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Ultrasonography as a minimally invasive method to assess pregnancy in the fire salamander (*Salamandra salamandra*)

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Ultrasound is an important tool for non-invasive assessment of pregnancy. It has been used in many species under wild and captive conditions (e.g., Haibel 1990, Hildebrandt et al. 2000, Hildebrandt et al. 2006, Martínez-Torres et al. 2006), but only few studies have been conducted on amphibians, as for instance, in the American toad, *Bufo americanus* (Johnson et al. 2002). Reproduction in amphibians is highly diverse. Urodelan amphibians display a wide range of reproductive strategies (Wells 2007), including oviparity (depositing eggs), viviparity (giving birth to fully metamorphosed terrestrial offspring) as is the case in the Alpine salamander (*Salamandra atra*) (Schorn & Kwet 2010, Riberon et al. 2001, Häfeli 1971) and in some fire salamander populations (Dopazo & Alberch 1994, Velo-Anton et al. 2012), and even ovo-viviparity (deposition of aquatic larvae) as in the fire salamander (*Salamandra salamandra*; Greven 1998, Thiesmeier 2004, Schorn & Kwet 2010). In early spring, fire salamanders deposit their larvae in small streams or ponds (Thiesmeier 2004, Steinfartz et al. 2007), after which the mating period begins. After hibernation during the winter, females start to deposit their larvae in spring again (Thiesmeier 2004). Despite this typical reproductive cycle, some female fire salamanders (*S. salamandra*) have been found to deposit their larvae already in autumn (Thiesmeier 2004).

Fire salamanders are frequently studied amphibians (e.g., Thiesmeier 2004, Schorn & Kwet 2010), and many studies on these animals are conducted on free-ranging individuals in the field (e.g., Bar-David et al. 2007, Schulte et al. 2007, Schmidt et al. 2007, Steinfartz et al. 2007, Lötters et al. 2012, Reinhardt et al. 2013). However, due to its specific reproductive mode, i.e. ovo-viviparity, fire salamanders are also suitable organisms for studies under seminatural or laboratory conditions (e.g., Weitere et al. 2004, Blaustein et al. 2006, Caspers et al. 2009, Segev et al. 2011, Caspers & Steinfartz 2011), as larvae can even be obtained from pregnant females in the laboratory (e.g., Cohen et al. 2005, Warburg 2009, Krause et al. 2011). However, assessing pregnancy in fire salamanders is quite difficult and so far relied mostly on a relatively subjective assessment by the observer, i.e., mainly on body condition parameters of females (e.g., body mass and belly shape). In the period from 2009 to 2012, we collected 117 potentially pregnant females in the wild and brought them temporarily to the laboratory for larval deposition, but only 39 of them (33.33 %) eventually deposited larvae under captive conditions. Thus, alternative or additional methods are needed to effectively assess the reproductive status in fire salamanders for experimental studies that require pregnant fire salamander females or for quantifying the reproductive status of individuals within a study population. We here introduce ultrasonography as an additional method for assessing pregnancy in this species.

For the examination of potentially pregnant females, we employed a real-time portable ultrasound unit, MyLab™ One (Fig. 1), which can be used in the laboratory as well as in the field. For performing the ultrasonography, coupling gel was used on the belly of the females and although it might be resorbed through the skin, no negative effects are known for amphibians (Schildger & Triet 2001). So far, ultrasonography is used in amphibians mostly for clinical diagnostics (Mutschmann 2010, Schildger & Triet 2001), as it is capable of picturing, for example, heart, liver, stomach and various other organs (Schildger & Triet 2001). At the time of this study, we had two possibly pregnant female fire salamanders available for inves-

tigation. These were the last two animals available from our field collections made in the Kottenforst, Germany (50°41'09" N, 7°07'03" E). For the purpose of larval deposition, they were housed individually in transparent Fauna™ boxes (41 × 14 × 18 cm) with a water compartment and stones and bark to hide under. Specimens were fed once a week with crickets and earthworms. For husbandry details see Caspers & Steinfartz (2011). Experiments were carried out in compliance with German laws for experimentation with live animals, and the salamanders were collected from the field and held captive for the course of the experiments with respective permits from the Untere Landschaftsbehörde der Stadt Bonn. All females were released after the experiments at the location where they had been captured originally.

We examined the ventral posterior region of the specimens, since larvae develop in the posterior region of the oviduct (Francis 1934). This 'uterine' region of the oviduct grows considerably in volume and length during pregnancy (Francis 1934). During the ultrasonography, one person gently held the female immobile in his/her hands and a second person placed the ultrasound scanner on the ventral body surface and moved it carefully over the belly. We kept the animals for at least six more months in the lab, and the procedure caused them no direct or lasting harm.

No larvae were detected in one female, for which reason we classified her as not pregnant. In the second, we clearly detected larvae (Fig. 2), which were readily identifiable by their skeleton and movement relative to the background.

To our knowledge, this is the first report on using ultrasonography to assess pregnancy in fire salamanders. The standard method of collecting pregnant females was based purely on body conditions, but it can definitely be improved by the ultrasonography approach, as it clearly enabled us to detect larvae at a progressed stage of pregnancy. Ultrasonography as applied in our case might be also a tool to unambiguously determine pregnancy in females of other salamandrid species with a fully internal viviparous mode of reproduction and development of juveniles, such

Figure 1. Photograph of the real-time portable ultrasound unit, MyLab™ One. For documentation, pictures can be saved as *.bmp files and exported from the device via a USB interface. Photo: Esaote.

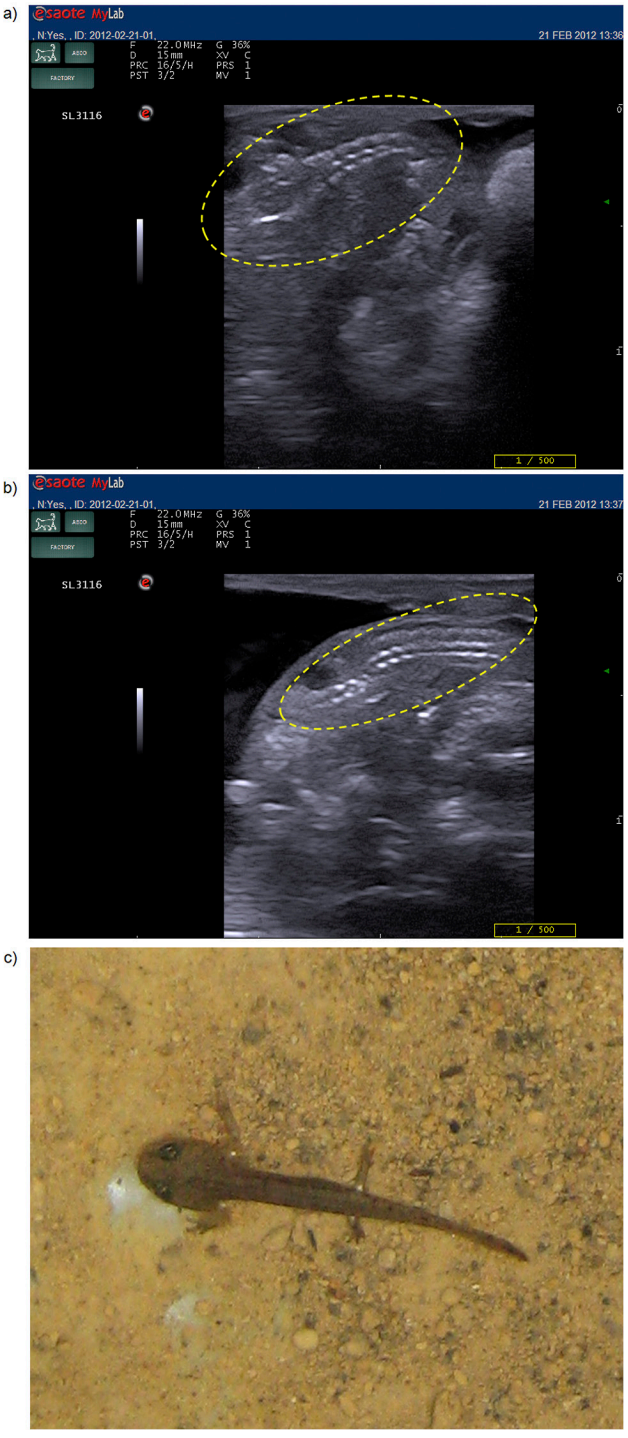

Figure 2. Two images (a, b) from the ultrasonography investigation, showing the structures of larvae inside the mother (indicated by yellow circles), and image (c) of a larva on the day of deposition.

as alpine salamanders (*S. atra* and *S. lanzai*) as well as *Lyciasalamandra* species.

Nonetheless, it remains unclear whether fertilized eggs or embryos at early developmental stages can be detected by ultrasonography. Accordingly, a case of non-detection with our method does not necessarily rule out pregnancy of a female if she is at an early stage. At which developmental stage this technique can detect pregnancy unambiguously in fire salamanders remains to be addressed in future experiments that are based on larger sample sizes. Furthermore, the detection of pregnancy certainly does not assure that these females will deposit their larvae under lab conditions. It may be possible that they retain them for a long period *in utero* or even absorb them. After being transferred to the lab, females are able to store sperm, fertilized eggs or larvae for at least two years *in utero* without larvae deposition (B. A. Caspers, E. T. Krause, unpubl. obs.). From this perspective, ultrasonography can therefore also be used to investigate reproductive strategies in amphibians, which are known to be highly variable between, and even polymorphic within, species, with ovipary and vivipary and individuals that may give birth to either aquatic or fully metamorphosed larvae (Häfeli 1971, Luiselli et al. 2001). Moreover, ultrasonography might allow to investigate the prenatal mechanisms (initial offspring number or embryo resorption) that cause variation within and between populations in numbers of juveniles produced per female (e.g., in the Alpine salamander; Häfeli 1971, Luiselli et al. 2001) and thus help to better understand reproductive strategies underlying population dynamics (Helfer et al. 2012).

In conclusion, we were able to demonstrate with the present pilot study that ultrasonography can be used to ascertain pregnancy at a progressed stage in fire salamanders. Future studies should assess whether it is also possible to use this method to collect quantitative data on the number of larvae and their antenatal development in the maternal oviduct. Females can easily be examined in the field, due to the small size and weight of a portable ultrasound unit. This extends the possibilities to investigate the reproductive cycle and its plasticity in amphibians, right in the field with only a minor impact on individuals. It will also increase the efficiency in selecting only the desired females for laboratory studies. We conclude that ultrasonography promises to be a useful and minimally invasive tool for future studies in salamander reproduction and life history both in the laboratory and in the field.

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