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Spottiness of European graylings (Actinopterygii, Salmonidae) correlates with environmental features and could signal the status of individuals

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Abstract
Skin spots play an essential role as a visual stimulus in the social behaviour of fishes, particularly in gregarious species. Several studies have demonstrated that the characteristics of some flank signals are used as conspicuous visual cues for different aspects of intraspecific communication, as well as shaping the interactions among individuals, and reflecting the features of individuals. Here, we analyse the spatial pattern of spots in the European grayling *Thymallus thymallus* (Actinopterygii, Salmonidae) (Linnaeus, 1758) (Salmonidae, subfamily Thymallinae), which are usually gregarious. Adult individuals are characterised by conspicuous black spots on their sides, generally restricted to the front half of the flank. By sampling 55 individuals in Slovenia, Croatia and Norway, we tested the possible influence of environmental factors (e.g. water velocity, water depth, position in the pool/stream) and/or individual characteristics (sex and length) on the characteristics and spatial pattern of graylings' spots. Spottiness did not show any relationship with sex or body size, but the numbers of both spots and spotted lines appeared to be correlated with some physical properties of the river: more highly spotted graylings seemed to inhabit faster and deeper waters, and were positioned at the head of pools and streams. We suggested that in gregarious fish, spottiness may signal the status (e.g. quality and/or dominance) of individuals, which has a role in determining the spatial arrangement of individuals along the river bed and, consequently, within the group.

Keywords: *Spots, Salmonidae, gregarious species,* Thymallus thymallus, *visual communication*

Introduction

In some fish species, spot, bar and stripe colours and patterns play a role in: (1) camouflage and crypsis from predators; (2) foraging strategies and behaviours; and (3) intraspecific communication and social challenges, such as territory defence and individual recognition; evidence of colour/pattern assortative mating has also been observed (Barlow 2000; Watanabe et al. 2006; Elmer et al. 2009; Greenwood et al. 2011; Kelley et al. 2013). In addition, during the last two decades, individual differences in various morphological and behavioural traits within a population have been highlighted as biologically meaningful adaptive traits (Wilson 1998; Koolhaas et al. 1999), with important consequences for evolutionary ecology (reviewed in Kittilsen et al. 2009).

There is some evidence demonstrating the essential role that spots play as a visual stimulus in the social behaviour of gregarious fishes living in groups. For example, the characteristics of some skin signals are used as conspicuous visual cues for intraspecific communication, and shape the behavioural interactions among individuals (e.g. Schroder & Zaret 1979; Martin & Hengstebeck 1981; Beeching 1993; Miyai et al. 2011). In addition, Karenina et al. (2013) recently showed that in some gregarious species, skin colouring and patterning signals the status of the individual. Indeed, the recognition of individuals within the same group through visual signals may be crucial for survival in gregarious fish (Karenina et al. 2013): gregarious species tend to have stripes and spots, which are used by individuals

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to establish preferences (Engeszer et al. 2004; Price et al. 2008). Yet a comprehensive understanding of the characteristics of skin visual signals for most freshwater fishes is still lacking (but see e.g. Archer et al. 1987; Fuller 2002; Gamble et al. 2003), especially when compared with the available studies on other vertebrate taxa (e.g. birds; Searcy & Nowicki 2005; Bradbury & Vehrencamp 2011). In particular, descriptive studies are scarce for freshwater fish species, even though they represent the first step toward the improvement of our knowledge concerning the role of colouring and patterning in fish visual communication, and they could potentially be the baseline for future experimental studies aimed at understanding the eco-evolution of fish communication systems.

Here, we analyse the patterns of spottiness in the European grayling *Thymallus thymallus* (Actinopterygii, Salmonidae) (Linnaeus, 1758) (henceforth grayling), a fish of the family Salmonidae (subfamily Thymallinae; Kottelat & Freyhof 2007), that is usually gregarious. Graylings are found from southern France (Loire basin) east to the Balkans, and as far south as the Montenegro (Jankovic 1962). In northern countries, they are found from Great Britain across most of Scandinavia east to the Urals (Weiss et al. 2002). This species has recently received increasing attention, particularly because the grayling is a culturally important salmonid species highly prized by anglers (Koskinen et al. 2001). Several basic issues, however, including phylogeography and taxonomy, remain uncertain (Koskinen et al. 2001; Froufe et al. 2005). For example, the Adriatic (Sušnik et al. 2001) and Loire Basin (France; Weiss et al. 2002) populations may represent distinct lineages worthy of species status. During the last few decades, grayling populations have been seriously affected by environmental degradation, over-fishing and increasing predation pressure by piscivorous birds (Bertok & Budihna 1999; Uiblein et al. 2000; Gum et al. 2009).

Adult individuals of this species are characterised by conspicuous black spots on their sides (Figure 1), generally restricted to the front half of the flank (Dunker 1960). These spots could play an important role in communication within the fish group. Spots of graylings are always located on the dark lines that separate two scale lines (see Persat 1982 and Figure 1). A previous study (Persat 1982) demonstrated that (1) adult spots are different from those on juveniles, which are spread all along the back of the fish; (2) the percentage of adult fish without spots is low; (3) the side-punctuation system (number and location of spots) seems to be stable; the amount and disposition of spots do not change over time nor with the size of the fish (larger graylings are neither more nor less spotted than smaller ones); and (4) the number and disposition of the spots are not symmetrical on the two sides (Figure 2). However, whether the individual's features (e.g. sex, length) influence the characteristics and spatial pattern of grayling spots remains unclear.

Here, we analyse the relationships between the characteristics of spottiness in graylings and environmental factors and individual features. We hypothesised that: (1) there is a sexual difference in flank spottiness, which may be the result of sexual selection. According to Williams and Mendelson (2013), spottiness: (i) could be used by males to intimidate or defeat competing males; and/or (ii) could have evolved if females prefer to mate with males that exhibit elaborate traits. We further hypothesised that: (2) spottiness differs as a function of the grayling's size, which could support the possibility that spots represent an honest signal of individual quality; and/or (3) spottiness is related to the (genetic) characteristics of the individual and varies as a function of the environmental variables (Stauffer & Gray 2004), with the most highly spotted fish representing the most dominant individuals that inhabit the most advantageous but costly sectors of the river.

Material and methods

Data collection

Graylings were sampled from five rivers in three different countries: Unec and Soča in Slovenia, Kupa and Kupica in Croatia, and Glomma in Norway (Figure 3). Fish were sampled in June 2013 by means of flyfishing (dry flies only and barbless hooks). Given that one of the aims of the present work was to relate spot patterns to environmental variables, catching on a fly only those graylings rising for insects allowed us to precisely locate the fish along the river bed. Caught fish were measured and photographed immediately after capture (pictures were taken with an Olympus Tough camera 4.5–18.0 mm), and then released (fish were not anaesthetised). We measured body length (from the mouth to the fork of the caudal fin) using a ruler. Sex was determined by external morphology, particularly the size and shape of the dorsal fin (Uiblein et al. 2001; Lucas & Bubb 2005): the large "sail-like" dorsal fin of the grayling is larger in males than in females, producing notable sexual dimorphism and generally allowing easy sex identification. From each picture (one per flank), we calculated: (1) the total number of spots; (2) the number of spots per scale line; and (3) the number of spotted lines. The spatial patterns of spots on both sides of an individual were also described by three dispersion indexes

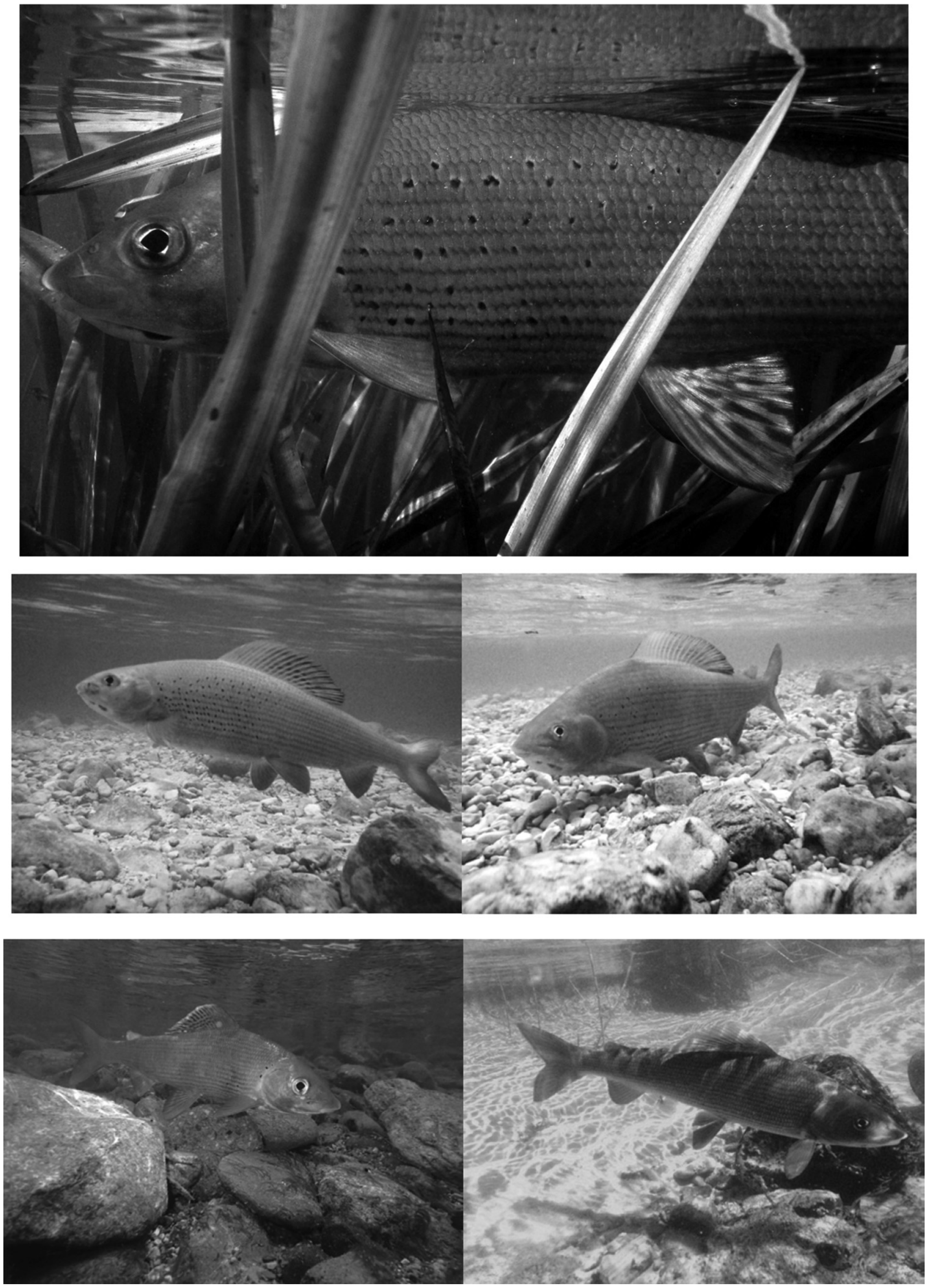

Figure 1. Top picture: Adult graylings are characterised by conspicuous black spots on their flanks, always located on the dark lines that separate two scale lines. Such spots are generally restricted to the front half of the flanks. Bottom pictures: four examples of differently spotted graylings, from a heavily spotted individual to one without spots.

calculated using the PASSaGE (Pattern Analysis, Spatial Statistics, and Geographic Exegesis) software program (version 2; Rosenberg & Anderson 2011). These indices, which represent three alternative methods for examining the deviation from a random distribution (Pielou 1969; Upton & Fingleton 1985; Ludwig & Reynolds 1988), included: (1) the index of dispersion, which under a random distribution of points is expected to equal 1; (2) the index of cluster size, which under a random distribution of points is expected to equal 0: positive values indicate a clumped distribution, whereas negative values denote a regular distribution (see also David & Moore 1954); and (3) Morisita's Index (Morisita

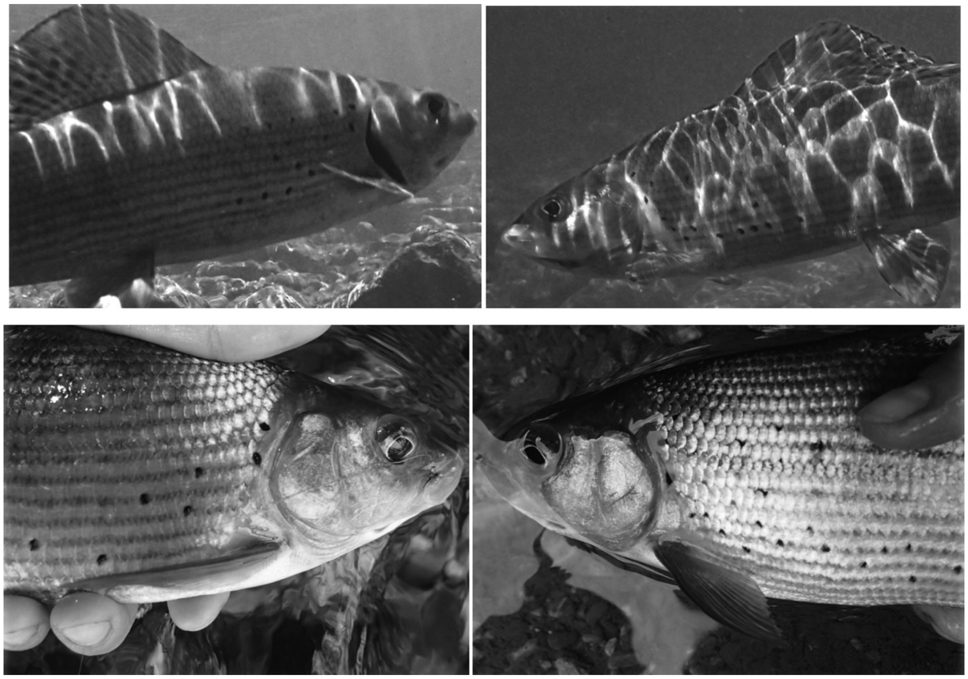

Figure 2. Two examples of flank spot asymmetry: both the right and left sides of the same individual are shown.

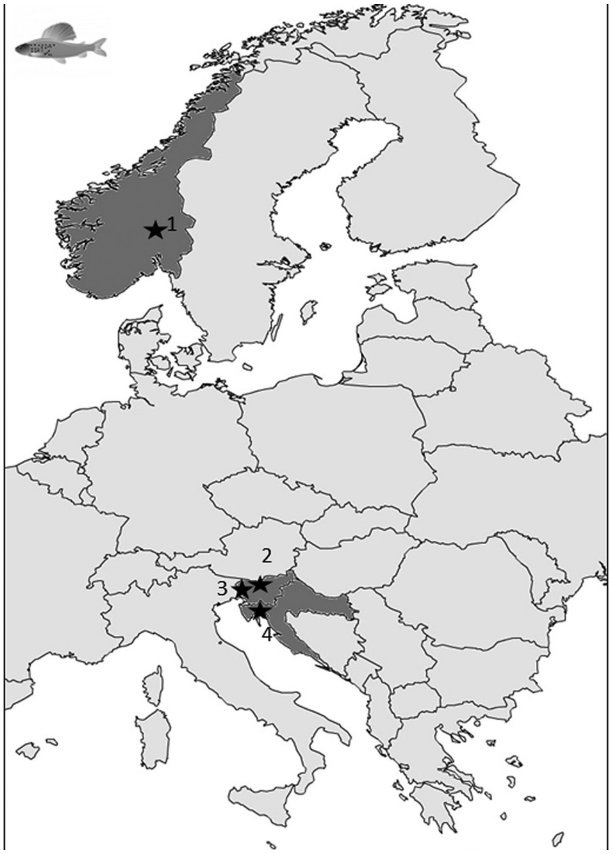

Figure 3. Locations (black stars) of the sampling areas in Norway (River Glomma = 1), Slovenia (River Unec = 2; River Soča = 3) and Croatia (River Kupa and Kupica = 4).

1959), which is the scaled probability that two points chosen at random from the whole population occupy the same space. For the latter index, the higher the value, the more clumped the distribution. The distribution of spots along the flanks was calculated as in Persat (1982).

For each grayling, we estimated six environmental variables. Except for water depth and distance from the river bank, all of the variables were three-level categorical factors (with an approximation of ~1 m around the exact point where the fish was caught). The following were measured: (a) water velocity, (1 = slow; 2 = medium; 3 = fast); (b) type of river bottom (1 = sand; 2 = sand with vegetation; 3 = rocks); (c) water depth (m); (d) distance from the river bank (m); (e) water transparency (1 = low; 2 = medium; 3 = high); and (f) fish position in the pool/stream (1 = head; 2 = middle; 3 = end). Due to the features of the rivers considered in the study, the head/end were considered as the very beginning/end of a pool/stream, i.e. ~1 m after the beginning or before the end of a pool/stream. The same person did all the environmental estimations, to avoid possible bias due to the observer's perception of the river features.

Statistical analyses

To explain the characteristics and spatial patterns of the graylings' spotted flanks, we ran two sets of linear mixed-effects models using different response variables. These two sets of models were related to: (1) the characteristics of the individual (body length and sex); and (2) the characteristics of the environment (i.e. the six environmental variables previously mentioned), as well as the location (i.e. both the river and the country), at which the individual was sampled. First, we conducted an analysis for the entire set of explanatory variables and checked for correlations (Spearman's rank correlation) among predictors, to eventually exclude those variables with $r_s \geq 0.6$. Then, we selected the optimal structure of the random component, which was the one containing two levels of random effects. These were generated by repeated measures of the distribution of spots on both flanks of the same grayling, which were in turn nested by the country. Number of spots, mean number of spots per line, index of dispersion and index of cluster size were log-transformed to achieve normality. Model simplification was performed by backward selection of variables from the full model, and models were compared using likelihood ratio tests until a minimal adequate model was obtained (Crawley 2007). The residuals of the final models were explored to verify the assumptions of normality and homogeneity. In addition, we conducted *t*-tests to explore the potential differences between the spot patterns from the two sides of the same grayling, i.e. if a directional difference for each of the analysed parameters exists – for example, do graylings always have more spots on the left side? The values presented are means ± standard deviation (SD). All statistical analyses were performed using R 2.10.1 statistical software (R Development Core Team 2009), and the nlme (Pinheiro et al. 2009) package.

Results

We obtained a sample of 55 graylings (26 males, 19 females and 10 individuals of unknown sex), ranging from 100 to 870 g in weight (average ± SD = 343.2 ± 147 g) and 18 to 43 cm in length (average ± SD = 31.8 ± 4.8 cm).

Although graylings generally showed bilateral asymmetry in the patterns of spots (Table I; Figure 2), there was no consistency in the direction of this asymmetry (Figure 4), neither for the number of spots (*t*-test = 0.08, p = 0.94, n = 50) nor for the number of spotted lines (*t*-test = 0.09, p = 0.93, n = 50). The analyses on the spatial patterns of spots (Table I) revealed that: (1) both the index of dispersion and the index of cluster size showed a distribution of spots close to random; and (2) Morisita's index was extremely homogeneous for the whole sample, displaying similar patterns of spot aggregation among graylings.

In the models we ran, length and sex of the fish did not show any effects on the characteristics and spatial distribution of spots. In addition, the spatial distribution of spots was never explained by environmental variables, which only explained (1) the number of spots, (2) the mean number of spots per line and (3) the number of spotted lines (Table II). Intriguingly, quantitative patterns of spots were always positively associated with the same environmental features, i.e. high spottiness was related to fast waters, greater depth and transparency (due to both water colour and type of river bottom), whereas the negative relationship between spottiness and fish position in the river demonstrated that the most highly spotted individuals tended to occupy the head of pools and streams (Figure 5).

Discussion

Spottiness did not show any relationship with sex or body size, but quantitative features such as the number of both spots and spotted lines appeared to be correlated with some physical properties of the river: more highly spotted graylings seemed to generally inhabit fast and deep waters, and were positioned at the head of pools and streams. Although skin colourations and, more generally, pigmented patterns are typically thought to result from sexual selection, by which differential mating success leads to differential reproductive success (e.g. Williams & Mendelson 2013), our data did not support this hypothesis. Similarly, we did not find any support for a link between spottiness and fish length.

Social and other environmental factors can demand plasticity and versatility in colouration and patterning (Rosenthal 2007): (1) rapid physiological changes in colours and patterns are involved in signalling motivational or reproductive state (e.g. Nelissen 1976; Baerends et al. 1986); and (2) during agonistic encounters, colouration and patterning changes may reduce aggression by the dominant opponent (Hurd 1997; Miyai et al. 2011). In addition, colouration can also be adjusted plastically to habitat characteristics, for example to convey camouflage against a given background (Maan & Sefc 2013). However, none of these scenarios seems to characterise the spottiness of grayling given that: (1) the spot patterns and numbers do not change over time (Persat 1982); and (2) as in the case of the rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Tack 1973), the number of spots may have a genetic origin (Persat 1982).

Table I. Characteristics of the spotted sides of graylings.

A. Quantitative characteristics

	Mean no. of spots	Range	Mean no. of spots per line[a]	Range	Mean no. of spotted lines	Range
Whole sample						
Left side	16.9	0–95	2.2	0–20	5.4	0–13
Right side	13.7	0–66	1.9	0–10	5.1	0–12
Total	15.3	0–95	2.0	0–20	5.2	0–13
By rivers						
Slovenia and Croatia[b]						
Left side	18.2	0–89	2.3	0–11	6.2	0–13
Right side	16.8	0–66	2.2	0–10	6.2	0–12
Total	17.5	0–89	2.3	0–20	6.2	0–13
Norway[c]						
Left side	0.9	0–4	0.5	0–2	0.7	0–3
Right side	0.8	0–4	0.44	0–2	0.6	0–2
Total[d]	7.6	0–95	1.1	0–10	1.7	0–11

A. Spatial features (average values calculated using PASSaGE v.2[e])

	Index of dispersion	Range	Index of cluster size	Range	Morisita's Index	Range
Whole sample						
Left side	1.05	0–3.63	0.05	−1–2.63	0.85	0–1.58
Right side	1.05	0.14–3.60	0.05	−0.86–2.60	0.87	0–2.04
Total	1.05	0–3.63	0.05	−1–2.63	0.86	0–2.04
By rivers						
Slovenia and Croatia[b]						
Left side	1.01	0–2.25	0.01	−1–1.25	0.84	0–1.58
Right side	1.04	0.14–3.6	0.44	−0.86–2.6	0.87	0–2.04
Total	1.03	0–3.6	0.03	−1–2.6	0.86	0–2.04
Norway[c]						
Left side	1.35	0.33–3.63	0.35	−0.67–2.63	0.92	0.67–1.25
Right side	1.13	0.5–1.75	0.13	−0.5–0.75	0.75	0–1.5
Total[d]	1.28	0.33–3.63	0.29	−0.67–2.63	0.87	0–1.5

[a] See Figure 1.

[b] Unec, Soča, Kupa and Kupica rivers, n = 41 individuals. Graylings from Slovenian and Croatian rivers were grouped because (i) they belong to the Adriatic basin and (ii) Slovenian and Croatian graylings belong to same population enclosed by Sava river system.

[c] Glomma river, n = 14 individuals.

[d] Total numbers also include those individuals for which it was not possible to sample both sides.

[e] See Methods for detailed information on the indices and PASSaGE software.

The recognition that individual differences within a population represent biologically meaningful adaptive traits (Kittilsen et al. 2009) has raised questions concerning the circumstances that benefit different phenotypes, as well as the costs and benefits of limited plasticity imposed by more or less fixed trait associations (e.g. DeWitt et al. 1998). Melanin-based colouration in vertebrates, such as the black spots dappling the skin of the Atlantic salmon *Salmo salar* (Linnaeus, 1758) and rainbow trout, and its association with behaviours, demonstrates that darker individuals are generally more aggressive, sexually active and resistant to stress than lighter individuals (Ducrest et al. 2008; Kittilsen et al. 2009). These findings might help explain the recorded observation that more highly spotted (and consequently darker flanked) individuals occupy the most advantageous places (the head of pools and streams where, for a fish mainly preying on drifting insects, the food arrives first and is generally more abundant; Rahel & Hubert 1991) and costly portions of the rivers (e.g. faster streams; Bisson et al. 1988; Aadland 1993). This could indicate that, in a gregarious fish, spottiness signals the status (e.g. quality and/or dominance) of individuals, which obviously plays a role in determining the spatial arrangement of individuals along the river bed and, consequently, within the group. Visual information and recognition are important for social learning in schooling fish and are widely used for communication between

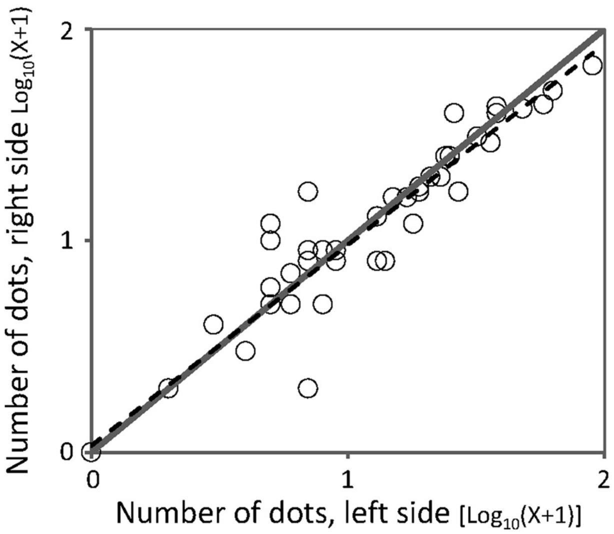

Figure 4. Relationship between the numbers of spots counted on both sides of each individual grayling. The broken line represents the regression line, while the thick grey line denotes the 1:1 relationship. The dispersion of the data shows that there is a certain level of bilateral asymmetry. However, the relationship is strong ($R^2 = 0.93$) and the regression line does not depart significantly from a 1:1 relationship [slope = 0.948, standard error (SE) = 0.038; intercept = 0.032, SE = 0.041].

mates (e.g. Pitcher & Parrish 1993; Brown & Laland 2003). For example, rapid recognition of social partners using visual cues appears to be crucial for individual survival in gregarious species (Karenina et al. 2013): when gregarious fish are preferably responsive to a specific visual signal, i.e. a particular element of the entire fish image which allows for mate recognition and the initiation of a certain reaction to it. The zebrafish *Danio rerio* (F. Hamilton, 1822), for instance, prefers to shoal with companions having the same pigment pattern, and thus individuals of this species ignore fish with a radically different pattern (Engeszer et al. 2007; Saverino & Gerlai 2008).

The environment should not affect the spottiness of grayling as: (1) there is little evidence that persistent melanin-based pigment patterns arising from melanophore aggregations in fishes are subject to environmental influence (Kittilsen et al. 2009); (2) there is strong heritability in the number of black skin spots (Kause et al. 2004); and (3) spot patterns and numbers do not change over time (Persat 1982); rather, spottiness may just reflect the status of individuals and, consequently, their position within the natural and social environment. In fact, eumelanin pigmentation seems to be commonly linked to individual features and personality: (1) in passerine birds (Järvi & Bakken 1984), it is important to control for immediate effects of social interactions; (2) the degree of eumelanism in the barn owl *Tyto alba* (Scopoli, 1769) has been linked to the ability to cope with different environments and stressful factors (Roulin et al. 2008); (3) melanin-based traits of the plumage provide information about personality traits of the bearer in siskins *Carduelis spinus* (Linnaeus, 1758), a highly sociable bird forming flocks (Mateos-Gonzalez & Senar 2012), in which darker individuals within the group are dominant and more aggressive; and (4) in salmonid fishes, dermal eumelanin pigment patterns provide a way to identify stress-sensitive and stress-resistant

Table II. Final linear regression models (from a backward model selection) showing the effect of the environmental variables on the characteristics of grayling spotted flanks. SE = standard error, Log = logarithm.

	Estimate	SE	t	P
Log (number of spots)				
Intercept	−5.66	1.10	−5.14	0.0001
Type of river bottom	0.78	0.20	3.86	0.0003
Water depth	0.90	0.30	2.96	0.005
Water transparency	1.88	0.27	6.96	0.0001
Position in the pool/stream	−0.23	0.10	−2.24	0.03
Log (mean number of spots per line)				
Intercept	−2.84	0.78	−3.65	0.0006
Speed of the stream	.52	0.21	2.49	0.02
Type of river bottom	0.40	0.13	3.11	0.003
Water depth	0.48	0.16	3.09	0.003
Water transparency	0.57	0.14	4.18	0.0001
Position in the pool/stream	−0.18	0.05	−3.33	0.002
Number of spotted lines				
Intercept	−13.67	3.54	−3.85	0.0003
Type of river bottom	2.07	0.66	3.16	0.003
Water depth	2.71	0.99	2.76	0.008
Water transparency	4.25	0.87	4.88	0.0001
Position in the pool/stream	−0.70	0.34	−2.08	0.04

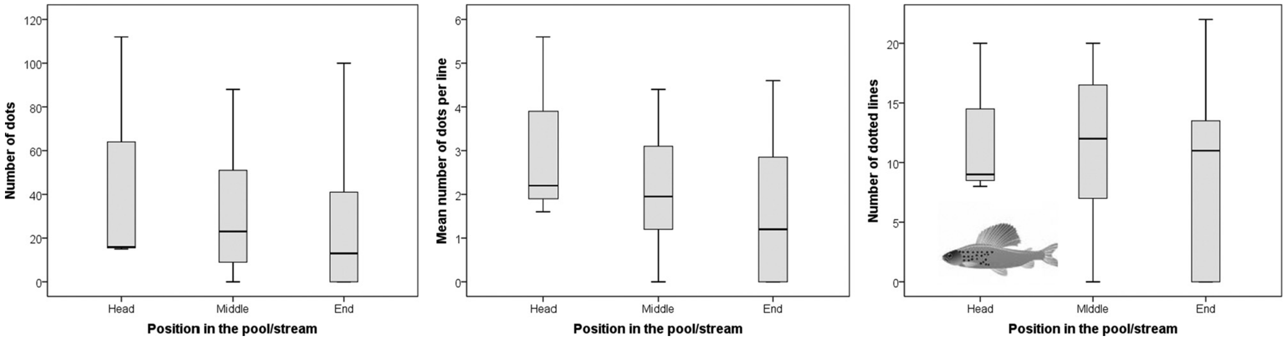

Figure 5. There is an inverse relationship between grayling spottiness and the position fish occupy in pools and streams, with the most highly spotted individuals located at the head of waters. Boxes define the upper and lower quartiles, horizontal lines within boxes represent medians and whiskers indicate minima and maxima.

individuals (Kittilsen et al. 2009). Foraging tactics, for example, have been found to be affected by personality, and the signalling of different personalities could shape these foraging tactics (Barnard & Sibly 1981; Kurvers et al. 2010). The possibility that spottiness might be a status signal should be considered an open question, as it is solely based on the results of the present correlative study. Further experimental and behavioural studies are needed to support or refute this supposition. Actually, it is still unclear how a temporally fixed pattern of colouration might imply that spots function as social signals, because social status may be dynamic: a dominant individual in one breeding season may not have the same social status in the next season (although high-quality individuals have the potential to have a high social status for longer than low-quality individuals).

As highlighted by Maan and Sefc (2013) for cichlid species, we expect that the next generation of research on fish visual communication will become more integrative, in which both genomics (which allows for both deeper and broader understanding of the mechanisms underlying colour variation) and ecological and behavioural studies are considered. In the specific case of graylings, experimental studies are highly welcome for elucidating the function of flank spottiness and its role in regulating life within a school.

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