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 evolutionarily 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
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 Ison (48°26′62.74″ north, 12°6′16.21″ east; April 1st 2019) and Mangfall (12°6′23.52″ east; 47°5′04.66″ north; April 1st 2019) and three females in the tributary Sims (12°9′1.02″ east; 47°51′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 stripping 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-lifesience.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

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

| 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             |
| ∑     |    |         |        |             |               | 62            | 428            |
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 comparison 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).

| 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                               |

Table 2: Description of the egg surface assessment criteria

![FIGURE 4](density_av.png) 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.

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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

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

ACKNOWLEDGEMENTS
We would like to thank E. Schulz, A. Huber (Kreisfischereiverein Rosenheim e.V.), R. Zillmer (Anglerbund Rosenheim) and M. Holzner (Bezirkfischereiverein Mühldorf Altötting e.V.) for their permission to conduct this study and their assistance with fieldwork. We are also grateful to B. Gum and L. Egg (Fischereifachberatung Oberbayern) for their support, to M. Mueller for her involvement in the planning phase of this project and to U. Raeder and W. Kuefner for technical assistance during REM analysis.

DATA AVAILABILITY STATEMENT
Data are available from the corresponding author upon reasonable request.

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How to cite this article: Nagel, C., Spiessl, C., Pander, J., & Geist, J. (2021). SEM images reveal intraspecific differences in egg surface properties of common nase (Chondrostoma nasus L.). Journal of Applied Ichthyology, 00, 1–9. https://doi.org/10.1111/jai.14233