately in
156 plastic boxes with about 160 ml volume and a continuous flow of water, as described by Rudolfsen et
157 al. (2005). This rearing method removed potential paternal effects on the offspring. When most of the
158 eggs in a particular sibling group hatched, the eggs and larvae were moved to a 2 l tank and for the next
159 3 - 4 weeks first fed *Artemia* nauplii and commercial dry feed (TetraMinBaby: Tetra, Melle, Germany),
160 and later dry feed and chopped Chironomidae larvae. The fry sibling groups were moved to larger 7.5 l
161 tanks 27 August, and from September – January given only Chironomidae larvae as food. In three of
162 the 15 sibling groups approximately half of the specimens were 6 January moved to another and
163 identical tank to avoid too high density of fish (offspring from the same sibling-group were kept in two
164 identical 7.5 l tanks). From 26 January to 16 April the offspring in the 18 tanks (15 sibling groups)
165 were fed dry feed containing carotenoids (astaxantin and β,β-carotene, see Appendix 1 for a recipe).
166 The specimens in each sibling groups were fed in excess, starting with three times a day the first 3 – 4
167 weeks from start feeding, and ending with once a day the last 6 months. Excess food was always
168 available in the tank the entire 12 months period. From October onwards, the sticklebacks experienced

the natural light regime in Bodø. This feeding of the reared offspring with carotenoids resulted in intensity of red of daughters and sons overlapping to a large degree with their wild caught parents, although the offspring were slightly more ornamented than their wild caught fathers' and mothers' (Appendix 2). Thirty-one offspring from the 15 sibling groups died from the start of feeding with carotenoids 26 January to the experiment terminated 5 ½ months later.

A potential genetic component of the intensity of red was estimated both by General Linear Models (GLM, see below) and as heritability ($h^2$). The latter, with standard error, was estimated by the classical parent-offspring regression method for the 15 fullsib groups and 301 offspring (after discarding non-mature offspring), and adjusted for unequal family-sizes as first suggested by Kempthorne & Tandon (1953). A potential sex effect of the intensity of red ($I_R$, see below) was handled by doing an overall regression between mother and the mean of daughters and likewise with the fathers and sons. The mean weight in each offspring group was used as a predictor in the model when estimating the regression coefficient.

Color measurement

~~Common for the field~~ study and the rearing experiment

To quantify intensity of red colour of the spine we took close up photos of the ventral part of the fish with raised spines including the red carotenoid-based skinfold at the basis of the pelvic spine. A standard red cardboard (227N, 1103 0964-Y-23R, Jotun A/S, Sandefjord, Norway) was included in all photos. We used a Nikon D2X digital camera (Nikon, Tokyo, Japan) with a Nikon ED AF Micro Nikkor 200 mm 1:D lens and Nikon Speedlight SB-80DX flash. The digital photos were analysed by Adobe Photoshop SC3 (San Jose, CA, USA) in Red-Green-Blue (RGB) modus. We started by drawing a line in Adobe Photoshop enclosing an area around each of the right and left pelvic spine. The average

193 density values for all three primary colours R, G, B (red, green, and blue) were quantified from all the
194 pixels enclosed by this area for each of the two spines (Villafuerte & Negro, 1998). This was repeated
195 for the standard cardboard in the photo. The mean R, G and B values of each of the two pelvic spines
196 were used in further calculations. The intensity of the red colour ($I_R$) of the skin folds of both the two
197 pelvic spines and the red cardboard was calculated according to the formulae
198
199 $$I_R = R/(R+G+B)$$
200
201 After calculating the $I_R$ from both the left and right pelvic spines for the first 267 fish, we estimated the
202 coefficient of correlation between the left and right spines as $R_S = 0.82$ (P =0.226, d. f. = 266,
203 Spearman's). Based on this relatively high correlation coefficient we decided to measure the right
204 pelvic spine solely for the remaining fish. We adjusted the final $I_R$-value of the skin fold of the
205 pelvic spines of each fish according to the $I_R$-value of the cardboard in each photo relative to the
206 average $I_R$-value of all photos. Similar methods to quantify colouration have previously been applied
207 by several authors who discussed this method of quantifying colour in ornaments, and gave more
208 details and estimates of repeatability (Yong et al., 2013; Nordeide et al., 2006; Villafuerte & Negro,
209 1998; Nordeide et al., 2008; Skarstein & Folstad, 1996; Skarstein, Folstad & Rønning, 2005; Neff et
210 al., 2008). An alternative method to quantify colour, spectrophotometry, was discarded. ~~This was~~
211 because the small size, difficult accessibility of the ornament, and (in some individuals) ~~non-even~~
212 distribution of the colour at different parts of the spine (Fig. 2) would impede the $I_R$-estimates. Red
213 coloration is caused by pteridines in some fishes (Grether, Hudon & Endler, 2001). Pteridines have
214 similar spectral properties as carotenoids, but pteridines are not extracted in acetone contrary to
215 carotenoids (Grether et al., 2001). In three spine sticklebacks the red colour at the pelvic spines is

caused by carotenoids and not pteridines, since the spines became colourless after extracting carotenoids by acetone (E. S. Egeland, unpublished data).

All fish were measured for total length (nearest mm) and total wet weight (nearest 0.001 g) and gonads were examined for sex and whether or not they were sexually mature. All fish were visually examined externally for the microsporidian *Glugea anomala* and in the body cavity for potential specimens of the tapeworm *S. solidus*.

Several people were involved in sampling the sticklebacks at the 17 different sites, and the time from death of the fish until they were frozen differed between sites. We carried out a small experiment to test if this time difference could affect our estimates of $I_R$. Thirty sticklebacks were captured from Lake Pallvannet (Table 1, site 9) May 16 2012, transported alive to the University of Nordland, and kept in a 300 L tank of freshwater containing hides made ~~out~~ of plastic tubes to reduce the stress level. The fish were not fed. On May 18 the fish were killed by MS-222, and photographed three times: immediately after death (0h), after one hour (1 h), and after four hours (4 h). The fish were held in a dark room at 4°C between photographs. Quantifying $I_R$-values (as explained above) revealed a mean (± S.D.) of 0.414 (± 0.0318) just after death (0 hours), 0.423 (± 0.0238) at 1 hour, and 0.414 (± 0.0200) four hours after death. The difference in $I_R$ during the time interval from 0 to 4 hours after death was non-significant (paired $t$-test: $t < 0.001$, $P > 0.99$, d.f. = 29).

The red ornament at the throat of the male sticklebacks clearly faded and nearly disappeared during handling and transportation as judged by the eye, during a period of 30 – 60 min (J.T. Nordeide, unpublished data). This observation concurs with ~~a report~~ that reported by Frischknecht (1993). Contrary, the red colour ~~at~~ of the pelvic spine ornament was much more stable and apparently not affected during the transport and handling (J.T. Nordeide, unpublished data).

Statistical analyses were carried out by General Linear Models (GLM) in SPSS version 20.0 (SPSS Inc. Chicago, Il, USA) according to Grafen & Hails (2002). None of the variables needed to be

transformed to meet the assumptions of independence, heterogeneity of variance, normality of error, and linearity (Grafen & Hails, 2002).

This study was carried out in accordance with ethical guidelines stated by the Norwegian Ministry of Agriculture through the Animal Welfare Act.

## Results

### Field study

Photos of the carotenoids-based pelvic spine ornament of three fish with $I_R$ 0.35, 0.37 and 0.46 are presented in Figure 2. Individuals with carotenoids based ornamented pelvic spines with an intensity of red ($I_R$) ≥ 0.37 were found in all the 17 examined populations (Fig. 3). The median $I_R$ of both males and females was higher than 0.37 for both sexes in all sites except three (no 3, 4, 7), whereas in another two sites (no 11 and 15) only males (not females) had median $I_R \geq 0.37$ (Fig. 3). The most elaborately ornamented individuals were found in population no 10 and no 14, where a few individuals had $I_R > 0.50$ (Fig. 3). "Sex" of the fish (entered the model as a "fixed factor") had the strongest effect on $I_R$ in a linear mixed model (GLM) including sticklebacks from all the 17 populations ($F = 101.417$, Table 2). Males had a higher $I_R$ as compared to females (Fig. 3 and 4). "Population" (entered the model as a random factor) had a strong effect on $I_R$ as well ($F = 64.114$, Table 2, Fig. 3). Body "length" of the sticklebacks (entered the model as a covariate) had an effect of $I_R$ (Table 2) and this association was positive (Fig. 4, Table 2). Finally, sticklebacks infected by the parasite *S. solidus* had a lower $I_R$ than non-infected fish (Table 2, Appendix 3). *S. solidus* were found in

264 ~~only~~ seven of the populations (no 6, 7, 8, 10, 12, 15 and 17) and one population (no 4) was infected by
265 *G. anomala*. Running the model again including all 17 populations but excluding all parasitized fish, or
266 only including (all) fish from the seven parasitized population only, both lead to only minor changes in
267 the estimated parameters.

268 As opposed to red pelvic spines, no sign of red colour at the throat was observed in any female
269 from any of the 17 populations. Most males in all populations sampled during the spawning season
270 (May – June/July) had at least some red colouration at their throat, although we did not quantify male
271 throat colour due to its volatile nature (Frischknecht, 1993).

272 To test for seasonality of the ornament, three spine sticklebacks from one of the lakes (Lake
273 Pallvatn) were sampled both in the spawning season (May 2012) and 3 – 4 months after the end of
274 spawning (October 2012, see Table 1). Including data from only these two samples from Lake Pallvatn
275 showed that both "Season" and "Sex" (as fixed factors) were significant, and they explained 30.1 % of
276 the variation of $I_R$ in a General Linear Model (Table 3, Fig. 5). The effect size of the $I_R$ between the
277 seasons was moderate especially for the females (Fig. 5), but still significant between seasons when the
278 model was run again with only females included ($F_{1,59} = 7.764$, $P = 0.007$). Re-running the same model
279 (as in Table 3) and adding body length of the fish as a covariate, gave a non-significant effect of length
280 ($F_{1,104} = 2.763$, $P = 0.099$) and only minor changes for the other predictors ("Season" and "Sex").

281 We then tested for association between condition of the fish and $I_R$ of the pelvic spines of the
282 three spine sticklebacks from the same lake (Lake Pallvatnet). Condition was estimated as the residuals
283 of fish weight (g) on length cm in a GLM, (weight needed no ln-transformation to be linearly
284 associated with length). None of the predictors "Residuals of weight on length" (as explained above) as
285 a covariate ($F_{1,43} = 2.555$, $P = 0.117$) or "Sex" as a fixed factor ($F_{1,43} = 2.882$, $P = 0.097$) was associated
286 with the response variable $I_R$ of the pelvic spines. Running the same model again after including (body)

"Length" (as a covariate) as a third predictor did not explain a significant part of the variation in the response variable either ($F_{1, 42} = 0.064$, $P = 0.802$).

Rearing experiment

No association was found between the mean intensity of red spines in each of the tanks with offspring groups (as the response variable), and the mean weight gain during the period when fed carotenoids which started 26 January and ended 16 April, as covariate ($F_{1,17} = 0.034$, $P = 0.855$, $R^2 = -0.060$). This indicates that the intensity of red of the specimens in each of the 18 tanks in total (15 sibling groups) was not a direct effect of the amount of carotenoid-feed consumed during this period.

In a GLM with "offsprings' $I_R$" as the response variable, and the predictors "sex", "length", "mother's $I_R$", and "father's $I_R$" as covariates, only the latter predictor ("father's $I_R$") was non-significant (see Appendix 4). This model explained 31.3 % of the total variance. Males had highest "offsprings' $I_R$" values (residuals after adjusting for "length") (mean ± S.E.: 0.36 ± 0.099), followed by immatures (-0.19 ± 0.146), and females (-0.203 ± 0.076). When running the same model after excluding immature offspring, the predictor "sex" was still significant ($p < 0.001$). Based on these results, we decided to analyse male and female offspring in separate models.

Starting with female offspring, "offspring $I_R$" was associated with "length" and with the interaction term between the two other covariates "father's $I_R$" and "mother's $I_R$" (Table 4). The p-values of "father's $I_R$" and "mother's $I_R$" were less than 0.031. The model explained 25.1 % of the variation, with "length" explaining 21.1 % and the remaining ("genetic") terms ("father's $I_R$", "mother's $I_R$", and "father's $I_R$" x "mother's $I_R$") explaining 4.0 % of the variance in the model. The

residuals of daughters' $I_R$ (adjusted for body length) plotted against their mother's $I_R$, is shown in Fig 6a.

Concerning male offspring, residuals of offspring $I_R$ was positively associated only with "length" ($F = 27.223$, $P = < 0.001$), whereas the p-value of "mothers $I_R$" was slightly non-significant ($F = 3.202$, $P = 0.077$)(Appendix 5). This model explained 23,1 % of the variation, with "length" explaining 21.4% leaving 1.7 % of the variation to be explained by the ("genetic") term "mothers $I_R$". Scatter plot of residuals son's $I_R$ (adjusted for length) against "mother's $I_R$" is shown in Fig. 6b. Running the model again after removing the one male offspring ("outlier") with a very drab mother ("mother's $I_R$" < 0.34), resulted in also "mother's $I_R$" becoming significant ($F = 2.068$, $P = 0.040$), and the model explained slightly more of the variance ($R^2 = 0.244$).

Finally, including only "immature" offspring, their $I_R$ as response variable was not significantly associated with neither "length" ($F_{1,54} = 0.432$, $P = 0.514$), or their "mother's $I_R$" ($F_{1,54} = 0.044$, $P = 0.835$) as covariates, and the model did not explain any variation ($R^2 = -0.029$).

The classical parent-offspring regression method (see Materials & methods) gave an estimated heritability of 0.14 (S.E. = 0.22) when data from all mature offspring and all parents were pooled. This heritability estimate was not significantly different from zero.

## Discussion

### Field study

333 This first extensive study of prevalence of female ornaments in three spine stickleback
334 populations suggested that carotenoids-based pelvic spine ornament is widespread among north-west
335 European populations. Females and males with red ornamented pelvic spines were present in all the 17
336 populations examined, abundant in most of them, and quite conspicuous in several populations. This
337 widespread occurrence of the red pelvic spines among populations has not been reported before, and
338 adds another interesting aspect to ~~the three-spine stickleback as~~ this a model species in studies of sexual
339 selection. Individuals with red spines have so far been reported in only three North-American
340 populations of sticklebacks (McKinnon et al., 2000; Yong et al., 2013) and one population from
341 Europe (Nordeide, 2002; Nordeide et al., 2006). The great variation in the exaggeration of carotenoids-
342 based ornaments in the 17 stickleback populations in the present study concurs with similar studies on
343 male and female House Finches (*Carpodacus mexicanus*) in North America (Hill, 1993a; b). The
344 variation between populations of House Finches was suggested to reflect local and regional variation in
345 dietary carotenoids pigments availability (Hill, 1993a). We have no data about carotenoids availability
346 in the 17 study cites and hence cannot confirm or dispute the importance of variation in available
347 pigments in the present study.

348 Some of the results from the present study concur with previous studies on stickleback
349 ornaments, whereas other results do not. For example, the red throat ornament (contrary to red spines)
350 was totally absent in all females from the 17 populations and this is contrary to ~~the three~~ findings or
351 abovementioned North American three spine stickleback populations (McKinnon et al., 2000; Yong et
352 al., 2013). We have also confirmed that the pelvic spines of males are more intensely red than the
353 spines of the females, as reported by Yong et al. (2013) for both throat and pelvic spine ornaments, and
354 McKinnon et al. (2000) for the throat ornament in sticklebacks. Body length of the fish was associated
355 with $I_R$ of the pelvic spines when all 17 populations were analysed together. This result agrees with
356 reports on female body size and throat colour by McKinnon et al. (2000) and both body size and throat

Unclear

357 colour and body size and pelvic spine colour by Yong et al. (2013). Presence and absence of the
358 parasite *S. solidus* explained a significant although minor part of the variation in $I_R$, supporting
359 previous studies suggesting a negative association between red (throat) ornament and infection of
360 several parasite species including *S. solidus* (see references in Introduction). The elaboration of the red
361 spine ornament was higher during the spawning season compared to 3 – 4 months after spawning for
362 both sexes, although many male and female sticklebacks were still red outside the spawning season
363 (Fig. 5). Similarly, McKinnon et al. (2000) and Yong et al. (2013) reported small and non-significant
364 differences in the throat ornament between ovulated and non-ovulated females, and they suggested that
365 $I_R$ of females does not signal readiness to spawn (McKinnon et al., 2000; Yong et al., 2013).
366 In the autumn sample from Lake Pallvatn we found no association between body condition of
367 the fish and the intensity of their red pelvic spines. Again our result concurs with the report by Yong et
368 al. (2013) on lack of association between red throat ornament and condition in three spine sticklebacks,
369 and these authors suggested sexual selection to be of limited importance in the evolution of female
370 ornaments. In female brook sticklebacks, however, Hodgson, Black & Hull (2013) reported a positive
371 association between pelvic spine colour and condition.
372 We cannot rule out the possibility that lack of detailed information about start and end of the
373 spawning season for each of the 17 populations and our sampling of populations over a several weeks
374 long period, might have influenced our estimates of $I_R$. Different stickleback populations were probably
375 sampled in different parts of their spawning period and the intensity of the ornaments may vary during
376 the spawning period. For example, fish in southern populations probably start spawning earlier than in
377 northern populations. On the other hand, we find large variation in $I_R$ between fish from different
378 populations located geographically close to each other and sampled within a few days, like for example
379 populations 3 – 6 and populations 9 and 11. These observations leave little doubt that ornaments from
380 different populations vary in exaggeration. This interpretation received support from the relatively

small effect size revealed by comparing $I_R$ during and 3 – 4 months after the spawning season for site number 9.

Rearing experiment

The results from the rearing experiment may be interpreted as the genetic component of red pelvic spines is weak or even perhaps not different from zero. The significant association between the response variable "daugther's $I_R$" and each of the predictors (*i*) "father's $I_R$", and (*ii*) "mother's $I_R$", and (*iii*) the interaction between "father's $I_R$" and "mother's $I_R$", together explain only 4 % of the variation in the model. Such a weak genetic component concurs with our low heritability estimate which was not significantly different from zero. The lack of significance in our heritability estimate was due to the large standard error which is evidently a direct function of the size of the experimental setup (e.g. Dupont-Nivet, Vandeputte & Chevassus, 2002). A larger data set would be needed to reveal a significant estimate when heritability is this low in order to avoid Type II-errors. Such a low genetic component concurs to previous estimates in other species like plumage coloration in blue tits (*Cyanistes caeruleus*) (Hadfield et al., 2006), canaries (*Serinus canaria*) (Muller, et al. 2012), and low correlation between carotenoid-based coloration of nestlings and that of their parent great tits (*Parus major*) (Pagani-Núñez et al., 2014). On the other hand, a significant genetic component has been reported for both ornaments and non-ornamental traits in fishes including sticklebacks (see Introduction). Fish utilize carotenoids poorly and retention of astaxanthin in the muscle of Atlantic salmon (*Salmo salar*) is less than 12 %, partly due to poor absorption from the gut (Bjerkeng 2008). Individual variation in the ~~efficiency~~ to utilize carotenoids may be a functional explanation for the

404 significant genetic component in carotenoids-based throat and flesh coloration in sticklebacks ~~and~~
405 ~~Chinook salmon~~, and ~~potentially to some degree in the~~ pelvic spine ~~ornament~~ in sticklebacks as well.

406 We cannot rule out the importance of assumptions not accounted for in this experiment. Firstly,
407 our estimate of a weak genetic component may be influenced by the ~~different~~ environmental conditions
408 ~~and potentially~~ different variants of carotenoids wild-caught ~~between parents'~~ and ~~their~~ reared
409 offspring. Secondly, our design removed potential effects of fathers on the offsprings' ornament (see
410 Material and methods). We cannot exclude potential effects of mothers although we do not expect
411 large maternal effects in $I_R$ of the offspring ornament. This is based on observations of the offspring's
412 spines initially being very drab. The spines turned red after the offspring were given feed containing
413 carotenoids from January onwards (see Material and methods) (J.T. Nordeide unpublished data).
414 Finally, some of the variation in the intensity of the ornamentation of the offspring may be due to
415 differences in the amount of food eaten, and hence amount of carotenoids consumed. On the other
416 hand, feed containing carotenoids were available to the fish in excess at all times, and no association
417 was found between weight gained during feeding with carotenoids and mean $I_R$ of their ornament (see
418 Material and methods).

419 To conclude, this study suggests that both male and female individuals with reddish and often
420 conspicuously red ornamented pelvic spines, are common in north-west European three spine
421 stickleback populations. Males were more ornamented than females, and the fish were more
422 ornamented during than after the spawning period. The genetic component of the intensity of red
423 spines seems to be low. This study gives little support for either red spines signalling spawning
424 readiness, or of sexual selection being important for the evolution of the ornament. The potential
425 adaptive function of the ornament, and how it evolved, remain largely unexplained.

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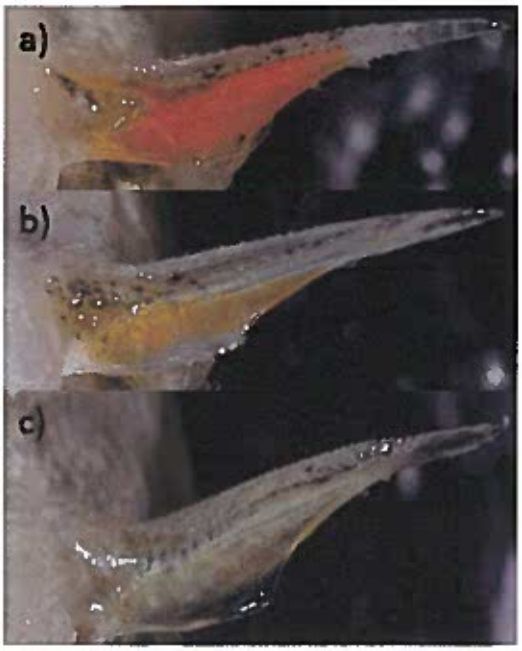


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664 Figure 2. Photo of the right pelvic spine from three different three spine sticklebacks from site number
665 10 (see Table 1). An elaborately ornamented male with intensity of red ($I_R$) = 0.46 is shown in a), an
666 intermediately ornamented female ($I_R$ = 0.37) is shown in b), and a drab female ($I_R$ = 0.35) is shown in
667 c).

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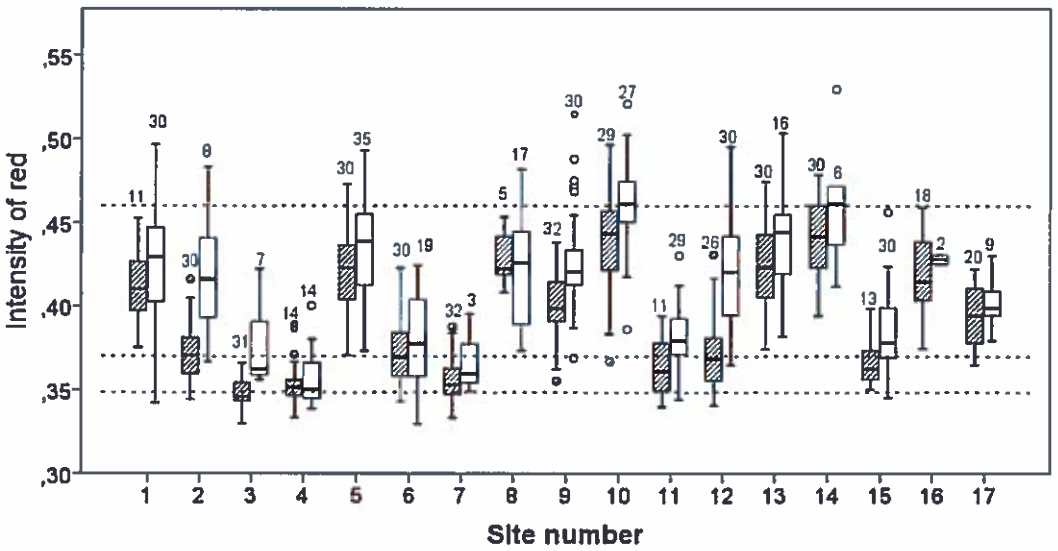


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681 Figure 3. Box-Whiskers plot of intensity of red ($I_R$) at the pelvic spines of three spine stickleback
682 males (open bars) and females (hatched bars), from 17 different sites. "Site number" refers to Table
683 1. The numbers in the figure show sample size. The three dotted horizontal lines are at $I_R$ -values
684 0.46, 0.37 and 0.35, which represent $I_R$-values of the pelvic spines from the three fish shown in Fig.
685 2.

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