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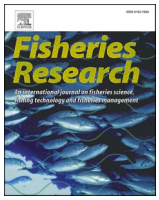

Effect of formalin fixation on size and weight of Atlantic wolffish (*Anarhichas lupus*) and spotted wolffish (*Anarhichas minor*) oocytes

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ABSTRACT

Oocytes were sampled from Atlantic wolffish (*Anarhichas lupus*) and spotted wolffish (*Anarhichas minor*) to investigate the effect of fixation and storage in formalin on oocyte size and weight over time. The effect of formalin on oocyte size was dependent on the original size of the oocyte. As the effect of fixation was similar for the two species, a common formula for the conversion of fixed oocyte size/weight to fresh oocyte size/weight was developed. The relationships were negative and nonlinear, the smallest oocytes increased ~11 % in diameter and ~15 % in weight when fixed in formalin, whereas the largest oocytes decreased about 4 % in diameter and 7 % in weight. After fixation, oocyte diameter was stable for up to 2, but not 10 years and for up to 10 years for oocytes at CA and VIT stage, respectively.

1. Introduction

There is an increasing focus on Stock Reproductive Potential (SRP) in fisheries assessment (Trippel, 1999), but for many species the required knowledge on the traits used to estimate SRP is lacking. Studies investigating these traits often sample fish gonads in the field, which are placed in preservatives for later laboratory examination. However, fixation of ovaries/oocytes in preservatives are known to impact oocyte size and(or) weight, therefore, to get information on the original size or weight of the preserved material, the effect of preservation should be known. There are a number of difficulties associated with this as the magnitude of change is specific to the preservative used, the species in question, and the developmental stage of the oocytes (Fleming and Ng, 1987; Frimpong and Henebry, 2012; Heins and Baker, 1999; Kjesbu et al., 1990; Klibansky and Juanes, 2007; Tan-Fermin, 1991). In addition, data on the effect of fixatives on fresh material is also sparse, likely due to logistical difficulties associated with measuring oocytes close to the time in which they are removed from the fish. With a limited number of previous studies and influencing factors on the impact of fixatives on gonadal tissue, the effect needs to be investigated on a case-by-case basis.

Atlantic wolfish (*Anarhichas lupus*) and its close relative, spotted wolffish (*A. minor*), are common demersal species in North Atlantic (Barsukov, 1959). These two species share many reproductive traits, with both being determinate, total spawners, which produce demersal eggs that are guarded by the male (Keats et al., 1985; Pavlov and Moksness, 1995; Tveiten and Johnsen, 1999). Both species halt oocyte development at the cortical alveoli (CA) stage for several years before spawning for the first time (Gunnarsson et al., 2008, 2006). After true vitellogenesis begins, ovary development of both species takes about 5–6 months (Barsukov, 1959; Tveiten and Johnsen, 1999) with spawning taking place from late summer to early winter for *A. lupus* and from midsummer to early winter for *A. minor* (Barsukov, 1959; Gunnarsson et al., 2016, 2008; Jónsson, 1982; Østved, 1963; Pavlov and Novikov, 1993; Templeman, 1986a, 1986b).

Just before spawning, three cohorts of oocytes are present in the ovary of *A. lupus* and *A. minor*: the vitellogenic (VIT) oocytes which will be spawned at the next spawning opportunity, oocytes at the CA stage that will be spawned the following year and primary oocytes that will be recruited to CA stage next year (Beese and Kändler, 1969). CA oocytes are typically 0.5–1.8 mm, while VIT oocytes vary from 2 mm to 6.5 mm, (Falk-Petersen and Hansen, 2003; Mazhirina, 1988; Pavlov and Novikov, 1993; Tveiten and Johnsen, 1999).

The most common fixative currently used in the reproductive biology of fishes is formalin. The aim of this study was to evaluate the effect, on diameter and weight of CA and VIT oocytes of long term storage in

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formalin in group, isolated oocytes and gonads of *A. lupus* and *A. minor*. These results can then be utilised in future studies on the reproductive biology of these species.

2. Material and methods

2.1. Sampling

In 2002 and 2009, oocytes from 27 *A. lupus* and 7 *A. minor*, which were caught in the commercial fishery in Icelandic waters, were sampled to investigate the effect of fixation in formalin on their diameter. Immediately after capture, the fish were put in ice and oocytes samples taken within 24 h. The aim was to collect 100 oocytes from each oocyte stage (CA and VIT) in each sampling occasion. To provide a wide range of oocyte sizes, ten oocytes were collected per gonad (fish). However, ten gonads were not always available to collect 100 oocytes, in such cases, except for the first sampling, replicates of 10 oocytes were taken from the gonads (Table 1). Oocyte samples were taken from the middle of the right gonad of each fish, photographed with Leica image Q500 MC and stored in 1.5 mL tube with 4 % formalin buffered with borax (3 g per liter of 4 % formalin).

In 2002 and 2009, a sample consisted of 10 oocytes that were stored in a single tube, in a few cases, the oocytes were so large that it was necessary to use two tubes to fulfil the adequate proportion in volume between sample (1/3) and preservative (2/3), but for simplification each of these two tubes were treated as a single sample. In 2002, a single sample of 10 oocytes was taken from each fish. In 2009, 7 fish were sampled with two replicates (i.e., 14 samples), 2 fish were sampled with 3 replicates, and 2 fish were sampled with 5 replicates.

To examine the effect of fixation in formalin on oocyte weight, two approaches were taken, 1) the preservation of oocytes individually and 2) the use of data that was originally used for the estimation of fecundity. For the effect of fixation on oocytes preserved individually, samples were taken exclusively from commercial landings and measurements of oocyte weight was performed in the laboratory. We aimed to collect a minimum of 80 oocytes, 20 oocytes for each species and maturity stage (Table 1). To achieve the 80 oocytes per species and stage, multiple samples of oocytes were sometimes taken from the same fish. In the case of individually preserved oocytes, one sample consists of one oocyte preserved individually in a 1.5 mL tube of formalin. Determination of development stage, either CA or VIT, was done macroscopically based on the maturity scale from Gunnarsson et al. (2006, 2008). For approach two, this provides information on change in weight when the oocytes are preserved as whole ovary. Data on 418 gonads from spotted and Atlantic wolffish that were originally sampled from commercial landings and scientific surveys (Gunnarsson, 2017) were used. Of these gonads, 311 were sampled in 2002–2006 from Atlantic wolffish and 107 from spotted wolffish in 2006–2010.

2.2. Measurement of oocytes

In the laboratory, following initial fixation, the oocytes were periodically (Table 1) taken from the tube and placed in a strainer and washed with water before being put on petri dish, photographed in a similar manner as prior to fixation (see above), and immediately returned to the formalin. This process took about 2–3 min for the group of ten oocytes and about 1 min for the single oocyte. The images of the oocytes were analysed using SigmaScan Pro 5. The oocytes of *A. lupus* and *A. minor* are generally spherical, however the average of the major and minor axes of each oocyte was taken as the oocyte diameter.

In 2019, in the laboratory, after the oocytes had been photographed for the measurement of diameter, individual oocytes at both VIT and CA stage were dried with paper towels before being weighed with a Mettler Analytical Balance AE240 (precision of 0.0001 g). The weighing of these individual oocytes was repeated on three occasions, up to 216 days after initial fixation (Table 1). The weights of the CA oocytes were excluded from the statistical analysis as most of the fresh oocytes at CA stages were in the range 0.0005–0.0010 g. As the precision of the balance was 0.0001 g, the data was not considered appropriate for examining changes in weight.

The gonads, which had previously been used for fecundity estimation (Gunnarsson, 2017), were weighed fresh (± 1 g) then fixed in formalin. After they had been stored in formalin for between 0.5 and 5 years, the number of oocytes in the ovaries was estimated gravimetrically. To estimate the proportion of non-oocyte components of total weight of fresh gonads, the ovary of 20 spotted wolffish were used. To do this, the ovary wall was cut longitudinally and flattened out, the oocytes were then scraped with the back of a knife to remove them from the ovary wall. After all the oocytes had been removed and weighed (± 0.1 g), the remaining tissue was weighed (± 0.1 g). Oocytes accounted for an average of 95.5 % of the ovary weight. Initial average weight of a fresh oocyte was estimated using the equation:

Average weight of fresh oocyte = (fresh ovary weight - weight of non-oocyte component)/fecundity·

During the estimation of fecundity, all the oocytes were removed from the ovary and weighed, the average weight of a fixed oocyte could be estimated using the equation:

Table 1
Species, sampling date, sampling number (S), number of oocytes stored in each tube, number of fish sampled at cortical alveolus (CA) and at vitellogenesis stage (VIT) and the total number of oocytes sampled. Some fish were sampled multiple times, hence the number of oocytes in tube multiplied with the number of fish does not always equal the number of oocytes. Zero days in formalin is when the oocytes were sampled and measured fresh and thereafter immediate put in formalin. The number in the parenthesis for each sample (S) represents the numbers of the equations in this study, showing which data each equation is based on. In Eqs. 2, 3 and 4 only oocytes at VIT stage were used.

				CA		VIT		
Species	Date	S	n oocytes in tube	n fish	n oocytes	n fish	n oocytes	Days in formalin
A. lupus	26.09.2002	1 (1)	10	7	70	3	30	0, 3, 30
A. lupus	03.12.2002	2 (1)	10	10	100	–	–	0, 4, 30
A. lupus	17.09.2009	3 (1)	10	–	–	4	100	0, 4, 57, 275, 405, 769, 3624*
A. minor	04.06.2009	4 (1)	10	2	100	5	100	0, 4, 57, 229, 385, 744, 3701*
A. minor	22.08.2019	5 (1)	10	10	100	14	140	0, 9, 31*
A. minor	22.08.2019	5 (1, 2, 3, 4)	1	10 * *	20	14 * *	28	0, 9, 31, 216 * **
A. minor	17.09.2019	5 (1)	10	–	–	2	20	0, 7, 38*
A. minor	17.09.2019	5 (1, 2, 3, 4)	1	–	–	2 * *	4	0, 7, 38, 182 * **
A. lupus	12.09.2019	5 (1)	10	5	100	5	100	0, 7, 45*
A. lupus	12.09.2019	5 (1, 2, 3, 4)	1	5 * *	20	5 * *	20	0, 7, 45, 182 ** *

*Final measurement was only done for oocytes at VIT stage.
**Same fish as in the row above.
*** At the latest date, the oocytes were only weighted.

Average weight of fixed oocyte = total weight of fixed oocytes / fecundity

To examine if the shape of the oocytes changed after fixation, aspect ratio (minor axis/major axis) was calculated for the single oocytes, fresh and after fixation (last size measurement, Table 1). This was also done for the ten oocytes in Fig. 1.

2.3. Statistical analysis

The effect of storage in formalin on the egg diameter was analysed, with all size measurement data (single and 10 oocytes), using a linear mixed effects model of the form:

$$\Delta m_{dsfvir} = \alpha_{ds} + \beta_{ds} m_{dsfv.0} + \gamma_{ds} t_{dsfvr} + \tau_r + \phi_{dsfv} + \omega_{dsfv|i} + \epsilon_{dsfvir}$$

where $\Delta m_{dsfvir} = m_{dsfvir} - m_{dsfv.0}$ is the difference between individual measurements of oocyte i from species s, development stage d, fish f in vial v from the average measurement prior to treatment repeated at timestep r. t_{dsfvr} is the number of days in the vial and α, β and γ denote the interaction effects between species and development stage on the average, initial size and number of days respectively, and τ_r is the effect of long-term storage (more than 10 years). The effect of fish and vials by fish were considered as random effects $\phi_{dsfv} \sim N(0, \sigma_\phi^2)$ and $\omega_{dsfv|i} \sim N(0, \sigma_\omega^2)$ respectively. $\epsilon \sim N(0, \sigma^2)$ is the i.i.d model residual.

The effect of storage on weight of individual oocytes at VIT developmental stage was analyzed using a linear model of the form:

$$\Delta w_{sfvr} = \eta_s + \theta_s w_{sfv0} + \kappa_s 1/t_{sfvr} + \xi_{sfvr}$$

where $\Delta w_{dsfvr} = w_{dsfvr} - w_{dsfv0}$.

The difference in weight distribution by species in the samples was tested using a Kolmogorov-Smirnov test (Birnbaum and Tingey, 1951).

The effect of storage on the average weight of oocytes stored as whole ovaries was analyzed using a model of the form:

$$log(w_{sfmy0}) = \mathfrak{a} + \mathfrak{b} log(w_{sfmy1}) + l + \mathfrak{S}_s|\mathfrak{M}_m + \mathfrak{Y}_y + \mathfrak{e}_{sfmy}$$

where w_{sfmy0} and w_{sfmy1} are the average oocyte weights before and after formalin of fish f of length l, species s and at month m and year y. The effect of species within the month $\mathfrak{S}_s|\mathfrak{M}_m$ and the year factor $\mathfrak{Y}_y$ were treated as random effects.

Model variables were selected from a maximal model in a stepwise manner using the Bayesian information criterion (BIC, Schwarz, 1978), while compared with the more lenient Akaike information criterion (AIC, Sakamoto et al., 1986). All analyses were conducted using the statistical software R (version 4.0.2, R Project 2020) using the lme4 package (Bates et al., 2015).

The analysis of the logit transformed aspect ratio was based on a mixed effect model of the form:

$$logit(\rho_{smvnt}) = \pi + \lambda_{sm} + \tau_t + \nu_n + \upsilon_v + \zeta_{smvnt}$$

where ρ_{smvnt} is the aspect ratio of eggs measured at timepoint t = 'before' or 'after', of species and maturity s and m respectively and number of eggs n (either 1 or 10). The effect of glas v is treated as a random effect υ. The significance of model parameters was tested using a likelihood ratio test.

3. Results

3.1. Descriptive statistic

All measurements of size and weight of fresh oocytes were combined to describe range and mean of the oocytes used in this study. A total of 1052 oocytes were measured on several occasions in this study, the smallest fresh oocyte was 0.637 mm and the largest 6.097 mm (Table 2).

The weight of individually measured oocytes at VIT stage varied from 0.0051 to 0.0858 g. In the analysis where the total weight of the fresh gonads was used, the average weight of the oocytes varied from 0.0020 g to 0.1692 g. In all instances, the range of values was larger for *A. minor* than *A. lupus* (Table 2).

There was a significant change in aspect ratio after formalin fixation ($\chi^2(1) = 13.222, p < 0.001$), but small difference in aspect ratio between fresh and fixed oocytes or a 2 % distortion after fixation. This seems to happen shortly after fixation as there was no difference between the distortion of the oocytes which had been in formalin for a few months, two years, and 10 years.

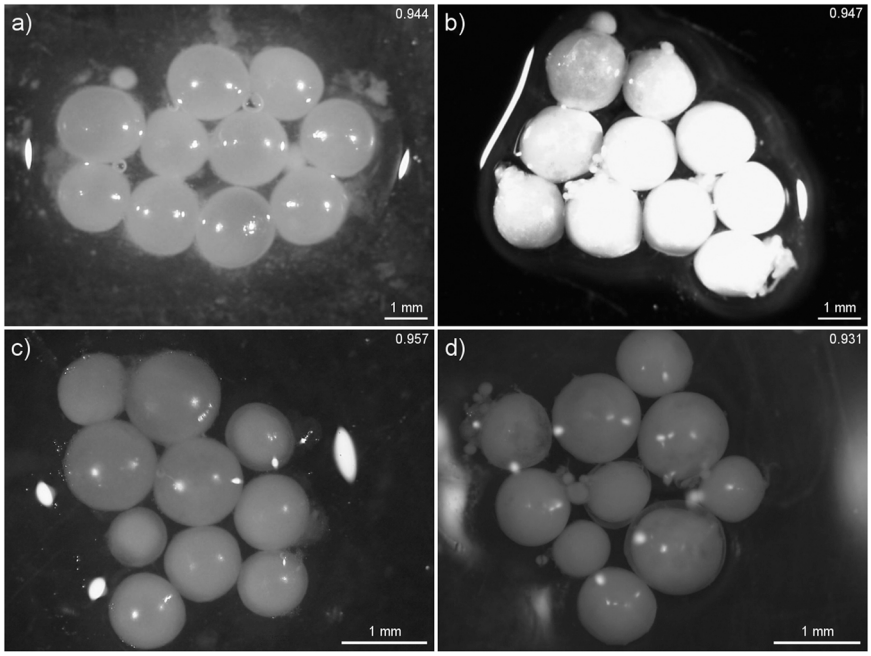

Fig. 1. Spotted wolffish oocytes at vitellogenesis stage a) fresh and b) after ten years in formalin and at cortical alveolus stage c) fresh and d) after two years in formalin. Mean aspect ratio is in the right corners in the figures. The oocytes at VIT and CA stages were collected from two fishes, both from sample 4 (Table 1).

Table 2
Range, mean ± s.d. in diameter (mm) and weight (g), for *A. lupus* and *A. minor* fresh oocytes at CA stage and VIT stage. Two datasets were used for weight measurements, one where only individual oocyte was used, the other was based on average weight of oocytes from fecundity estimate (gonad).

		CA			VIT		
	Species	Range	Mean	n	Range	Mean	n
Diameter	*A. lupus*	0.637–1.244	0.941 ± 0.116	290	3.272–5.309	3.914 ± 0.513	250
	A. minor	0.750–1.786	1.200 ± 0.190	220	2.004–6.097	4.068 ± 0.980	292
	Combined	0.637–1.786	1.0535 ± 0.1991	510	2.004–6.097	3.997 ± 0.801	542
Weight individual	*A. lupus*	0.0002–0.0012	0.0006 ± 0.0003	20	0.0153–0.0269	0.0204 ± 0.0029	20
	A. minor	0.0005–0.0016	0.0011 ± 0.0003	20	0.0051–0.0858	0.0447 ± 0.0180	32
	Combined	0.0002–0.0016	0.0008 ± 0.0004	40	0.0051–0.0858	0.0353 ± 0.0185	52
Weight gonad	*A. lupus*				0.0020–0.1293	0.0264 ± 0.0224	311
	A. minor				0.0061–0.1692	0.0377 ± 0.0341	107
	Combined				0.0020–0.1692	0.0293 ± 0.0263	418

3.2. Effect of formalin on oocyte size

The analysis of the effects of formalin on size measurements was based on all data (Table 1), where measurements prior to treatment were treated as reference to calculate ΔD_{dsfvir}, the difference between the original size D_0 and size in formalin D_1. After model selection, the model with the lowest BIC value was the model that omitted the interaction between the effects of species and development stage ($\alpha_{ds} = \alpha = 0.11722$) and the effect of the initial size $\beta_{ds} = \beta = e\ -0.06067$. Other effects, such as the effect of time in particular, τ_r, that is the effect of long-term storage (about 10 years) did not improve the BIC score of the model. In comparison, the AIC score for this best model was only slightly higher than that of the model with lowest AIC score while having three fewer parameters. In addition, no significant differences were detected between the measurements of the individual oocytes and those stored in groups of 10 ($\chi^2_1 = 0.44, p = 0.833$). The conversion formula from this analysis was (Table 3).

$$D_0 = -0.1172 + 1.0646 D_1 \quad (1)$$

Where D_0 is the original size of the oocyte and D_1 its size in formalin. According to this equation the smallest oocyte at CA stage is estimated to increase in size about 11 % in formalin, where the largest oocyte at CA stage is estimated to increase about 0.1 %. The smallest oocytes at VIT stage estimated decrease is about 0.6 % and the largest 4.3 % (Table 2). Thus, the diameter of oocytes at CA stage is estimated to increase in formalin, while oocytes at VIT stage decrease (Fig. 2a).

3.3. Effect of formalin on oocytes weight

Only oocytes at VIT stage were used in this analysis. Two analysis were conducted to estimate the effect of fixation and storage time in formalin on weight of oocytes of *A. lupus* and *A. minor*. The first analysis concerned single oocytes which were measured on three occasions during their storage in formalin. The best model, according to the BIC, used neither the fresh weight of the oocytes nor time in formalin to explain the difference between weights of fresh and fixed oocytes, but species difference was however included, see Eq. 2 for *A.lupus* and 3 for *A. minor* (Table 3).

Table 3
Estimate of coefficients ± s.e. in the equation in this study and *P* value. See statistical analysis for further details for the coefficient.

Equation nr.	Coefficients	Estimate	*P*
1	α	0.1172 ± 0204	< 0.001
1	β	0.0646 ± 0062	< 0.001
2	η	-0.0041 ± 0.0003	< 0.001
3	η	-0.0026 ± 0.0002	< 0.001
4	η	-0.0032 ± 0.0002	< 0.001
5	a	0.1617 ± 0.053	0.008
5	b	1.0547 ± 0.0074	< 0.001

$$W_0 = W_f - 0.004129 \quad (2)$$

$$W_0 = W_f - 0.002570 \quad (3)$$

Where W_0 is the weight of fresh oocyte and W_f its weight in formalin. However, there was a significant difference in the weight range of these two species (D = 0.86, p < 0.01), and the relationship between the weight of fresh oocytes and in formalin seems to be similar between these two species at same weight range (Table 2 and Fig. 3). Therefore, it was decided to exclude the species effect from the analysis, see Eq. 4 (Table 3).

$$W_0 = W_f - 0.003153 \quad (4)$$

According to this analysis the difference in weight between a fresh and fixated oocyte is a constant and neither initial weight of the oocytes nor time after fixation has any effect.

The second analysis was based on measurements from the whole ovaries. The best model, based on the BIC score, for the effects of formalin on the average weight of an oocyte stored as a whole ovary was model that included the log (w) as a linear term ($\chi^2_1 = 1628, p < 0.01$), the monthly random effect $\mathfrak{M}_m$ and annual random effect Y_Y:

$$W_0 = 1.173 \times W_f^{1.05} \quad (5)$$

According to Eq. 5 the lightest oocytes increased about 15 % in formalin and the heaviest decreased about 7 % in formalin (Fig. 2b, Tables 2 and 3).

4. Discussion

This study demonstrates that changes in size of oocytes preserved in formalin of *A. lupus* and *A. minor* is dependent on its original size and weight. Common formulas, one for diameter and another for weight, were found which can estimate the original size of oocytes in formalin for these two species. Both these formulas showed negative nonlinear relationship between fresh size or weight and change in formalin. According to the formula for diameter, most of the oocytes at CA and VIT stages increase and decrease respectively in formalin.

Regarding changes in the weight of the oocytes after preservation in formalin, the two methods utilised in this study (oocytes preserved individually and preservation of the whole ovary) produced differing results. When the ovary was preserved whole, the initial weight influenced the change in weight, whereas when the oocytes were preserved individually, the initial weight had no influence on the change. The reason for this could be statistical, as a greater number of samples in the analysis where ovaries were stored whole which covered a greater range in oocyte weights (0.0020–0.1747 g, n = 411) than where oocytes were stored individually (0.0051–0.0858 g, n = 52). Further the effect on weight on single oocytes and ovary when fixation in formalin was similar (Fig. 3).

In the present study, the smallest oocytes gained weight in formalin

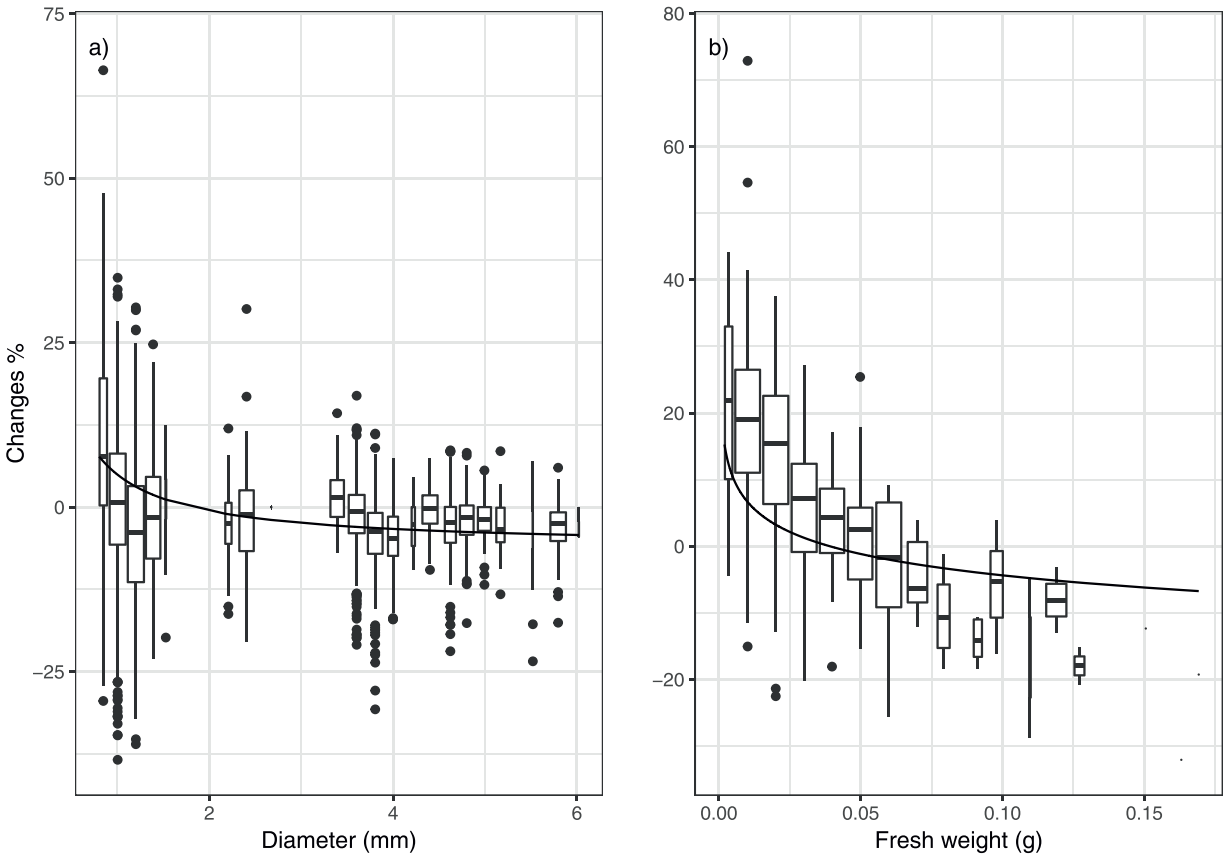

Fig. 2. Change in % in diameter and average weight of oocytes stored as whole ovaries, before and after being preserved in formalin. The black lines represent predicted values based on Eq. 1 on a) and Eq. 5 on b).

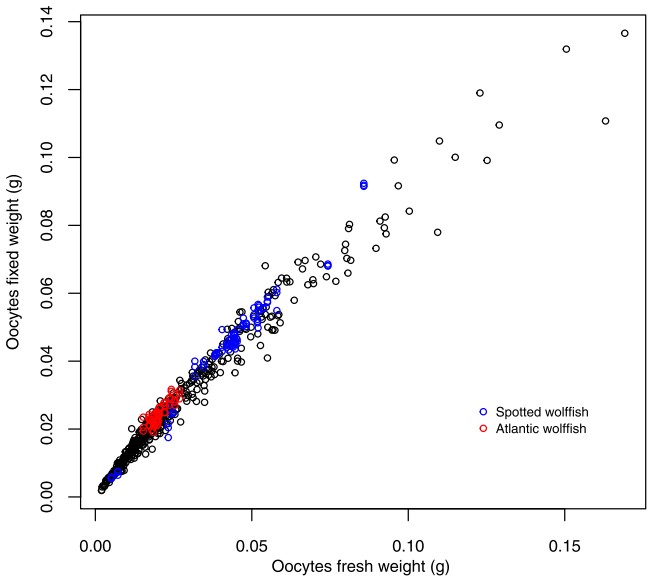

Fig. 3. Relationship between fresh weight and weight in formalin of oocytes of Atlantic wolffish and spotted wolffish. Each oocyte was weighted on three occasions in formalin with the fixed weight referring to the first measurement for each sample. The black circles represent the data from ovary sampling, previously used in fecundity estimation.

whereas the larger oocytes lost weight. The reason for this is likely the changing composition of the oocytes as they develop. Osmosis is the main process which mediated the changes in size or weight of fresh oocytes after fixation in formalin. Osmosis is influenced both by the concentration gradient of solutes between the inside of the oocytes and the formalin, and the permeability of the oocyte's membrane. Therefore, changes in weight/size are specific to tissues involved e.g., some organs of rabbit increase in weight when fixed in formalin while others decrease and anchovy larvae generally decrease in size when fixed formalin, but their eyes increase in size (Fraser, 1985; Theilacker, 1980). As the oocyte moves from the cortical alveoli stage, yolk production switches from an endogenous process taking place within the oocyte, to exogenous process where the oocyte absorbs the vitellogenin transferred from liver via the blood to the oocyte (Wallace and Selman, 1981). As an oocyte develops, the composition and concentration of solutes within the oocyte will vary as well as the volume of the oocyte; the volume of largest oocyte in this study was 877 times larger than the volume of the smallest oocyte and its surface 92 times larger (Table 2). The permeability of the oocyte membrane also changes during the development process, becoming more permeable in order to absorb vitellogenin (Tyler and Sumpter, 1996). These changes in the oocyte composition and permeability likely explain why the change in size/weight is not constant and not always negative.

Ovaries sampled for oocytes size measurement and individual weight were kept on ice for less than 24 h before being measured and placed in formalin, which may have resulted in water loss and thus have decreased in weight before the "fresh weight" was measured. The air temperature in Iceland is relatively low, in summer and winter it is rarely more than 15 °C and 7 °C respectively. In addition, the ovaries were stored on ice and storage of ovaries for 24 h on ice is known to have only a negligible impact on weight (Klibansky and Juanes, 2007). Based upon the low air temperature and being stored on ice, we consider this storage to have only a minimal impact on our study. However, this is something that should be considered in future studies, especially when

carrying out similar studies in warmer climates.

Hydration time when the fixated oocytes are taken out of formalin and washed with water before size measurements, might influence its size or weight. Regarding size this seems not to be the case, there was no difference of the effect on fixation in formalin between single and ten oocytes although the hydration time was 2–3 times longer for the ten oocytes than for the single oocytes. Similar for weight single oocytes hydration time was about 1 min but for the oocytes from the fixed gonad it was about 5–10 min. Despite this difference in hydration time there was no difference in the effect of fixation in formalin on weight between the single oocytes and the oocytes from the gonads (Fig. 3, Gunnarsson, 2017).

It was attempted to measure the circularity of the oocytes using ImageJ to examine the effect of fixation and duration in formalin on circularity. Major changes in shape would indicate poor fixation and affect the measurements of diameter if they are no longer circular. Before the oocytes were photographed, they were washed with water and put on a petri dish, however there were usually some traces of water that resulted in imageJ being unable to determine the outlines of the oocytes. As an alternative, a subset of the oocytes were examined for changes in aspect ratio which gives an indication of circularity. The influence of fixation was low with a 2 % distortion, with no effect from the duration in formalin, thus, changes in circularity was considered unlikely to affect the results.

There are only a limited number of studies which examine the impact of fixation in formalin on oocyte diameter, where the original oocyte diameter is measured. Those studies have reported no practical change (Kennedy, 2018; Óskarsson et al., 2002). Oocytes of anchovy (*Engraulis encrasicolus*) and sardine (*Sardina pilchardus*) shrunk by an average of 4.7 % and 3.6 % respectively, following one month of storage in formalin, but this was based on oocytes which had been stored in formalin for ca. 3 h before the initial measurements (Rakka and Ganias, 2015). The current study is in line with these previous studies regarding oocytes at VIT stage with a decrease of ~0–4 % in diameter. To our knowledge no study hitherto has been done on effect of formalin on oocytes at CA stage. Previous studies on the effect of formalin on oocytes diameter or weight have not incorporated original size or weight and have described the change with a single percentage increase/decrease, therefore, comparison to other studies is difficult. Svåsand et al. (1996) is the exception where oocytes of cod were showed to increase about 1–3 % in formalin depending on their original size. Oocytes of Atlantic salmon (Salmo salar) have been reported to not change in weight in formalin while ca. 250 g of gonad from cod (*Gadus morhua*) and haddock (*Melanogrammus aeglefinus*) increased about 1.4–6.8 % in weight in formalin (Fleming and Ng, 1987; Klibansky and Juanes, 2007). However, it is difficult to compare these results with current study where weight change was at the range of 22 % for oocytes at VIT stage depending on original weight of the oocytes.

After fixation in formalin, oocyte diameter of both CA and VIT oocytes was stable for at least 2 years. The final measurement of oocyte diameter of some samples was after almost 10 years in storage. After this length of time, oocytes at VIT stage were unstable and excluded from the analysis and the oocytes at CA stage had shrunk considerably and were judged to be unmeasurable. Thus, the maximum reliable storage time for oocytes at CA stage is 2 years pending further investigations. The reason that the oocytes at CA stage didn't tolerate such a long time in formalin might be due to changes in pH value. From the penultimate measurement to the last one about 8 years past, in the long-term, formalin is known to change its pH value and most likely it did in this study, but it wasn't measured (Tucker and Chester, 1984). The mean diameter of the oocytes at VIT stage in the last measurement was not different from the mean diameter from previous measurement, accordingly the reliable storage time for oocytes at VIT stage is at least 10 years. Most of the oocytes at VIT stage seemed to tolerate such a long time in formalin, but not all of them, few of them had damage before the last measurement. The samples were not attended for during the period from 2 to 10 years in formalin and in some vials most of the formalin had evaporate and in 4 vials, 1 or 2 oocytes were damaged and unmeasurable. Therefore, it is perhaps more appropriate to state that the result suggests that oocytes at VIT stage can be preserved in formalin up to 10 years without change in its diameter.

Studies looking at the stability of oocyte or egg size/weight usually encompass a total period of <1 year (Fleming and Ng, 1987; Lowerre-Barbieri and Barbieri, 1993; Rakka and Ganias, 2015). Due to logistical or economic reasons, the time between collection of ovary samples and actual analysis could vary from many months to many years. With the development of new methodologies and equipment, it may also be desirable to re-analyse historical samples, it is thus important to ensure that long term storage would not impact the resultant measurements. The current study is, to the authors knowledge, the only study examining stability over such an extended period and demonstrate that for vitellogenic oocytes, long-term storage in formalin is possible without impacting results.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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CRediT authorship contribution statement

Ásgeir Gunnarsson: Supervision and writing the draft of the manuscript as data handling, James Kennedy: Validation as writing the manuscript, Bjarki Elvarsson: Formal analysis and Methology. Writing the method section, Árni Magnússon: Formal analysis and Methology, Birkir Bárðarsson: Data handing and reviewing the manuscript.

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