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A potential antioxidant role for melatonin and AFMK in plasma, ovarian fluid, and eggs during reproduction in rainbow trout

Konrad Pomianowski [a,*], Magdalena Gozdowska [a], Stefan Dobosz [b], Rafał Różyński [b], Ewa Kulczykowska [a]

[a] Department of Genetics and Marine Biotechnology, Institute of Oceanology, Polish Academy of Sciences, Powstańców Warszawy 55, 81-712 Sopot, Poland
[b] Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Rutki, 83-330 Żukowo, Poland

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ABSTRACT

Melatonin (Mel; *N*-acetyl-5-methoxytryptamine) is recognized in fish as both a biological time-keeper and regulator of many physiological processes, including reproduction. Beyond its endocrine functions, Mel acts as an antioxidant, either by directly scavenging reactive radicals and forming N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), or by influencing the activity of antioxidant enzymes. This study examined whether Mel contributes to the protection of post-ovulated eggs of farmed rainbow trout against oxidative stress. Mel and AFMK levels were measured in plasma, ovarian fluid, and eggs, while the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) were determined in eggs. Plasma Mel concentrations did not significantly differ among pre-ovulation, ovulation, and post-ovulation stages, nor did they correlate with ovarian fluid levels. AFMK was undetectable in both plasma and ovarian fluid, whereas both Mel and AFMK were consistently present and positively correlated in eggs. This suggests that Mel directly reacts with reactive oxygen species within the eggs, resulting in AFMK formation. Mel concentrations in eggs did not correlate with the activities of SOD, CAT, or GST, implying that Mel primarily contributes to the antioxidant defense of post-ovulated eggs through non-enzymatic radical scavenging. Mel and AFMK levels were lower in eggs from four-year-old females compared to three-year-old, whereas SOD activity showed the opposite trend, suggesting a compensatory upregulation of enzymatic defense mechanisms in response to an age-related Mel decline. The concentration of Mel in eggs was approximately three times higher than in plasma or ovarian fluid, suggesting possible local synthesis within oocytes.

1. Introduction

Melatonin (Mel; *N*-acetyl-5-methoxytryptamine) is an indoleamine hormone first identified in the late 1950s as a potent agent inducing skin blanching in frogs (Lerner et al., 1958), thereby revealing its role in vertebrate pigmentation. Shortly thereafter, Mel was recognized in mammals as a central circadian signal, transmitting time-of-day information from the brain to peripheral tissues. Studies in the early 1990s on isolated pineal glands of rainbow trout elucidated how salmonids regulate Mel secretion in response to light and water temperature fluctuations, enabling adjustment to changing environment (Max and Menaker, 1992). Over recent decades, Mel has been demonstrated to have a broad range of metabolic and physiological roles, including in fish reproduction (Falcón and Munoz-Cueto, 2024). Furthermore, Mel has gained increasing attention for its potent antioxidant properties. Experimental evidence indicates that Mel and its metabolites, particularly N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), act as highly effective preventive antioxidants and free radical scavengers, mitigating oxidative damage in diverse organisms (Galano et al., 2013; Galano and Reiter, 2018; Tan et al., 2007). This antioxidant capacity is regarded as one of the most evolutionarily conserved roles of Mel, potentially dating back to early life forms, including prokaryotes (Zhao et al., 2019). However, uncertainties remain regarding the extent and precise mechanisms of these protective effects in vertebrates (Boutin et al., 2024). Our recent investigations have explored Mel's role in oxidative stress responses in fish, proposing elevated Mel concentrations as an adaptive response to oxidative stress in fish skin (Gozdowska et al., 2022; Pomianowski et al., 2023). The present study aims to investigate whether Mel contributes to oxidative stress protection in post-ovulatory eggs of female rainbow trout.

* Corresponding author.
E-mail address: kpomianowski@iopan.pl (K. Pomianowski).

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The quality of fish eggs determines reproductive success. Salmonids, including rainbow trout, are particularly noteworthy because they can retain ovulated eggs within the body cavity for several days or even weeks (Springate et al., 1984). Their ovaries lack an ovarian lumen (Romer, 1970), so ovulated eggs are released directly into the body cavity, where they are surrounded by a fluid known as coelomic fluid (CF) or ovarian fluid (OV). This fluid, secreted by the ovarian epithelium and derived from blood plasma (Coffman et al., 2000; Hirano et al., 1978), contains a complex mixture of components with specific biological functions that influence egg quality and fertilization success (Gueho et al., 2024; Johnson et al., 2014; Lahnsteiner, 2002). However, egg quality may deteriorate as a result of the over-ripening process, also termed post-ovulatory ageing (POA), defined as a progressive loss of oocyte viability after ovulation until fertilization (Aegerter and Jalabert, 2004; Rime et al., 2004). In rainbow trout, ovulated eggs retained in the body cavity show a time-dependent decrease in fertilization potential: in some females, viable oocytes persist for up to three weeks, while in others, egg quality decreases markedly within two weeks (Aegerter and Jalabert, 2004). In mammals, reactive oxygen species (ROS), primarily generated by mitochondrial metabolism, are established contributors to oocyte ageing (Goud et al., 2008; Lord and Aitken, 2013; Takahashi et al., 2013; Wang et al., 2021). Oxidative stress triggers a cascade of molecular events that promote oocyte deterioration, including a decline in maturation-promoting factor (MPF) activity, disruption of calcium homeostasis, mitochondrial dysfunction, and oxidative damage to critical intracellular targets such as lipids, proteins, and DNA (Lord and Aitken, 2013). However, to the best of the authors' knowledge, corresponding evidence in fish is still lacking. To mitigate oxidative stress, organisms rely on complex antioxidant defense systems composed of enzymatic and non-enzymatic components that maintain cellular redox homeostasis, as recently reviewed by Jomova et al. (2024). Lord et al. (2013) have demonstrated that supplementation of in vitro culture medium with Mel protects mammalian oocytes from post-ovulatory ageing. Treatment with Mel delays apoptosis, reduces fragmentation, prolongs the fertilization-competent window, and improves embryo quality relative to untreated aged oocytes. These beneficial effects are attributed to a reduction in oxidative stress, as Mel-treated aged oocytes exhibits significantly lower levels of ROS (Lord et al., 2013). Whether similar protective mechanisms operate in fish oocytes remains uncertain. The specific pathways underlying Mel's antioxidant action are also not fully elucidated; they may involve direct radical scavenging and formation of its metabolite AFMK, or indirect upregulation of key antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) (Rodriguez et al., 2004; Tan et al., 2007; Zhang and Zhang, 2014). Therefore, the aim of this study was to examine two potential modes of Mel action: direct free radical scavenging and indirect modulation of key antioxidant enzymes.

Mel accumulation in the ovaries is widely recognized to date, no direct evidence confirms Mel synthesis by fish oocytes, although the hormone is consistently detected in eyed-stage embryos (Yamada et al., 2002). The most plausible source is maternal transfer, but endogenous synthesis within the egg yolk cannot be excluded, since both Mel and its biosynthetic enzymes have been identified in avian eggs (Olszańska et al., 2007; Olszańska and Stępińska, 2008). These findings suggest a potential yolk-based Mel production that warrants further investigation in fish. Numerous studies have reported the presence of Mel and its biosynthetic enzymes at very early stages of fish ontogenesis, indicating that Mel synthesis begins during embryogenesis (Kazimi and Cahill, 1999). It is generally assumed that the primary role of Mel during early development, including the oocyte stages, is mainly associated with free radical protection, whereas its hormonal functions emerge later in ontogenesis (Kalamarz et al., 2009; Maitra and Hasan, 2016). Although the roles of Mel in promoting cell proliferation, accelerating embryonic development, and supporting larval and post-larval maturation are well established in fish (Danilova et al., 2004), our understanding of its source, presence, and specific functions within fish oocytes remains unknown.

This study aims to determine whether Mel contributes to protecting post-ovulated eggs against oxidative stress in farmed rainbow trout. We analyzed: (1) plasma Mel and AFMK concentrations 14 days before ovulation, at the time of egg collection (stripping), and 12 days after ovulation; (2) Mel and AFMK levels in collected eggs and ovarian fluid collected after ovulation; and (3) relationships between Mel and AFMK concentrations and the activities of key antioxidant enzymes, SOD, CAT, and GST, in post-ovulatory eggs. These analyses seek to clarify whether Mel's antioxidant action involves direct free radical scavenging with AFMK formation or/and indirect modulation of enzymatic antioxidant defenses.

2. Materials and methods

Both experiments were conducted at the breeding facility of the Department of Salmonid Research, Inland Fisheries Institute (IFI, Rutki, Poland), using the same spring-spawning rainbow trout (*Oncorhynchus mykiss*, Rutki selected strain) broodstock. The department maintains salmonid stocks through full breeding cycles and is equipped with a hatchery, rearing hall, and concrete ponds. Water supply from the Radunia River provides a flow rate of 450 L s^{-1}. Adult broodstock were maintained under natural photoperiod and temperature regimes (0–20 °C) in Radunia River water and fed a commercial trout diet (Aller Aqua, Svendborg, Denmark) twice daily at 09:00 and 15:00. Fertilized eggs and larvae were reared in the hatchery using groundwater with a stable thermal regime.

2.1. Experiment 1

Five three-year-old female rainbow trout (mean body weight ± SD: 0.91 ± 0.28 kg) that had ovulated within 24 h were selected from the main spawning stock and held in separate tank. Following sedation with MS-222, approximately 2000 eggs were stripped from each female divided into two experimental batches. One batch remained unrinsed, while the second was rinsed in a 1:10 solution of OvaFish™ extender (IMV Technologies, France) to remove contaminants, faeces, and residual ovarian fluid. The rinsing solution was equilibrated to the holding water temperature (7 °C). Approximately 200 eggs were washed once, in 5 replicates, using a tray containing 1 L of the solution. Eggs were gently stirred with a goose feather for 30 s and then separated from the solution using a sieve.

Approximately 300 rinsed and 300 unrinsed eggs from each female were used for fertilization trials and further subdivided into three equal replicates. Each replicate, consisting of 100 eggs, was fertilized using pooled milt from three males. Fertilization was performed by gentle mixing for 30 s, followed by addition of 100 μL of milt and rinsing with freshwater (7 °C). The fertilized eggs were then transferred to a separate rearing unit. Embryonic development was monitored daily, and eggs showing whitening, indicative of mortality, were recorded. Developmental progress was assessed at the eyed stage (70°days post-fertilization), hatching (80°days post-fertilization), and swim-up stage (80°days post-fertilization). All egg collection and handling procedures were performed as part of routine hatchery procedures during the spawning period. Remaining rinsed and unrinsed eggs were immediately frozen on dry ice and stored for subsequent analyses.

2.1.1. Experiment 2

Twelve four-year-old female rainbow trout (mean body weight ± SD: 0.97 ± 0.10 kg) were captured from the same main spawning stock as in Experiment 1, transferred to a separate breeding tank, and individually tagged with PIT markers for identification throughout the experiment. The study included three blood sampling events and one collection of eggs and ovarian fluid from each female. Prior to blood and egg collection, fish were anaesthetized in a bath containing MS-222. Blood samples (2 mL) were collected using lithium heparin S-Monovette

syringes (Sarstedt, cat. no. 04.1920) at three time points: two weeks before the expected ovulation (water temperature 7.2 °C), at post-ovulation (before egg collection, 8.8 °C), and twelve days after egg collection (12.4 °C). All samples were taken at the same time of day (12:00). Plasma was obtained by centrifugation (4000 x *g*, 5 min) and immediately frozen on dry ice. On the same day, eggs were collected from twelve females between two and four days post-ovulation. Approximately 2000 eggs stripped from each female were divided into two experimental batches: one left unrinsed and the other washed under identical conditions to those described in the previous experiment. At the time of egg collection, 3 mL of ovarian fluid were non-invasively collected from each female. Egg and ovarian fluid samples were immediately frozen on dry ice and transported to the Institute of Oceanology, Polish Academy of Sciences (PAS), for subsequent analyses.

2.2. Determination of Mel and AFMK

Mel and AFMK concentrations were determined using high-performance liquid chromatography (HPLC) with fluorescence detection, following solid-supported liquid extraction (SLE), as described by Gozdowska et al. (2022).

2.2.1. Egg samples

Five egg grains were homogenized in phosphate buffer (0.05 M, pH 6.8) using a Bead Ruptor Elite homogenizer (Omni International, USA). Homogenates were centrifuged at 10,000 x*g* for 15 min at 4 °C. Supernatants (1 mL) were applied to SLE columns (SLE NOVUM, 100 mg/5 mL; Phenomenex), and compounds were eluted with 6 mL dichloromethane:ethyl acetate (1:1, *v*/v). After evaporation, residues were dissolved in 0.2 mL methanol:PBS buffer (pH 7.4, 1:1 v/v) and analyzed by HPLC.

2.2.2. Plasma and ovarian fluid samples

Plasma and ovarian fluid samples (1 mL each) were mixed with phosphate buffer (1 mL, 0.05 M, pH 6.8), centrifuged at 8000 x*g* for 10 min at 4 °C, and processed by SLE following the same protocol as for egg samples.

2.2.3. Chromatographic analysis

Analyses were carried out using an Agilent 1200 Series Quaternary HPLC system with fluorescence detection. Separation was performed on a ZORBAX Eclipse Plus C18 column (150 × 4.6 mm, 3.5 μm; Agilent, USA). Mobile phases: A – 10 mM ammonium acetate (pH 5), B - methanol. A gradient (20–50% B over 20 min) was applied at a flow rate of 1 mL/min, at 20 °C. Injection volume: 20 μL. Fluorescence detection was performed at 230 nm excitation and 350 nm emission. Identification of Mel and AFMK was based on retention times of standards (Sigma, Germany).

2.3. Antioxidant enzymatic activity assays

2.3.1. Catalase (CAT) activity assay

CAT activity was determined using the Catalase Assay Kit (Cayman Chemical Co., Ann Arbor, Michigan, USA). Samples were prepared by homogenizing three rinsed egg grains in 0.5 mL of cold buffer (50 mM dipotassium phosphate, pH 7.0, containing 1 mM EDTA) at 5 m/s for 30 s with the Bead Ruptor Elite homogenizer (Omni International, USA). Homogenates were centrifuged at 10,000 x*g* for 15 min at 4 °C, and absorbance of supernatants (diluted 1:1.5) was measured at 540 nm. Enzyme activity was calculated according to the manufacturer's instructions, using a formaldehyde standard curve with corrections for sample dilution.

2.3.2. Superoxide dismutase (SOD) activity assay

Total SOD activity (Cu/ZnSOD, MnSOD, and FeSOD) was determined using the Superoxide Dismutase Assay Kit (Cayman Chemical Co., Ann Arbor, Michigan, USA). Samples were prepared by homogenizing three rinsed egg grains in 0.5 mL of cold HEPES buffer (1 mM ethylene glycol tetraacetic acid, 210 mM mannitol, 70 mM sucrose; pH 7.2) at 5 m/s for 30 s with the Bead Ruptor Elite homogenizer (Omni International, USA). Homogenates were centrifuged at 1500 x*g* for 5 min at 4 °C, and absorbance of the supernatants (diluted 1:20) was measured at 450 nm. Enzyme activity was calculated according to the manufacturer's instructions, using the SOD standard curve with corrections for sample dilution.

2.3.3. Glutathione S-transferase (GST) activity assay

GST activity was determined using the Glutathione-S-Transferase (GST) Activity Assay Kit (DTNB Method) (Elabscience Bionovation Inc., Houston, Texas, USA) in accordance with the manufacturer's instructions. Samples were prepared by sonicating two rinsed egg grains in 1 mL of cold PBS buffer (0.01 M, pH 7.4) on ice with the Microson XL sonicator (Misonix, USA), applying six 10-s cycles. Products were centrifuged at 10,000 x*g* for 10 min at 4 °C, and absorbance of the supernatants (diluted 1:50) was measured at 450 nm. Enzyme activity was calculated using the glutathione standard curve with corrections for sample dilution.

2.3.4. Protein assay

Protein concentration was measured using two commercial Protein Determination Kits (Cayman Chemical, USA). The bicinchoninic acid (BCA) method was applied for quantifying proteins in egg homogenates used for Mel and AFMK determinations, as well as for CAT and GST activity assays. The Bradford method was employed for homogenates analyzed in the SOD activity assay.

2.4. Statistical analysis

Statistical analyses were performed using Statistica 13.3 software. Data are presented as mean ± standard deviation (SD). The normality of data distribution was assessed using the W Shapiro-Wilk test. For data that did not meet the assumption of normality, the nonparametric Mann-Whitney *U* test and Spearman's rank correlation were applied. Mel levels and SOD, CAT and GST activity in eggs from three- and four-year-old female rainbow trout (Experiments 1 and 2) were compared using Student's *t*-test, whereas AFMK levels were compared using the Mann-Whitney U test. Correlations between Mel levels and antioxidant enzymes activities (SOD, CAT, GST) in eggs from three-year-old and four-year-old rainbow trout females were examined using Pearson's correlation coefficient (*r*). The relationship between Mel and AFMK levels in eggs was analyzed using Pearson's correlation in Experiment 1 and Spearman's rank correlation in Experiment 2. Differences in plasma Mel levels were evaluated using one-way repeated-measures ANOVA. Survival rates at three developmental stages for rinsed and unrinsed eggs were analyzed separately for each female using the Mann–Whitney U test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Mel and AFMK concentrations in plasma and ovarian fluid

Mel concentrations in plasma of four-years-old female rainbow trout measured at three time points: 14 days before expected ovulation, at egg collection (2–4 days after ovulation) and 12 days after egg collection are presented in Table 1. These values did not differ significantly among the three reproductive stages ($P = 0.34$, Table 1). Plasma Mel levels showed no correlation with those measured in ovarian fluid collected during stripping (2–4 days after ovulation, $r = 0.45$). AFMK was undetectable in both plasma and ovarian fluid.

Table 1
Melatonin concentrations (pmol/ml, ± SD) in plasma and ovarian fluid (OV) of four-year-old rainbow trout females measured at three time points: 14 days before expected ovulation (I); at egg collection (2–4 days after ovulation, II); 12 days after egg collection (III). The number of fish analyzed is indicated in brackets.

	I	II	III
Blood plasma	0.63 ± 0.16 (n = 11)	0.51 ± 0.09 (n = 12)	0.51 ± 0.26 (n = 12)
Ovarian fluid (OV)	×	0.61 ± 0.2 (n = 11)	×

3.2. Mel and AFMK concentrations in eggs

Fig. 1 shows Mel and AFMK concentrations in rinsed eggs from three- and four-year-old female rainbow trout (Experiments 1 and 2, respectively). Both Mel and AFMK levels were significantly lower in eggs from four-year-old females than in those from three-year-old females. For unrinsed eggs, three-year-old females (n = 5) had Mel and AFMK levels of 1.35 ± 0.31 and 0.94 ± 0.34 pmol/mg protein, respectively, whereas four-year-old females (n = 12) showed Mel levels of 0.81 ± 0.26 pmol/mg protein and AFMK levels of 0.25 ± 0.13 pmol/mg protein.

The concentration of Mel in eggs of four-year-old females was approximately three times higher than in plasma or ovarian fluid collected during stripping. The mean Mel concentration in eggs was 2.03 pmol/g, compared with 0.60 pmol/g in ovarian fluid and 0.51 pmol/g in plasma, assuming a density of 1.025 g/mL for both plasma and ovarian fluid. Mel and AFMK levels in eggs from four-year-old females showed a strong positive correlation (n = 12; r = 0.82), whereas no statistically significant correlation was observed in eggs from three-year-old females (n = 5; r = 0.21). Fig. 2 presents the Spearman's rank correlation coefficient between Mel and AFMK concentrations in rinsed eggs from four-year-old rainbow trout females, shown as a scatterplot of raw values. All analyses were performed on both rinsed and unrinsed eggs; however, only results from rinsed eggs were reported to eliminate the influence of ovarian fluid on the outcomes.

3.3. Antioxidant enzyme activities in eggs

The activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) in rinsed eggs from three- and four-year-old female rainbow trout (Experiments 1 and 2, respectively) are presented in Table 2. SOD activity differed significantly between eggs from three- and four-year-old females (Student's t-test, $P < 0.001$), with higher values in eggs from four-year-old females, whereas CAT (P = 0.74) and GST (P = 0.37) activities showed no significant age-related differences. Pearson's correlation coefficients (r) and corresponding P values between Mel levels and antioxidant enzyme activities (SOD, CAT, GST) in eggs from three- and four-year-old female rainbow trout are presented in Table 3. No statistically significant correlations were observed in either age group (all $P > 0.05$).

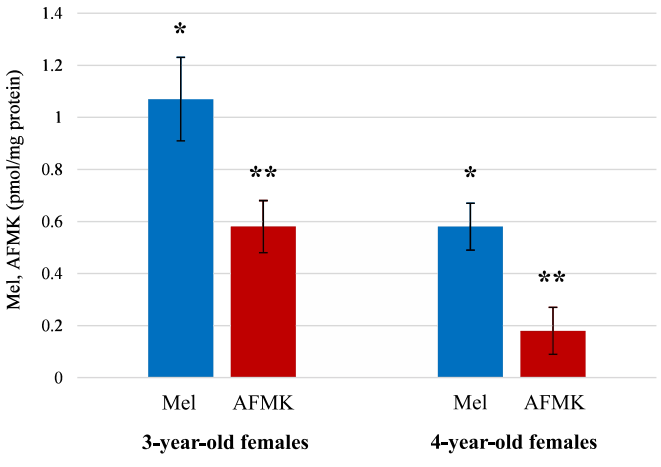

Fig. 1. Mel and AFMK concentrations in rinsed eggs from three-year-old (n = 5) and four-year-old (n = 12) rainbow trout females. Values are means ± SD. Significant differences: *$P < 0.001$ (Student's t-test); **$P < 0.001$ (Mann-Whitney U test).

3.4. Fertilization test results

Fig. 3 presents effect of rinsing rainbow trout eggs with the OvaFish™ solution prior to fertilization on survival rates (%) at the eyed-egg, hatching, and swim-up stages in Experiment 1. Eggs were obtained from three-year-old females (n = 5). Rinsing the eggs before fertilization with OvaFish™ did not improve embryo or larval survival at any of the developmental stages ($P > 0.1$).

4. Discussion

Recent studies have proposed that Mel participates in the oxidative stress response in the skin of two fish species, the three-spined stickleback and the European flounder (Gozdowska et al., 2022; Kulczykowska, 2019; Kulczykowska et al., 2018; Pomianowski et al., 2023). Building on these findings, the present study investigates whether Mel contributes to the protection of post-ovulated eggs against oxidative stress in farmed rainbow trout.

In our study on rainbow trout, Mel was detected in plasma, ovarian fluid and eggs; however its metabolite AFMK was not measurable in plasma or ovarian fluid, while both Mel and AFMK were consistently present in eggs (Fig. 1.). The concentration of Mel was three times higher in eggs than in plasma and ovarian fluid. This elevated level may indicate Mel production by the oocytes, which warrants further investigation to confirm. It should be noted that the gonads are not only a target for Mel in fish, but also a potential source of Mel: mRNA of the enzymes engaged in Mel biosynthesis, Mel itself and its receptors are present in the gonads (Falcón and Muñoz-Cueto, 2026). We have also found transcripts of Mel biosynthesis enzymes in gonads of the European flounder (Pomianowski et al., 2021). Nevertheless, regardless of its origin, Mel may serve as an intrinsic molecular defense in eggs, protecting them from oxidative damage during retention within the ovarian cavity.

A strong positive correlation between Mel and AFMK concentrations observed in eggs during Experiment 2 (Fig. 2) suggests a possible link between Mel presence and AFMK formation within the eggs. Furthermore, the activities of key antioxidant enzymes, SOD, CAT or GST, were measured in eggs (Table 2); however none showed a significant correlation with Mel levels (Table 3). These findings indicate that Mel does not modulate enzymatic antioxidant defenses in eggs. Therefore, it is most likely that Mel contributes to the antioxidant protection of post-ovulatory eggs primarily through the direct neutralization of free radicals and subsequent AFMK formation, rather than by upregulating enzymatic defense mechanisms. It should also be noted that the primary antioxidants in fish eggs include vitamins E and A, as well as provitamin A carotenoids, which originate from yolk reserves dependent on maternal diet as fish cannot synthesize them de novo (Palace and Werner, 2006).

It is worth mentioning that Mel and AFMK levels in oocytes were significantly higher in three-years-old females (Experiment 1) than in four-years-old individuals (Experiment 2). This observation aligns with previous reports showing that Mel concentration decreases with organismal age, a pattern well-documented in both mammals and fish (Reiter, 1994; Zhdanova et al., 2008). Age-related decreases in Mel are associated with the accumulation of oxidative damage and weakened antioxidant defenses. In the same study, superoxide dismutase (SOD) activity was higher in oocytes from four-year-old females than in those

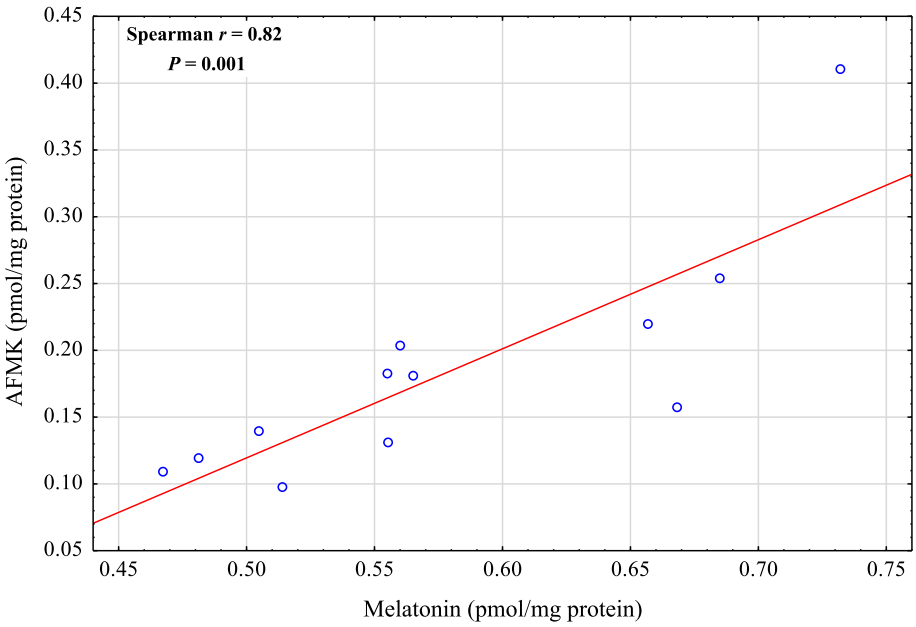

Fig. 2. Spearman's rank correlation coefficient between Mel and AFMK concentrations in rinsed eggs from four-year-old rainbow trout females (n = 12). The relationship is shown as a scatterplot of raw values.

Table 2
Activity of antioxidant enzymes (SOD, CAT, GST) in rinsed rainbow trout eggs, normalized to protein content. Values are means ± SD. *SOD activity differed significantly between three- and four-year-old females (Student's t-test, P < 0.001), whereas CAT (P = 0.74) and GST (P = 0.37) activities showed no significant age-related differences. The number of fish analyzed is given in brackets.

	SOD (U/mg protein)	CAT (nmol/min/mg protein)	GST (U/μg protein)
3-year-old-females (n = 5)	4.31 ± 2.35 *	2.78 ± 1.48	10.8 ± 7.98
4-year-old-females (n = 12)	11.5 ± 3.42 *	3.01 ± 1.27	7.3 ± 5.27

Table 3
Pearson's correlation coefficients (r) and corresponding significance levels (P values) between antioxidant enzyme activities (SOD, CAT, GST) and melatonin (Mel) levels in eggs of three- and four-year-old rainbow trout females. No statistically significant correlations were observed in either age group (all P > 0.05). The number of fish analyzed is indicated in brackets.

	SOD	CAT	GST
Mel 3-year-old-females (n = 5)	r = 0.259 P = 0.673 (n = 5)	r = −0.297 P = 0.627 (n = 5)	r = 0.7984 P = 0.411 (n = 3)
Mel 4-year-old-females (n = 12)	r = − 0.182 P = 0.57 (n = 12)	r = 0.242 P = 0.447 (n = 12)	r = 0.017 P = 0.960 (n = 11)

from three-year-old. Despite decades of research, the relationship between SOD activity and ageing or lifespan remains inconclusive (Hussain et al., 1995; Warner, 1994). Notably, the higher SOD activity in eggs of older females may reflect a compensatory enhancement of enzymatic antioxidant defenses as Mel levels decline; thus, enzymatic antioxidants such as SOD may become increasingly important for mitigating oxidative stress as Mel's direct radical scavenging diminishes.

All analyses were conducted on both rinsed and unrinsed eggs, but only results from rinsed eggs were considered to avoid the influence of ovarian fluid on the outcomes. The OvaFish™ extender was developed to rinse faeces, blood and coelomic fluid from the salmonid egg surface to provide optimal environmental conditions for sperm motility. When rainbow trout eggs were rinsed prior to fertilization with OvaFish™ extender, fertilization success increased from 70% to 80% (Haffray et al., 2008). In our study, however, no significant differences were observed between rinsed and unrinsed eggs in terms of fertilization and developmental success, including the eyed-egg, hatching, and swim-up stages.

As aquaculture develops, identifying robust biomarkers for fish egg quality remains a key objective. Current research focuses on oxidative stress markers given the critical impact of ROS on egg viability across taxa. Studies in turbot (*Scophthalmus maximus*) indicate that lipid peroxidation measured by malondialdehyde (MDA) concentration, and total antioxidant status (TAS), are promising predictors of egg quality (Ramos-Júdez et al., 2025). Similarly, increased thiobarbituric acid reactive substances (TBARS), a product of lipid peroxidation, correlate with post-ovulatory ageing in Atlantic salmon eggs (Clarkson et al., 2024). The observation that Mel concentration corresponded to that of AFMK in rainbow trout eggs suggests both compounds may serve as useful biomarkers for POA. However, broader validation is required to establish their diagnostic and practical utility in routine aquaculture.

5. Conclusions

Taken together, these findings suggest that Mel directly reacts with reactive oxygen species within the eggs, leading to the formation of AFMK. Mel concentrations in eggs did not correlate with the activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), or glutathione-S-transferase (GST), indicating that Mel contributes to the antioxidant defense of post-ovulated eggs primarily through direct neutralization of free radicals rather than by enhancing enzymatic activity. Mel and AFMK levels were lower in eggs from older females, whereas SOD activity showed the opposite trend, suggesting a compensatory upregulation of enzymatic defense mechanisms in response to an age-related decline in Mel. The concentration of Mel in eggs was approximately three times higher than in plasma or ovarian fluid, which may indicate endogenous Mel synthesis by the oocytes. All

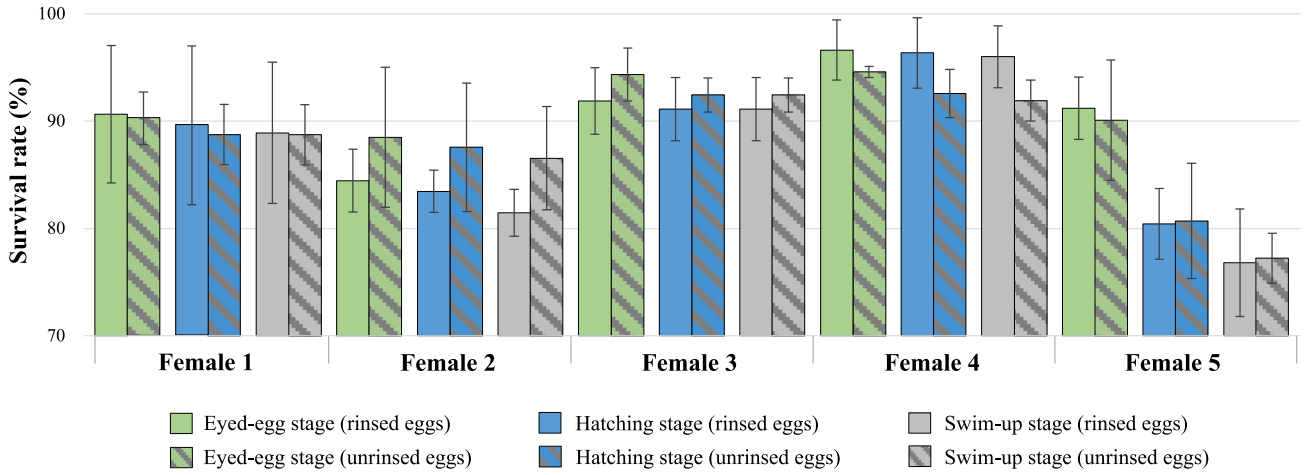

Fig. 3. Effect of rinsing rainbow trout eggs with the OvaFish™ solution prior to fertilization on survival rates (%) at the eyed-egg, hatching, and swim-up stages in Experiment 1. Eggs were obtained from 3-year-old females ($n = 5$). Rinsing the eggs before fertilization with OvaFish™ did not improve embryo or larval survival at any of the developmental stages assessed ($P > 0.1$, Mann-Whitney U test).

analyses were performed on both rinsed and unrinsed eggs; however, only results from rinsed eggs were considered to eliminate the influence of ovarian fluid on the outcomes. Tests confirmed that rinsing did not affect fertilization and developmental success, including the percentages of eyed embryos, hatch rate, and swimming larvae.

CRediT authorship contribution statement

Konrad Pomianowski: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Magdalena Gozdowska:** Validation, Methodology, Investigation, Formal analysis. **Stefan Dobosz:** Supervision, Methodology, Conceptualization. **Rafał Różyński:** Methodology, Investigation. **Ewa Kulczykowska:** Writing – review & editing, Writing – original draft, Supervision, Investigation.

Ethics statement

All experiments complied with the Directive 2010/63/EU of the European Commission for animal experiments and with the guidelines and approval of the Ethics Committee for Animal Experimentation of Bydgoszcz University of Science and Technology, Poland; Protocol No. 16/2024.

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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.

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Data availability

The raw data associated with this project (Experiment 2) were deposited in the IOPAN GeoNetwork repository and are available at the following link: Doi: 10.48457/IOPAN.2025.491.

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