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SEM images reveal intraspecific differences in egg surface properties of common nase (*Chondrostoma nasus* L.)

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Abstract

Scanning electron microscopy (SEM) has been widely used to describe interspecific differences in egg quality of teleost freshwater fish, but potential intraspecific differences are poorly studied. Eggs of many rheophilic cyprinids are covered with adhesive structures such as attaching villi facilitating egg attachment at substrates of spawning grounds with high currents. Recent findings indicate that the egg quality of the rheophilic cyprinid common nase (*Chondrostoma nasus* L.), a target species of conservation, differs in the adhesiveness between spawning populations, potentially explaining differences in recruitment success. In this study, a SEM image-based standardized protocol was established to assess egg surface quality of *Chondrostoma nasus* eggs. Multivariate statistics detected significant differences of egg surface properties among individual females and among three different populations. These differences were mainly attributed to length variability and merging of adhesive villi as well as to coating and filament-like connections of these structures. The findings of this study highlight the need for further investigations to better understand the relationship of egg surface properties, egg stickiness and hatching success to understand the recruitment ecology and performance of early life stages in freshwater fish.

KEYWORDS

cyprinids, early life stages, fish biology, fish stock management, freshwater fish conservation, scanning electron microscopy, spawning ground restoration

1 | INTRODUCTION

Effective conservation of freshwater fish requires knowledge on species-specific traits of each step of the life cycle (Geist, 2011; Pander & Geist, 2013), which holds particularly true for the sensitive egg and larval stages (Schiemer et al., 2002). The life cycle of a fish starts with the release and fertilization of eggs. The size and structure of fish eggs as well as the timing of release is highly species-specific and evolutionary shaped towards the abiotic and biotic habitat conditions (Bagenal, 1971). While there is a wealth of knowledge on interspecific differences in fish egg properties for a broad set of species of teleost freshwater fish, from temperate to neo-tropical regions (e.g. Brooks et al., 1997; Riehl & Patzner, 1998; Rizzo et al., 2002), very little is known about potential intraspecific variation (but see Keckeis et al., 2000). Also, the egg envelope has been shown to be a sensitive biomarker for environmental pollutants such as xenoestrogens, which may threaten fertilization and protection of the embryo during development (Arukwe & Goksøyr, 2003; Arukwe et al., 1997). This clearly emphasizes the need for a systematic analysis of the ultrastructure of

fish eggs, particularly on an intraspecific level, as water chemical effects on egg development is likely to vary between populations spawning in different rivers.

A widely implemented tool for studying egg surface properties is the use of scanning electron microscopy (SEM) (Riehl & Patzner, 1998; Rizzo et al., 2002). Although the ecology of a species cannot be automatically deduced from structure of the egg surface, eggs of most gravel spawning cyprinid species are characterised by a coverage with adhesive structures such as attaching villi (e.g. Patzner et al., 2006; Petz-Glechner et al., 1998; Riehl et al., 2002). This ensures that a large proportion of the eggs laid adhere to surfaces at spawning sites, which in these species are often characterized by medium to rapid current velocities (Bartoň et al., 2021; Melcher & Schmutz, 2010). This is particularly evident in common nase (*Chondrostoma nasus* L.) which spawns its eggs at current velocities of up to 1 m/s or even greater (Melcher & Schmutz, 2010; Nagel et al., 2020b). *Chondrostoma nasus* is a specialist among riverine fish and has formerly constituted a large portion of the fish community in many rivers of Central and Eastern Europe (Kottelat & Freyhof, 2007). There, this species plays an important role in the food web for lower trophic levels by grazing on benthic algae (Gerke et al., 2018) as well as for higher trophic levels by providing an important food source for apex predators (Šubjak, 2013). Yet, the degradation and fragmentation of habitats has led to severe population declines of this species (Mueller et al., 2018; Peňáz, 1996). As a result, *Chondrostoma nasus* is listed in several conservation lists (Bohl et al., 2003; Kirchhofer et al., 2007; Wolfram & Mikschi, 2007) and has become a flagship species for river conservation (Schiemer et al., 2002). Spawning of nase occurs in schools in which the sex ratio can reach up to 1 female on 25–30 males (Harsanyi & Aschenbrenner, 1995). During spawning, females scatter large numbers of eggs on the substrate surface of shallow gravel banks where several males immediately fertilize them (Peňáz, 1996). Eggs that are not able to adhere at the surface or the substrate interstices (Duerregger et al., 2018) of the spawning ground drift downstream (Hofer & Kirchhofer, 1996; Nagel et al., 2020b), where development success remains uncertain. Since these eggs are likely to face a higher mortality rate owing from potentially unfavorable development conditions and their availability for drift feeding and weak-swimming predators (Šmejkal et al., 2017), a high proportion of less adhesive and consequently off-drifting eggs may ultimately reduce recruitment potential.

There are anecdotal reports of fish breeders and anglers that suspect great differences in recruitment success of different nase populations. Recent findings also indicate that egg adhesive quality differs between spawning populations of *Chondrostoma nasus* (Nagel et al., 2020b), stressing the need for studying egg surface properties also on an intraspecific level. Consequently, the aim of this study was to investigate potential differences in egg quality of *Chondrostoma nasus* by comparing egg surface properties of seven females originating from three different spawning populations. A standardized protocol sheet for the analysis of SEM images was developed to test our hypothesis that surface structure of *Chondrostoma nasus* eggs shows a significant intraspecific variability.

2 | MATERIAL AND METHODS

2.1 | Female spawners

Seven females of *Chondrostoma nasus* were caught in April 2019 during their spawning migration in tributaries of the Inn River (Bavaria, Germany), the largest tributary of the Danube River in Germany (Figure 1). Two females each were caught in the tributaries Isen (48°26′62.74″ north, 12°66′16.21″ east; April 1st 2019) and Mangfall (12°6'23.52" east; 47°50'46.66" north; April 1st 2019) and three females in the tributary Sims (12°9'1.02" east; 47°51'4.20" north; April 2nd 2019). All fish used for this study were caught in the course of breeding and re-stocking initiatives of local angling clubs using a 1.5 kW electrofishing device (Grassl). Prior to striping of eggs, fish were anesthetized with MS-222 (Tricaine methanesulfonate; concentration according to Adam et al., 2013). Subsequently, total length (TL) of each specimen was measured to the nearest cm and total weight (TW) was determined to the nearest gram. Scales were used to identify the age of each female by counting the annuli. Immediately after egg release, subsamples of ~10 ml unswollen and unfertilized eggs from each female were preserved in 96% ethanol without any contact to water or other substances. Eggs were fixated for at least 10 days prior to further handling.

2.2 | Egg size and SEM imaging

Egg size was determined by measuring the diameter of 15–20 preserved eggs of each female (±0.01 mm) with a stereo-microscope Olympus SZX10 (Olympus Deutschland GmbH) using a magnification of 20.0 and the cellSens-Software (OLYMPUS CORPORATION; www.olympus-lifescience.com). Eggs that were used for these measurement were not taken for subsequent scanning electron microscope (termed SEM hereinafter) imaging to avoid potential bias on egg surface analysis owing from mechanical damage caused by handling of the eggs.

Nine eggs from each female were randomly selected for SEM imaging. First, egg moisture was removed using a vacuum (0.05 mbar) freeze dryer (Alpha 1–4, Christ,) at −47°C for 120 s. Second, eggs were fixed to a SEM sample holder with conductive carbon adhesive pads and gold-coated using a Polaron SC502 Sputter Coater (Fisons Instruments).

Subsequently, eggs were examined with a SEM (S-2300, Hitachi) at a voltage of 25 kV, a geometric working distance of 10 and a magnification of 1,500. Nine photographs of the egg surface from each egg were taken, following the pattern displayed in Figure 2. Technical settings of the SEM remained constant during imaging of all photographs.

Since the image quality of some photographs was not sufficient for a reliable assessment, which was especially true in the S3 sample, these were excluded. In order to obtain an equal number of images for each egg, seven images from each egg were randomly selected from the remaining photographs. This resulted in a total number of

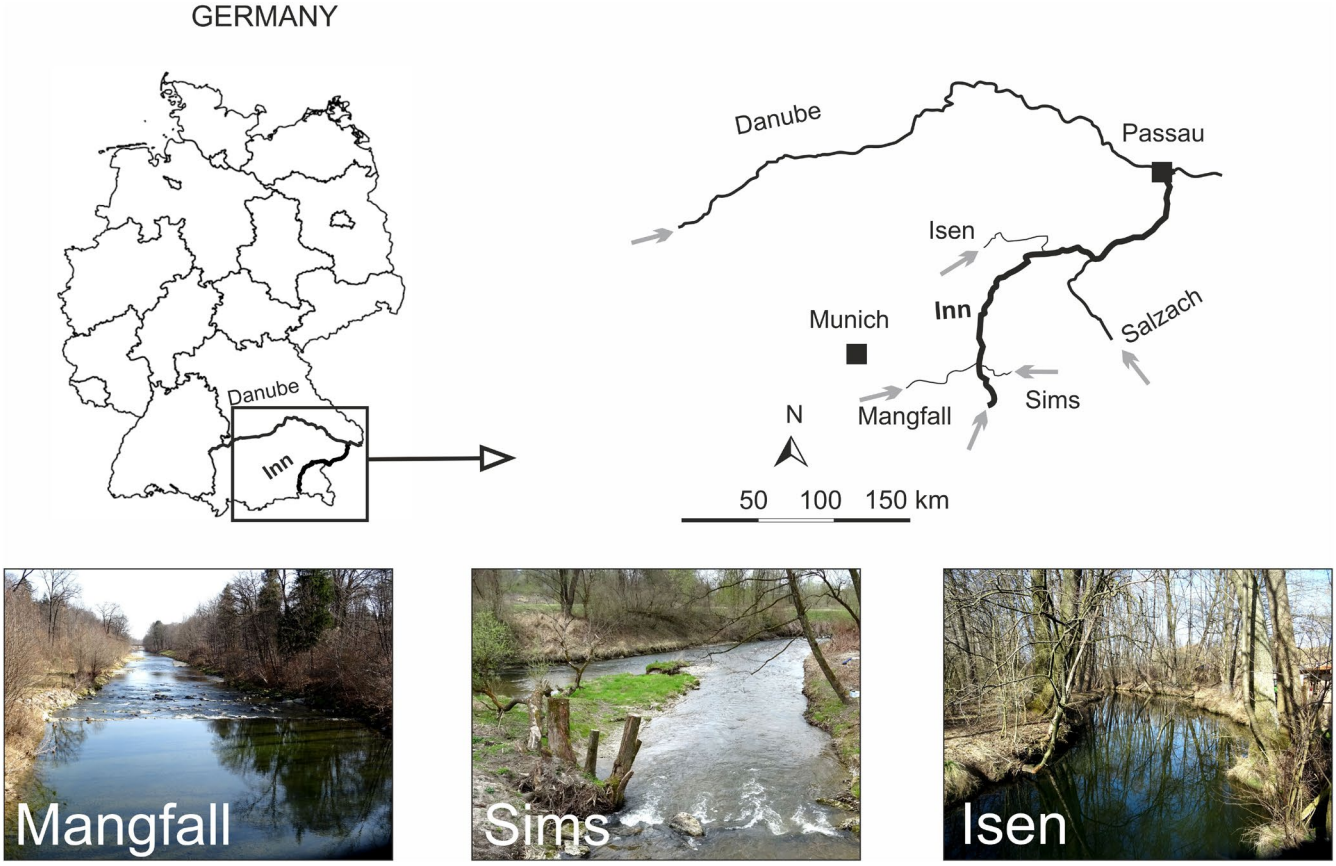

FIGURE 1 Map of the study area and photographs of the rivers with studied spawning populations of nase

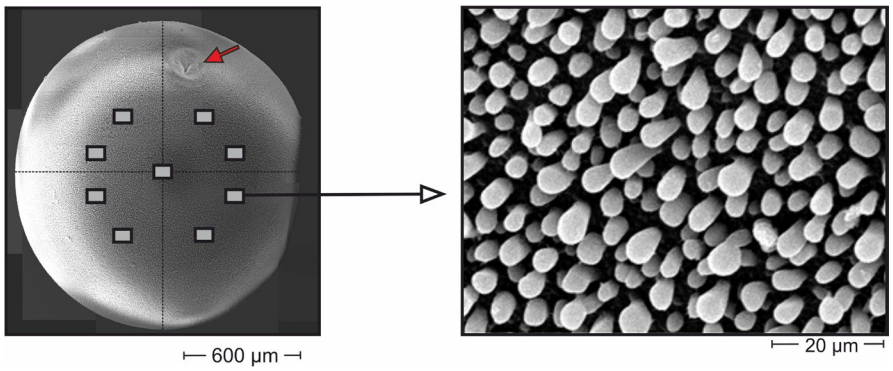

FIGURE 2 Left side: Egg of *Chondrostoma nasus* (×20) with visible microphyle (red arrow) and an overlaid schematic indicating the areas where photographs were taken. Right side: Magnification (×1,500) used to assess egg surface properties; note the adhesive villi covering the zona radiate externa of *Chondrostoma nasus* eggs

		Female attributes				SEM imaging	
River	ID	TL (cm)	TW (g)	Age (years)	Egg size (mm)	Eggs used (*n*)	Images used (*n*)
Isen	I1	49	1,384	9+	1.95 ± 0.13	9	63
Isen	I2	47	966	9+	1.81 ± 0.08	9	63
Mangfall	M1	49	1,090	9+	2.22 ± 0.04	9	63
Mangfall	M2	49	1,335	9+	2.11 ± 0.07	9	63
Sims	S1	53	1,660	10+	2.02 ± 0.08	9	63
Sims	S2	54	1,850	11+	2.09 ± 0.07	9	63
Sims	S3	51	1,420	11+	2.21 ± 0.06	8	50
Σ						**62**	**428**

TABLE 1 Origin, ID, female attributes and egg size (mean ± *SD*) for each specific *Chondrostoma nasus* female used in this study as well as number eggs used for SEM imaging and number SEM images used for egg surface assessment. Note: All measurement of egg sizes was done with preserved eggs, which causes a volume reduction of ~ 25% (Patzner et al., 2006). TL = total length; TW = total weight

428 images for the assessment (Table 1). In a final step, all images were encoded and put into a randomized order by an external person. Subsequently, these images were reviewed by the same person and then recoded to their original ID.

2.3 | Assessment of egg surface properties

First, density of adhesive villi (AV) per image was determined by counting the number of AV on the egg surface. For each egg, only the image from the centered photograph was evaluated (Figure 2), as only this shooting angle allowed an accurate counting of all AV. Only fully visible AV were counted.

To systematically assess further egg surface properties, six criteria were defined and rated at a level of 0 (low), 1 (medium) or 3 (high). This rating scheme was adapted from a protocol that has been established to assess external injuries in fish and is capable of distinguishing possible differences between groups as well as to identify the underlying causes when combined with multivariate statistics (Mueller et al., 2017). Assessment criteria were defined according to a combination of results from a literature search (Patzner et al., 2006; Riehl & Patzner, 1998; Rizzo et al., 2002) and own observations on egg surface characteristics. The criteria were: (1) equality of distribution of adhesive villi, (2) length variability of adhesive villi, (3) coating of adhesive villi, (4) merging of adhesive villi, (5) filament-like connections between adhesive villi and (6) globule structures covering adhesive villi (Figure 3, Table 2).

2.4 | Statistical analysis

Univariate statistics were used to test for differences in the densities of AV between individual females and spawning populations likewise. Prior to tests for significance, data distribution was checked for normality using the Shapiro-Wilk test. Since none of the data were normally distributed, significances were tested with the Kruskal-Wallis test, followed by pairwise comparisons using the Mann-Whitney *U* test. All univariate statistics were performed in R (version 3.6.3; R Core Team, 2017).

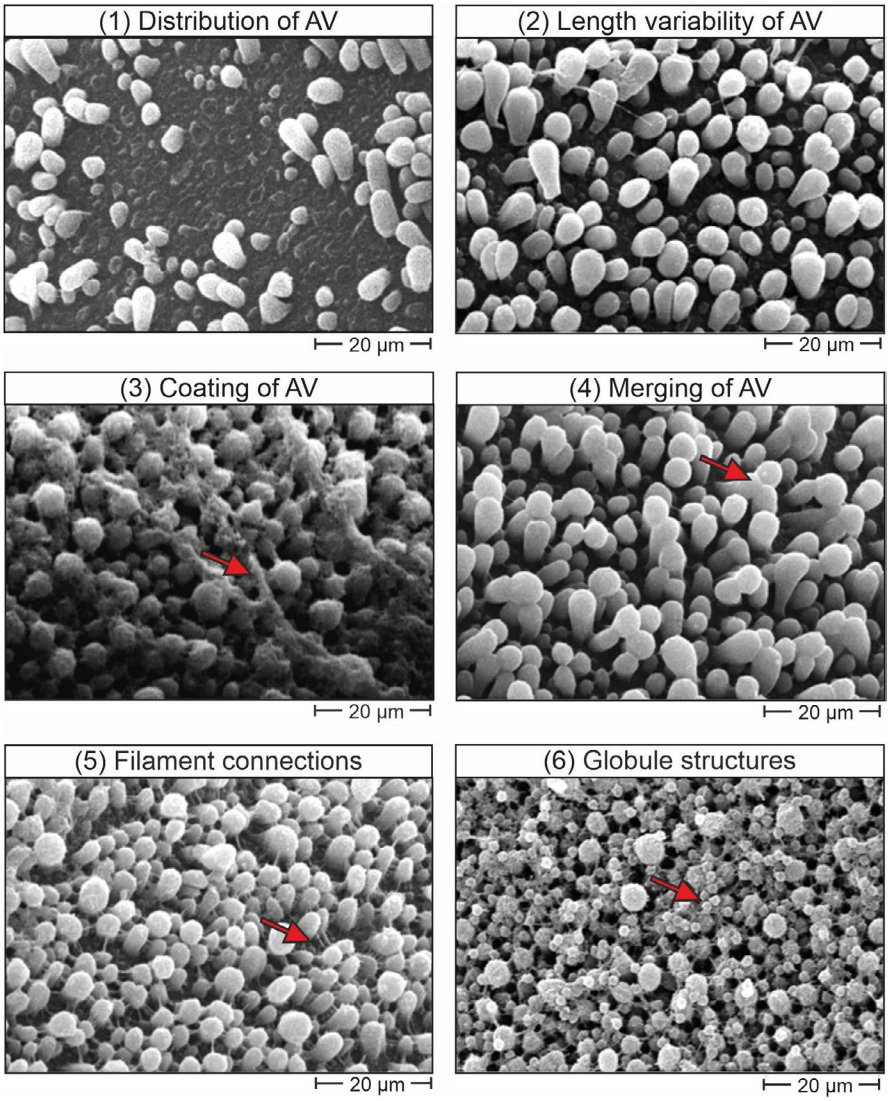

FIGURE 3 Criteria defined for assessment of *Chondrostoma nasus* egg surface properties. All images represent category 3 (=high occurrence). Red arrows highlight characteristics of criteria 3–6. Definition of the criteria follows Table 2. AV = adhesive villi

Multivariate statistics were used to compare egg surface properties according to the criteria of the assessment protocol described above. First, a resemblance matrix based on Bray-Curtis similarities (Bray & Curtis, 1957) was computed using each image as a sample and each assessment criterion as a variable. Non-metric multidimensional scaling (nMDS) was performed to visualize differences in egg surface properties. The one-way analysis of variances (ANOSIM) was used to check for significances in egg surface differences of individual females and spawning populations. Subsequently, a similarity percentages analysis (SIMPER) was performed to reveal the criteria causing similarities and differences in and between the groups. All multivariate analysis were conducted in Primer v7 (Plymouth Marine Laboratory). For all analysis, significant differences were accepted at $p < .05$.

3 | RESULTS

From a total of 59 SEM-images analysed, density of AV varied from 150 to 379 per image, which equals 31,250 to 78,832 AV per mm^2. Significant differences were detected on the level of individual females (Kruskal-Wallis-Test: $\chi^2 = 29.058$; $df = 6$; $p < .001$) and populations (Kruskal-Wallis-Test: $\chi^2 = 7.650$; $df = 2$; $p < .05$). When comparing individual females, AV density was lowest in M1 (171 ± 10) and significantly higher in all other *Chondrostoma nasus* (Figure 4).

Based on a total of 428 images assessed according to the criteria of the protocol, ANOSIM detected significant differences between eggs from different populations (Global *R*: .32; $p < .001$) and overall females likewise (Global *R*: .34; $p < .001$; Table 3, Figure 5). Differences were most pronounced among eggs from the Mangfall population with the Sims and Isen populations, but only small differences occurred in the comparison of females originating from the Isen compared to the River Sims, as reflected by the widely overlapping ordination of the symbols in Figure 5 and the low *R* value of this group comparision of only .064 (Table 3).

Egg surface images from the Isen population revealed an average similarity of 49.8%, to which filament-like connections between AV contributed most (contribution: 57.02%; average rating: 1.62), followed by length variability of AV (contribution: 17.36%; average rating: 0.66). Eggs from the Sims population showed an average similarity of 51.33%; mainly caused by a high prevalence of filament-like connections between AV (contribution: 56.73%; average rating: 2.16) and coating of AV (contribution: 14.82%; average rating: 0.96).

TABLE 2 Description of the egg surface assessment criteria

Criterion	Description
Distribution of AV	Equality in the distribution of adhesive villi on the zona radiate externa (0 = AV are equally distributed)
Length variability of AV	Estimated variability in the length distribution of adhesive villi (0 = AV show a similar length)
Coating of AV	Adhesive villi are coated with a jelly-like structure
Merging of AV	Merging of several adhesive villi on the distal ends
Filament connections	Filament-like connection between adhesive villi
Globule structures	Small globule structures coat adhesive villi

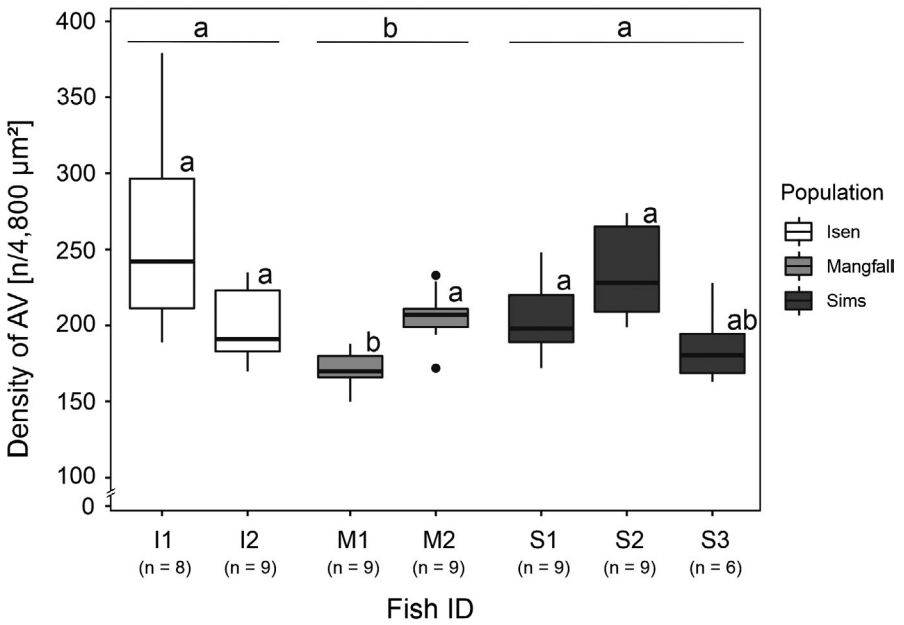

FIGURE 4 Density of adhesive villi (AV) on the zona radiate externa. Spawning populations are indicated by different shades of grey. Outliers are marked with black dots. Unequal small letters above boxes and indicate statistically significant differences between different females and spawning populations respectively ($p \leq .05$). Abbreviation of the IDs follow Table 1

TABLE 3 Group comparisons of the different populations and individual females. *R*- and *p*-value are based on the one way analysis of similarities (ANOSIM). Average dissimilarity (AVDIS) and ranked criteria contribution (given in %) is based on the results of the similarity percentages (SIMPER) analysis

	ANOSIM			Ranked criteria contribution [%]		
Comparison	***R***	***p***	**AVDIS**	**1st**	**2nd**	**3rd**
Population						
Isen versus Mangfall	.316	**<.001**	60.37	Length variability [26.6]	Filament connections [25.3]	Distribution of AV [16.2]
Isen versus Sims	.064	**<.001**	51.82	Filament connections [27.7]	Coating [18.6]	Length variability [16.8]
Mangfall versus Sims	.353	**<.001**	62.47	Filament connections [29.2]	Length variability [21.6]	Coating [14.8]
Individual females						
I1 versus I2	.088	**<.001**	51.57	Filament connections [30.9]	Length variability [17.7]	Distribution of AV [14.6]
I1 versus M1	.445	**<.001**	62.24	Filament connections [31.3]	Length variability [27.7]	Merging of AV [14.8]
I1 versus M2	.390	**<.001**	58.70	Filament connections [30.2]	Length variability [20.2]	Distribution of AV [18.5]
I1 versus S1	.158	**<.001**	47.63	Coating [31.0]	Filament connections [26.0]	Length variability [15.5]
I1 versus S2	.181	**<.001**	51.81	Filament connections [29.1]	Length variability [21.1]	Merging of AV [17.9]
I1 versus S3	.315	**<.001**	46.62	Globule structures [33.1]	Filament connections [27.0]	Length variability [20.6]
I2 versus M1	.330	**<.001**	63.09	Length variability [32.7]	Filament connections [19.9]	Merging of AV [16.0]
I2 versus M2	.223	**<.001**	57.43	Length variability [32.7]	Distribution of AV [19.7]	Filament connections [19.6]
I2 versus S1	.192	**<.001**	53.67	Filament connections [26.9]	Coating [26.6]	Length variability [16.0]
I2 versus S2	.074	**<.01**	53.95	Length variability [24.0]	Filament connections [19.9]	Merging of AV [18.2]
I2 versus S3	.432	**<.001**	57.77	Filament connections [33.4]	Globule structures [26.0]	Length variability [11.4]
M1 versus M2	.061	**<.01**	46.48	Length variability [30.7]	Distribution of AV [23.3]	Merging of AV [18.6]
M1 versus S1	.493	**<.001**	63.78	Filament connections [28.7]	Coating [24.5]	Length variability [23.5]
M1 versus S2	.153	**<.001**	53.03	Length variability [29.4]	Filament connections [23.4]	Merging of AV [18.8]
M1 versus S3	.855	**<.001**	76.81	Filament connections [33.6]	Globule structures [22.1]	Length variability [20.1]
M2 versus S1	.390	**<.001**	57.72	Filament connections [29.3]	Coating [23.0]	Length variability [19.5]
M2 versus S2	.087	**<.001**	48.61	Length variability [26.2]	Filament connections [23.5]	Distribution of AV [20.4]
M2 versus S3	.839	**<.001**	74.14	Filament connections [32.6]	Globule structures [22.3]	Length variability [15.7]
S1 versus S2	.161	**<.001**	49.99	Filament connections [27.0]	Coating [25.0]	Length variability [20.6]
S1 versus S3	.492	**<.001**	51.33	Globule structures [29.0]	Filament connections [24.9]	Coating [17.0]
S2 versus S3	.630	**<.001**	62.18	Filament connections [27.1]	Globule structures [26.0]	Length variability [15.6]

Average similarity in the Mangfall population was highest (54.65%) and, contrasting to the Sims and Isen populations, mainly caused by length variability of AV (contribution: 49.53%; average rating: 1.80) and merging of AV (contribution: 19.72%; average rating: 0.87). Consequently, these criteria also caused the differences in the comparisons between the populations and individual females (Table 3).

4 | DISCUSSION

The findings of this study point at distinct differences in the surface structure of *Chondrostoma nasus* eggs among populations and individuals, which likely affect adhesiveness and thus recruitment success in this species. The reasons for these differences may be explained by genetic effects such as local adaptation, by maternal effects or ambient environmental conditions which needs to be clarified in future studies. The protocol developed in this study has demonstrated its applicability to assess egg surface properties and, when used in combination with multivariate evaluation methods, its ability to identify potential intraspecific differences in the egg surface structure of *Chondrostoma nasus*. Egg quality in general is affected by several components, ranging from endocrine status and diet composition of the female during growth of the oocyte, nutrient composition of the oocyte to female attributes such as size and age as well as physico-chemical water conditions affecting egg incubation after egg release (Brooks et al., 1997; Keckeis et al., 2000). Yet, an effect of the latter can be excluded in our study, as eggs were directly striped and fertilized without any contact to water. However, a variety of reasons remain that could explain the differences observed. Keckeis et al. (2000) found that egg size and to a lesser extend also the chemical composition of the egg is highly influenced by the age of the female spawner. As female *Chondrostoma nasus* of the Sims population were older (10–11 years) than females from the Mangfall and

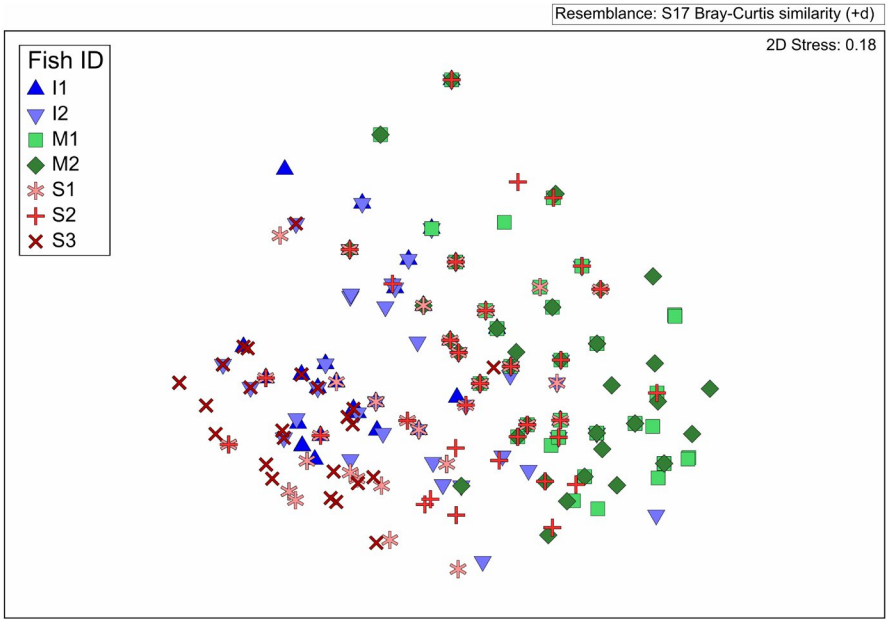

FIGURE 5 Non-metric-multidimensional-scaling (nMDS) of egg surface properties for different females (indicated by different symbols) and spawning populations (indicated by different colors). The distance between the symbols corresponds to the dissimilarity in egg surface properties (small distance = large similarity). Abbreviation of the IDs follow Table 1

Isen population (all 9 years), this could also explain the differences observed in our study, which were mainly caused by higher occurrence of filament-like connections, coating of the egg surface as well as a lesser length variability of AV in the Sims population. Yet, the rather small differences in age of 1–2 years suggests that this is unlikely to be the case and stresses the need for further investigations. Future research should also include endpoints such as stickiness and hatching success, as it remains unclear if these are related to the differences in the observed eggs surface properties. However, previously observed differences in adhesive abilities (Nagel, et al., 2020b) and hatching success between the Mangfall and the Sims population in the wild (Duerregger et al., 2018) suggest that this is likely the case. This stresses the need of linking observations on egg surface properties to general egg quality expressed by egg stickiness and hatching success and other important incubation conditions such as physico-chemistry of the water (Kincheloe et al., 1979; von Westernhagen, 1988) and substrate composition (e.g. Nagel et al., 2020a; Sternecker & Geist, 2010). This is of particular importance as severe recruitment problems may arise from a combination of stressors such as a poor egg quality, a reduced adhesive ability resulting in higher off-drift of eggs and deteriorated habitat conditions on spawning grounds. Additionally, recent findings demonstrate that egg adhesiveness at spawning grounds can be extremely reduced in rivers with hydropeaking effects (Bartoň et al., 2021). In turn, improvement of spawning ground quality might partially compensate for reduced egg quality as a loose and porous interstitial as well as low fine sediment infiltration rates positively contribute to hatching success (Nagel et al., 2020a; Nagel et al., 2020a). In addition, a porous spawning substrate can incorporate a higher share of laid eggs, even if they have less adhesive abilities, and eggs infiltrating to the hyporheic zone are incubated in more sheltered conditions compared to those, that could not adhere at spawning sites (Duerregger et al., 2018; Persat & Olivier, 1995).

In light of still declining *Chondrostoma nasus* populations and intensive efforts to conserve and restore this species, future research is needed to better understand the relationship between egg surface properties and constraints for recruitment success in the early life history of this species. Assessing differences in egg surface properties in relation to adhesiveness and recruitment success in species with similar eggs other than nase may also be an important future direction in understanding fitness differences and resilience among individuals, populations and species in relation to changes of their habitats.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request.

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