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# Great crested newt *Triturus cristatus*, smooth newt *Lissotriton vulgaris*, and other amphibians in an acidified area of southern Norway surveyed using eDNA and other methods

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**ABSTRACT** - Acid rain for many decades has led to severe acidification of waters in southern Norway. Acidic water can be fatal to gill-breathing vertebrates (i.e. fish and larval amphibians). Great crested newt *Triturus cristatus* (GCN) - seems to be less tolerant of acidic water than other Norwegian amphibians. Not until 2015 was GCN recorded in Agder, the southernmost county in Norway, when the larvae of this species were found in two ponds. The aim of our investigation, in late spring and summer 2021, was to find out whether GCN was still present in these two ponds and ten others in the same area, which are surrounded by peat bogs and forest. Since this is a marginal and acidic area with probably low numbers of individuals and low detectability, we used three survey methods in combination (funnel traps, nets, and eDNA) and also measured water conductivity and pH. At the same time, the occurrence of other amphibians in the area were investigated; the smooth newt *Lissotriton vulgaris*, the common toad *Bufo bufo*, the common frog *Rana temporaria* and the moor frog *Rana arvalis*. Using traps and nets, GCN was found in four ponds but in only two of these ponds by eDNA. However, GCN eDNA was detected in three other ponds, showing that a combination of methods gave the most complete result. eDNA of the common toad and the common frog were detected in (almost) all samples but there were few records from traps or nets. Smooth newts were detected in almost all ponds by traps, nets and eDNA, while none of the methods detected the moor frog. Especially when a species is rare at a location, eDNA analysis may be the most efficient method of detection. However, only trapping and netting can give information about breeding. Water pH in late spring and early summer varied from 4.7 to 5.6 (median pH 5.1), which makes this area marginal for amphibian reproduction.

## INTRODUCTION

Amphibians are in decline globally with populations disappearing from locations where they were once abundant (Halliday, 2008). Some of the factors behind these declines include the destruction of habitats, invasive alien species, diseases, and acid rain (Dolmen, 2018). The great crested newt *Triturus cristatus* (GCN) is found in the northern parts of Europe and is categorised as 'Near Threatened' (NT) in Norway (Artsdatabanken, 2021). It is far less common than the smaller smooth newt *Lissotriton vulgaris* and vulnerable to further reduction in population size (Dolmen, 2008). It is therefore important to map the distribution of this species so that it can be better protected. Surveying of rare species like the GCN with traps and hand nets can be time consuming and challenging, with the risk of not detecting the presence of the species in areas with few individuals. By combining these methods with environmental DNA (eDNA) analysis of water samples, it may be possible to detect species in a shorter time and improve the overall detection rate. eDNA analysis has been used successfully to study the presence and distribution of a wide range of species (for a review see Ruppert et al., 2019), including aquatic species and amphibians (Ficetola et al., 2008; Rees et al., 2014; Ruso et al., 2019). eDNA normally becomes undetectable in fresh water less than one month after the target species has left the water (Dejean et al., 2011). However, acidic water might accelerate the degradation of eDNA (Seymour et al., 2018).

Many decades of acid rain has led to acidification of water and watercourses, especially in the southernmost parts of Norway. The decline in freshwater fish populations in parts of southern Norway is associated with increasing acidity in rivers and lakes. The chief cause of increased acidity is acid precipitation which is the product of the emission, oxidation and long-distance transport of air pollutants, particularly sulphur dioxide (Leivestad & Muniz, 1976). There have been a number of liming projects to prevent fish deaths in lakes and rivers (Sandøy & Romundstad, 1995). Water with a very low pH is fatal to gill-breathing vertebrates (i.e. fish and larval amphibians), as it causes ion loss over the gills, and exposure to toxic aluminium compounds which can be dissolved from the bedrock. Tolerance for acidic water varies between different species of fish and amphibians, but the problems start at around pH 5.

In Norway, the smooth newt is found breeding in water as acidic as pH 4.6 (Strand, 2002), and larval GCN have been found at pH 4.9 (Dolmen, 1980; Strand, 2011). However, while the smooth newt is commonly found in waters within

the range of pH 5.0–5.4, GCN is more sensitive to acidic water and is only rarely found breeding in water at lower than pH 5.5. The common frog *Rana temporaria* and the moor frog *Rana arvalis* are less sensitive to acidic water and are often found breeding at pH 4.5–5.0. The common toad *Bufo bufo* seems to avoid breeding below pH 5 (Strand, 2002; 2010). In contrast to the other species, prior to 2015 GCN had never been documented in Agder, the southernmost county of Norway, but it is possible that the species had a larger and undetected distribution before the period of acid precipitation.

To our surprise, during a mapping project in Agder in 2015, GCN was found breeding in two forest ponds in Gjerstad municipality in the northern part of Agder (Strand & Stornes, 2015) (Fig. 1). This finding led to new investigations during the following two years, expanding the survey area to include eight municipalities southwest of Gjerstad. However, GCN was not encountered in the expanded area. The new surveys covered 124 bog and forest ponds and in 47 of them aquatic vertebrates (i.e. the smooth newt, the common frog, the common toad and fish) were found. The ponds with aquatic vertebrates had a median pH of 5.7, slightly higher than that for the other ponds (pH 5.0); a statistically significant difference ($p < 0.001$ Mann-Whitney U-test) (calculated from data in Strand & Stornes, 2016; Strand, 2017).

The aim of the present study was to see whether GCN was still present in the two original ponds six years later, and whether the species was found in other ponds within the same area. We also aimed to compare the efficiency of funnel traps and nets with analysis of eDNA for detection of all amphibians in the area, which include the smooth newt, the common toad, the common frog, and possibly the moor frog. In addition, the potential presence of the chytrid fungus *Batrachochytrium dendrobatidis* (BD) that is a global threat to amphibians was also examined in the water samples with the use of eDNA analysis. Finally, we set out to investigate to what extent the acidic water in the area limits the reproduction of amphibians.

## MATERIALS & METHODS

### Area description and selection of ponds

This study, undertaken in summer 2021, included twelve ponds (Fig. 1), of which ponds #1–#10 were previously investigated in the 2015 survey (Strand & Stornes, 2015). The pond 'Stemtjern' is divided into two by a peat bog and is, as in the 2015 survey, treated as two separate ponds (#8 Stemtjern East and #9 Stemtjern West). These ponds lie within an area of 5 km² in a landscape dominated by forested hills with peat bogs in the lower parts; details of the location, area and altitude of these ponds are presented in Table 1S (see Supplementary Material). In Gjerstad (Agder county), the marine level (i.e. the highest sea level after the Ice Age) is 90–110 m above today´s sea level. The soil below this level is characterised by clay, with sediments of sandy soil where rivers flow out during the ice melt. The altitude of the ponds investigated in this study are at 144–236 m (Table 1S), where the soil is dominated by less fertile moraine (Thorsnæs & Lauritzen, 2021).

### Field methods

We visited the area on four occasions during late spring and summer 2021 (29 & 30 May, 25 June, 18 July, and 11 August). During the mating season in the spring, the newts are very active and are easily caught with traps. In May, we used collapsible traps, with funnel-shaped entrances at each end, to catch adult newts. The traps, measuring 25 cm in diameter and 60 cm in length, originally designed for removing minnows *Phoxinus phoxinus* from lakes, are used for monitoring GCN in Norway (Dervo et al., 2019). Ten traps were set in each pond and taken up after 24 h, after which the species and numbers of amphibian captured were recorded.

For the detection of amphibian larvae and adults, at all visits we made 10 or more horizontal hauls along the pondbank with long-handled hand nets. The nets were fine-meshed and suitable for catching aquatic animals down to the size of planktonic crustaceans. The mesh frame was approximately 25 cm by 25 cm.

In 2021, water samples were collected from the ten ponds that had been investigated in 2015. Specific conductivity (total ionic score) was measured with a 'Delta Scientific model 1014' conductivity meter and read as µS/cm at 25 °C (K25). The conductivity measures the ionic concentration (hardness) of the water and indicates its buffer capacity (acid-binding capacity). The acidity (pH) was measured electrically with a 'Polymetron 55N' pH meter, with an electrode suitable for low-ionic water.

### Collection of samples for eDNA analysis and qPCR

Samples for eDNA analysis were collected in May and July 2021. One litre of water was sampled from each pond by collecting sub samples of 100 ml at locations evenly distributed around the pond and combined in a sterile bottle. Samples were then transported to the laboratory and filtered through a cellulose nitrate filter (0.45 µm pore size). DNA was extracted and purified from the filters by using DNeasy® Blood & Tissue Kit (Qiagen) following the manufacturer's protocol.

Traps and/or nets were used in 11 of the 12 ponds while eDNA samples were taken from all ponds, which means that one pond was only investigated with respect to detecting the amphibians and *B. dendrobatidis* by using eDNA. Also, one of the eDNA samples was a combination of three adjacent ponds (the Rossmyr ponds, ponds #5–#7).

TaqMan qPCR assays with species specific primers and probes (Table 2S, in Supplementary Material) were used for detection of the various species in the isolated eDNA. The 20 µl PCR-mix consisted of 1 x TaqMan Environmental Master Mix (Applied Biosystems), 0.9 µM of each PCR primer, 0.55 µM probe and 3 µl of template eDNA. The qPCR was conducted on a StepOnePlus Real time PCR system (Applied Biosystem) with temperature profile of 50 °C for 2 min and 95 °C for 10 min, followed by 60 cycles of 96 °C for 15 s, 60 °C for 60 s, with fluorescence detection after each cycle. We analysed every sample in triplicate. Genomic DNA from the species and dd $H_2O$ was added as a template in positive and negative controls respectively.

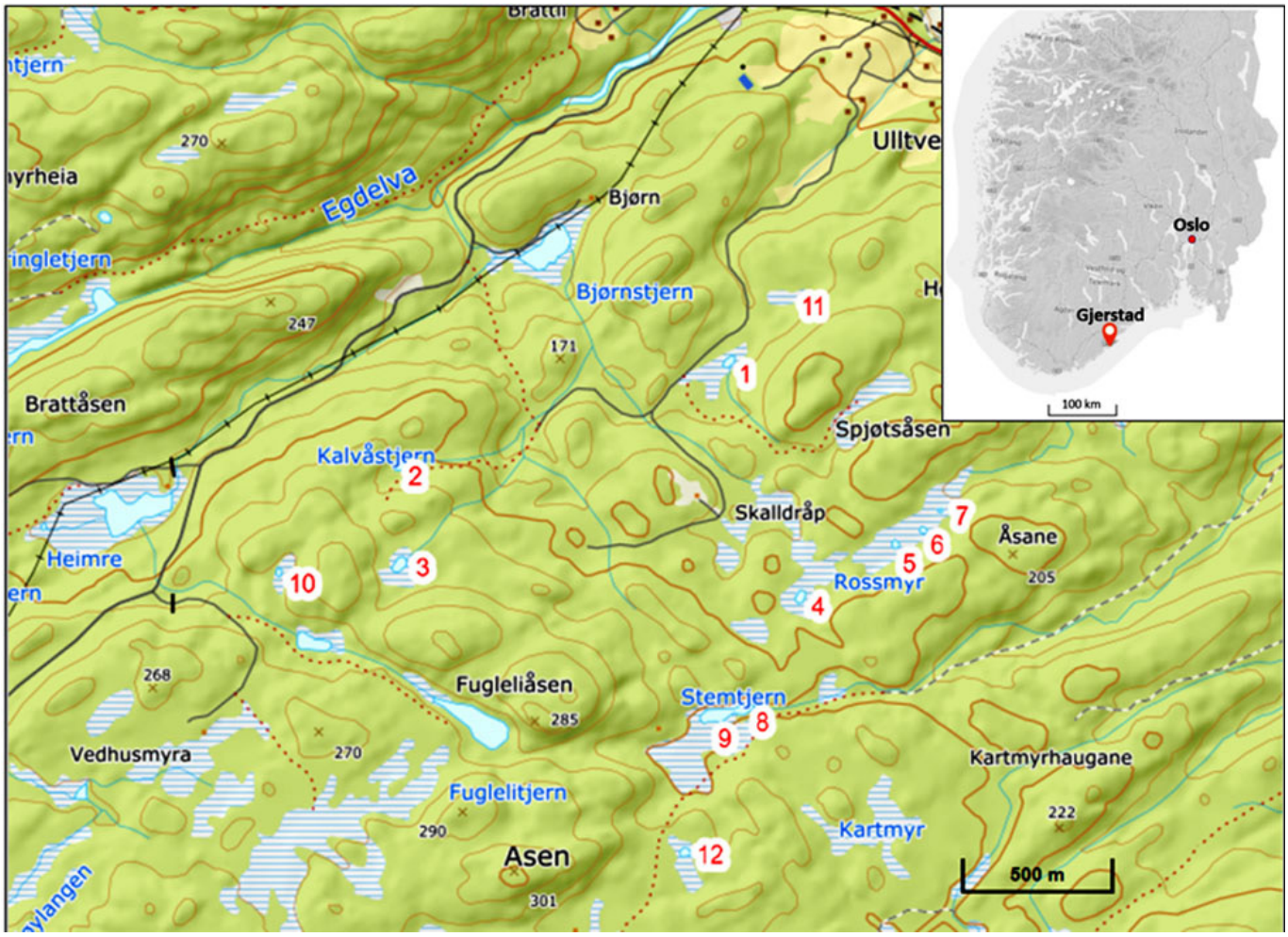


**Figure 1**. Map of the study area. The numbers indicate the locations of ponds.

## RESULTS

**Newts**

In May 2021, adult GCN and smooth newts were retrieved from the two ponds (#2 & #3) in which they had been detected in 2015 using hand nets (Table 1). Traps were set in six of the other ponds from 2015, and the smooth newt was caught in all of these ponds, in addition, one female GCN was found in pond #10. In June and July 2021, using hand nets, we were able to detect larvae of the smooth newt in only two of the six ponds in which adults had been trapped earlier. Larval GCN were found in one pond investigated that had given a negative result in 2015 (#9), but we were not able to detect larvae of this species in the pond where we had trapped the female. The traps also caught the adult smooth newt in one of the new ponds (#11). In August, larvae of the smooth newt were found in another two of the six ponds where traps were used, which means that reproduction was detected in four of the six ponds where adults were found in May.

eDNA from GCN was detected in half of the 10 eDNA-samples (Table 2), of which three samples came from ponds with negative results using traps and/or nets. One of these samples was a mixture of water samples from the three Rossmyr ponds (#5–#7). The result was negative for one pond where larvae were found both in June and July (#9). eDNA from smooth newts was detected in all but one of the samples (pond #4).

**Anurans and chytrid fungus *Batrachochytrium dendrobatidis***

In May 2021, newly hatched tadpoles of the common toad were found in one pond (#1), visually and by net. This species was not found in any of the other ponds in this survey. The common frog was observed in four ponds, of which tadpoles were caught by nets in ponds #6 and #7. In August, adult frogs were observed visually in ponds #4 and #10, jumping into the water as we approached. The moor frog was not found at any location.

eDNA of the common toad was detected in all samples while eDNA from the common frog was detected in seven (Table 1). Pond #8 (Stemtjern East) was not analysed for the eDNA of the common frog. No eDNA of the moor frog or *B. dendrobatidis* was detected in the surveyed ponds.

**Measurements of specific conductivity and pH**

The conductivity ranged was 14–19 µS/cm and showed some increase during the investigation period (Table 1). The largest increases were seen in the three Rossmyr ponds (#5–#7). The pH values were correspondingly low, and in May were pH 4.7–5.3. There was a generally small increase – 0.2

**Table 1**. Counts of amphibians, using traps and nets, and water chemistry from ponds in Norway, from the first investigation in 2015 and the follow-up investigations in 2021

| Pond No. | 28 June 2015 | | | 29–30 May 2021 | | | | 25 June 2021 | | | 18 July 2021 | | | 11 August 2021 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Net | pH | k25 | Net | Traps | pH | k25 | Net | pH | k25 | Net | pH | k25 | Net | pH | k25 |
| 1 | Lv3, [Lv]1, Bb1 | 5.5 | 17 | Bb | [Lv]55♂, 22♀ | 5.2 | 16 | - | - | - | Lv1 | 5.7 | 17 | Lv40, [Lv]1♀ | 5.6 | 17 |
| 2 | Lv6, Tc4 | 6 | 19 | [Tc],[Lv] | - | 5.5 | 18 | - | - | - | - | - | - | - | - | - |
| 3 | Lv21, Tc2 | 5.9 | 16 | [Tc],[Lv] | - | 5.3 | 16 | - | - | - | - | - | - | - | - | - |
| 4 | Neg. | 4.8 | 17 | [Lv]1♀ | [Lv]1♂ | 4.7 | 19 | Neg. | 4.9 | 18 | Neg. | 5 | 17 | [Rt]1 | 4.9 | 18 |
| 5 | [Lv]1 | 5 | 16 | [Lv] | [Lv]14♂, 7♀ | 5 | 14 | - | - | - | Neg. | 5.5 | 19 | Lv1 | 5 | 24 |
| 6 | Lv2 | 5 | 17 | [Lv], Rt | [Lv]40♂, 14♀ | 5 | 16 | Neg. | 5 | 21 | Neg. | 5.2 | 21 | Lv1 | 4.9 | 26 |
| 7 | Neg. | 4.9 | 19 | [Lv], Rt | [Lv]14♂, 5♀ | 5 | 16 | - | - | - | Neg. | 5.2 | 19 | Neg. | 4.8 | 26 |
| 8 | Lv9, [Lv]1 | 5.6 | 16 | - | - | - | - | Lv10 | 5.6 | 18 | Lv1 | 6 | 17 | Lv4 | 5.3 | 20 |
| 9 | Lv4 | 5.6 | 19 | - | - | - | - | Tc2, Lv25, [Lv]1♀ | 5.6 | 19 | Tc1 | 5.8 | 18 | Lv6, [Lv]1♂, 1♀ | 5.4 | 18 |
| 10 | Neg. | 5.2 | 15 | Neg. | [Tc]1♀, [Lv]15♂, 14♀ | 5 | 16 | Neg. | 5.4 | 15 | Lv1 | 5.3 | 16 | [Rt]1 | 5.3 | 16 |
| 11 | - | - | - | - | [Lv]2♂ | - | - | - | - | - | - | - | - | - | - | - |
| 12 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

Abbreviations: Lv - *Lissotriton vulgaris*; Tc - *Triturus cristatus*; Bb - *Bufo bufo*; Rt - *Rana temporaria*. Abbreviations in brackets refer to adults. Numbers refer to the number of individuals. K25- conductivity (µS/cm). Neg. = investigated with no finding,- = not investigated/no water sample taken

to 0.5 pH units - from May to July, and a small decrease was seen thereafter.

## DISCUSSION

### Methodology agreement and discrepancy

Using funnel traps and nets, the smooth newt was found in 11 of the 12 ponds included in our study, while GCN was observed in four ponds (#2, #3, #9 and #10). eDNA from GCN was only detected in two (#2 and #3) of those four ponds. On the other hand, its eDNA was detected in samples from two additional ponds (#1 and #8), as well as in a combined sample comprising three discrete ponds (#5, #6 and #7), i.e. from ponds where the species was not detected by the two other methods. The detection of GCN by eDNA analysis in ponds where it was not detected by other methods demonstrate the sensitivity of eDNA analysis. Three replicates of all eDNA analyses were used in this study in order to avoid false negatives where species detection probability is low, e.g. because of few individuals in the population. Suggestions of how many replicates to use in such studies varies depending on environmental factors and population size. Ficetola et al. (2015) suggest using up to eight replicates, eventually combined with several eDNA extractions, to avoid false negatives where detection probability is low. In our study, all eDNA analysis replicates were found positive for GCN in ponds where this species was detected by traps or net, while fewer than the three replicates were positive where other methods of detection failed. One could speculate that including a higher number of replicate analyses might have led to the detection of GCN in some of the ponds that were eDNA negative in this study. However, a higher probability of false positives should be taken into consideration in such an analysis scheme. Negative result for traps and nets may be due to the fact that the animals were scarce and so had a low probability of being caught, stayed too far from the shore, or in too deep water so that they were out of reach of traps and nets. However, the negative test with eDNA for the two ponds where the species was detected by traps and nets give rise to speculation: in one of these ponds (#10), only one adult (female) was found in May, but reproduction was not detected. As this pond is situated close to the two ponds where GCN was found in 2015, maybe only a few stray animals were present, releasing too little eDNA into the water for detection. A low abundance and a more restricted spatial movement of the newts during the spawning period could be the reason why we did not detect GCN eDNA in this pond. As shown by Buxton et al. (2017) various environmental factors, population size and breeding and larval development, affect the amount of

**Table 2**. Quantitative PCR (qPCR) tests for various amphibian species and the chytrid fungus *Batrachochytrium dendrobatidis* from 12 ponds in Norway. Each sample was tested three times, where one or more positive replicates were considered a positive eDNA detection. Each + or – represents one replicate. The positive samples had a Ct-value (cycle threshold) between 29 and 40. '+' eDNA detected (positive sample);'-' eDNA not detected (negative sample)

| Pond no. & location | *L. vulgaris* | *T. cristatus* | *R. temporaria* | *R. arvalis* | *B. bufo* | BD |
|---|---|---|---|---|---|---|
| 1. Spjøstjenn | + + + | - - + | + - - | - - - | + + + | - - - |
| 2. Kalvåstjenn | + + + | + + + | - - + | - - - | + - + | - - - |
| 3 .Grønbergtjenn | + + + | + + + | - - - | - - - | + + + | - - - |
| 4. Igletjenn | - - - | - - - | - + + | - - - | + + + | - - - |
| 5., 6., & 7. Rossmyra combined | + + + | + - + | - + + | - - - | + + + | - - - |
| 8. Stemtjern East | + + + | + + - | Not measured | - - - | + + - | - - - |
| 9 .Stemtjern West | + + + | - - - | + - + | - - - | - + + | - - - |
| 10. Little Grønbergtjenn | + + + | - - - | - + - | - - - | + + + | - - - |
| 11. Torgrimsmyr | + + + | - - - | - - - | - - - | + + + | - - - |
| 12. Bjørnåsen | + + + | - - - | + + + | - - - | - - + | - - - |

eDNA released into the water. In the other ponds that were negative for GCN eDNA (Stemtjenn West, #9), GCN larvae were found on two occasions but only at the same place just 15 meters upstream from its adjacent pond (Stemtjenn East, #8), which was eDNA-positive for GCN. We speculate that perhaps the eDNA had drifted the few meters from the western to the eastern pond.

The smooth newt was found in all 11 ponds investigated with traps and/or nets. eDNA was found in all samples except in the one taken from the most acidic pond in this investigation (which is probably not suitable for reproduction, see below), showing a high degree of agreement between the survey methods.

At their breeding sites, tadpoles of toads and frogs are normally present in greater numbers than larvae of the newts, and their presence is easily detected using traps and nets during spring and early summer. Despite this, only three anuran breeding sites were found. The low recording rate with funnel traps and nets is in sharp contrast to the detection of common toad eDNA which was found in all the ponds investigated and common frog eDNA that was present in seven of the nine samples taken. A potential explanation for recording eDNA from these species in ponds where no larvae was collected, might be that adult common toads and common frogs moved around in the area, on land as well as in the ponds, and although not successful in breeding left cells that were detected by eDNA-analysis.

Several studies have shown that eDNA analysis is more sensitive than other survey methods when it comes to detecting aquatic species (Sard et al., 2019; Hallam et al., 2021). This might partly be an explanation for some of our findings where eDNA recorded the presence of a given species while the species was not detected with traps or nets. Especially when the given species is rare at a location, eDNA analysis might be the most efficient method. Biggs et al. (2015) demonstrated that GCN was significantly more efficiently recorded by eDNA analysis compared to standard methods as bottle traps, torch counts and egg searches.

The moor frog was not found in this study by either traps, nets or eDNA, giving good agreement between these methods.

Use of eDNA is a sensitive method for detecting animals in nature when they are otherwise hard to observe. This was the case in our study where we detected eDNA of the common frog and the common toad when hardly observing them in the wild. Nevertheless, the use of eDNA has its uncertainties. The risk of false positive eDNA detection due to contamination or unspecific amplification is a concern worth acknowledging. Negative controls and positive controls were included in all our qPCR runs (except for positive control for BD), and the species specificity of the qPCR assays has been validated in other studies and found to be good. It is also possible that the eDNA in some of our samples could have derived from dead amphibians or animal faeces containing amphibian-DNA.

The eDNA analysis used here does not contribute to any quantitative measurements, nor does it give any information concerning the life stage of the individuals. Thus, ideally to maximise the amount of information collected, eDNA analysis should be used in combination with methods such as trapping and netting.

**Amphibians and water acidity**

The conductivity measurements showed very low values (and most often below 20 µS/cm), which is normal for waters influenced by peat bog. The acid-binding capacity is very poor and explains the very low pH values measured in this survey. All ponds are situated above the marine level and were influenced by peat bogs. The peat (*Sphagnum* spp.) acts like an ion exchanger, by removing $Ca^{++}$ from the water in exchange for $H^{+}$, which reacts with water to form acid substances. In 2015, smooth newts were found in 8 of the 10 ponds included in our updated study, while the investigation in 2021 found individuals of the newt in

all these ponds. Reproduction (evidenced by the presence of larvae) was confirmed in eight of the ponds, and the two ponds apparently without larvae were among the most acidic: Pond #4, where water was at pH 4.7–5.0, and pond #7 where it was pH 5.0–5.2. The water in these ponds was probably too acidic for larvae development. GCN was detected at two new locations, one of which yielded an adult using traps. Despite great effort, larvae were not found at this locality, and we assume that the water was too acidic for the species to reproduce. In the other pond, larval GCN were found at pH 5.6–5.8, which is quite similar to the acidity of two ponds in which the species was found in 2015. Those observations are in line with previous findings (Strand, 2002; 2010).

The number of larvae detected roughly depends on water acidity: in the ponds with pH above 5.5, several larvae of the smooth newt were found, while at lower pH, only one was detected. Another factor which might play a role is the temperature of the surface water. We think that the somewhat meagre larvae detection in June and July might be due to high temperature of surface water, which can drive the larvae to deeper, colder and more oxygen-rich water. In August, after heavy rainfalls, the surface water had cooled, and larvae of the smooth newt were found in two more ponds.

A study by Seymour et al. (2018) found that water as acid as pH 5.3 accelerated the decay of lotic multispecies eDNA. The acidic waters in our study area do not seem to have degraded the eDNA to undetectable levels, as we detected amphibian eDNA in water at pH 4.7–5.0.

In conclusion, reproduction of the newts in Gjerstad is highly restricted by acidic water. GCN was still present in the two original ponds and was found in two additional ponds using traps and nets. However, GCN eDNA was detected in two samples from localities for which the results were negative with traps and nets, and vice versa; was not detected in samples from two ponds where GCN was actually caught by traps and nets. This disagreement between the methods is in contrast to the high agreement between the methods for the smooth newt (which was detected in nearly all ponds) and the moor frog (which was not detected in our study). For the common frog and the common toad, which were found in only a few ponds using traps and nets, and whose eDNA were detected in (almost) all samples, the sensitivity of the eDNA-method makes it better suited to detect a species if only few individuals are present. Since eDNA analyses does not give us information about life stages, sex ratios, body conditions etc., it should ideally be combined with trapping and netting for best results.

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