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RESEARCH ARTICLE

Rapid growth causes abnormal vaterite formation in farmed fish otoliths

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ABSTRACT

Sagittal otoliths are essential components of the sensory organs that enable all teleost fish to hear and maintain balance, and are primarily composed of calcium carbonate. A deformity, where aragonite (the normal crystal form) is replaced with vaterite, was first noted over 50 years ago but its underlying cause is unresolved. We evaluated the prevalence of vateritic otoliths from two captive rearing studies which suggested that fast growth, due to environmental rather than genetic control, led to vaterite development. We then tested this by varying light and temperature to create phenotypes with different growth rates, which resulted in fast growers (5 times larger) having 3 times more vaterite than slow growers. A decrease in either the ratio of otolith matrix proteins (otolin-1/OMM-64) or $[Ca^{2+}]/[CO_3^{2-}]$ may explain why fast growth causes vaterite deposition. As vaterite decreases hearing sensitivity, reducing growth rates in hatcheries may improve the welfare of farmed fish and increase the success of conservation efforts.

KEY WORDS: Aberrant otolith, Aquaculture, Crystalline otolith, Deformity, Fish welfare, *Salmo salar*

INTRODUCTION

The bony structures of the inner ear of vertebrates aid hearing and balance, with deformities in these structures typically causing sensory impairment (Merchant and Nadol, 2010). In teleost fish, the most abundant and diverse vertebrate group, the sagittal otolith is a primary part of the hearing organ and is composed of calcium carbonate in the crystal form aragonite (Carlström, 1963). A deformity in which the aragonite is replaced by vaterite crystals is abnormal in the wild, occurring in 1–24% of otoliths. However, it is extremely common in farmed fish, with on average a 3.7 times higher prevalence than in their wild counterparts and, in the most recent study, affecting 100% of harvest-size farmed Norwegian salmon (Reimer et al., 2016). Vaterite formation is irreversible once begun, and vaterite replacement results in otoliths which are larger, lighter, more brittle and less regularly shaped than their aragonite counterparts. Because of these differences, replacement of aragonite by vaterite probably causes severe hearing loss by reducing otolith function, potentially impacting fish welfare and restocking efficiency (Reimer et al., 2016).

Despite over 50 years of evidence of vateritic otoliths (Palmork et al., 1963; Mugiya, 1972; Strong et al., 1986; Bowen et al., 1999), the cause(s) of their formation is unknown. Previous attempts to induce vaterite formation by temporarily exposing juvenile fish to different temperatures for short periods of time were unsuccessful (Gauldie, 1996), and there is no correlation between vaterite prevalence and fish sex or early maturation rates (Sweeting et al., 2004), or the prevalence of other skeletal deformities (Tomás and Geffen, 2003). The recent discovery of marked differences in prevalence between wild and farm-reared fish suggests that the cause(s) of vaterite formation is a consistent difference between these two groups or the environments they experience (Reimer et al., 2016).

There are several factors that differ in universal ways between farmed and wild settings which may affect otolith development. First, as a result of domestication, farmed fish such as Atlantic salmon (*Salmo salar* L.) now display a range of genetic differences from their wild conspecifics (Glover et al., 2017), so a genetic predisposition to vaterite deformation is possible. While vaterite prevalence in wild-origin fish raised in captivity is high (see fig. 6 of Gauldie, 1996), its heritability has not been explicitly tested. Second, the composition of wild and commercial feeds differs, especially in the proportions of terrestrially sourced nutrients important for proper development (Naylor et al., 2009). Third, hatcheries often use continuous light to enhance growth and suitability for sea-cage transfer (Saunders et al., 1985), which can affect physiology and development (Oppedal et al., 2003). Finally, temperature fluctuations within hatcheries may influence vaterite formation (Sweeting et al., 2004), which may be particularly important in early life (Wargelius et al., 2005). As otolith formation is a complex interaction between genetic and environmental factors (Radtke and Shafer, 1992), it is important to evaluate the separate and interacting effects of all possible factors.

Determining the cause of the vaterite otolith deformity in cultured fish has broad significance to aquaculture and conservation. As >50% of farmed fish have ∼50% loss in hearing sensitivity due to vaterite (Reimer et al., 2016), pinpointing the cause of this deformity could drive measures to improve the welfare of billions of farmed fish worldwide. Furthermore, over 100 countries release hatchery-raised fish for restocking wild fisheries (sea ranching or supportive breeding). Reducing the incidence of vaterite formation could increase the generally poor success rates of these activities (Moore et al., 2012).

Here, we conducted three experiments to determine whether the separate and interacting effects of diet, rearing temperature, light regime and genetic origin might be influencing vaterite prevalence in hatchery-reared fish. As the two initial experiments suggested a relationship between fish size (i.e. growth rate) and vaterite

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formation, we conducted a final experiment to isolate and test the effect of growth rate on vaterite prevalence.

MATERIALS AND METHODS

This work was conducted in accordance with the laws and regulations of the Norwegian Regulation on Animal Experimentation 1996. The protocol was approved by the Norwegian Animal Research Authority (permits 7121 and 6546). All experiments were conducted at the Institute of Marine Research field research station in Matre, Norway (60°N). Both otoliths were removed from Atlantic salmon from each experiment, cleaned, dried, and photographed under a dissecting microscope at 10× magnification. As vateritic otoliths from Atlantic salmon are easy to distinguish visually from aragonitic ones (Sweeting et al., 2004; Oxman et al., 2007; Reimer et al., 2016), they were visually scored as 'vaterite' if any vaterite crystals were visible (indicating that the switch to vaterite formation had been made), or 'aragonite' if they were not. Vaterite prevalence was defined as the proportion of vaterite otoliths in each replicate tank.

Experiment 1 – effects of genetic origin and diet on vaterite prevalence

Salmon eggs (wild-caught broodstock, commercial strain A, and their F1 hybrids) were reared together in ∼6°C/complete darkness, and hatched in January 2014 (see Harvey et al., 2016, for experiment details). In March, 2700 fry were transferred to six freshwater tanks (450 fry per tank, n=2 tanks per diet treatment).

Fry were fed to satiation with a standard commercial diet (NutraXP, Skretting, Preston, UK), a carbohydrate-rich diet (Coarse Fish 23, Skretting), or a 'natural' diet mimicking the food available within spring spawning rivers: a mix of insect larvae; black mosquito larvae Culicidae and glassworms, i.e. transparent larvae of the phantom midge Chaoboridae, as well as freshwater copepods Cyclopidae and water fleas Daphniidae (Ruto Frozen Fishfood).

At ∼130 days post-feeding (dpf), all fish were killed, weighed, measured for fork length, and DNA tested using parentage analysis to identify genetic origin. Otoliths were extracted from 45 fish per replicate tank (15 individuals per genetic origin, randomly selected after their genetic origin had been identified). Final replicate numbers were n=2 for each genetic origin/diet treatment combination (n=270 fish assessed in total). Results were analysed with a two-way ANOVA (diet and genetic origin as fixed factors, as well as their interaction term). *Post hoc* Tukey HSD tests assessed differences between individual treatments.

Experiment 2 – effects of temperature shock and light regime on vaterite prevalence

Salmon eggs (commercial strain B) were reared at 6°C in complete darkness, and hatched in January 2012 (see Wargelius et al., 2005, for experiment details). Heat-shock treatments exposed alevins to 12°C for 24 h at 20 degree-days (20dC), 220dC or no heat-shock (control). In March, fry were transferred to 18 freshwater tanks (∼295 fry per tank, n=3 tanks per treatment). Nine tanks, three for each heat-shock treatment, were exposed to standard hatchery conditions of continuous light (LL), and an additional nine tanks were exposed to 12 h of light and 12 h of dark per day (LD 12:12), mimicking average spring conditions.

At ∼285 dpf, 40 fish from each replicate were killed. Some groups were too degraded for otolith extraction. Final replicate numbers were: n=3 for LD 12:12/control, LL/control and LD 12:12/220dC; n=2 for LD 12:12/20dC and LL/20dC; and n=1 for LL/220dC (n=560 fish assessed in total). As there was only one replicate tank for the LL/220dC treatment, results were analysed using a two-way ANOVA (light regime and heat shock as fixed factors, without the interaction term). As maturation, which affects growth, differed between light regime treatments, a one-way ANOVA (maturity by light regime treatment as a fixed factor) and *post hoc* Tukey HSD test were performed to assess the effect of maturation on the observed response to the light regime treatment.

Experiment 3 – effects of temperature and light regime (growth rate) on vaterite prevalence

Salmon eggs (commercial strain B) were incubated at ∼6°C in complete darkness, and hatched in January 2015. In March, 2700 fry were transferred to 12 freshwater tanks (225 fry per tank, n=3 tanks per treatment).

Light treatments consisted of the hatchery standard of continuous light (LL) or 18 h of light and 6 h of darkness per day (LD 18:6), which was designed to reduce growth rate without inducing maturity. Light treatments were combined with either high (13°C) or low (6°C) temperatures to create four treatment groups (LL-high, LL-low, LD 18:6-high, LD 18:6-low).

At 155 dpf, 40 fish from each tank were killed, weighed and their otoliths extracted. Final replicate numbers were n=3 tanks for each light/temperature treatment (n=480 fish assessed in total). Results were analysed using a two-way ANOVA (light and temperature as fixed factors), and *post hoc* Tukey HSD tests assessed differences between treatment groups. Based on the results of experiments 1 and 3, a linear regression was used to test whether vaterite prevalence was related to average fish growth rate (final weight divided by dpf).

RESULTS

Experiment 1 – effects of genetic origin and diet on vaterite prevalence

Fish fed a standard commercial diet were 2−3 times more likely to have vaterite otoliths than those fed natural or carbohydrate-rich diets. Vaterite prevalence varied with the interaction between genetic origin and diet ($F_{4,9}$=5.3, P=0.018); thus, the effect of diet upon vaterite prevalence differed between the farmed, hybrid and wild salmon (Fig. 1A). There was no main effect of genetic origin ($F_{2,9}$=0.03, P=0.98), and vaterite prevalence was more common in fish fed a commercial diet than in those fed a natural or carbohydrate-rich diet ($F_{2,9}$=49.2, P<0.001). Pairwise comparisons showed that the higher vaterite prevalence in individuals fed a commercial diet compared with the alternative diets was only significant for farmed and hybrid fish.

Experiment 2 – effects of temperature shock and light regime on vaterite prevalence

Vaterite prevalence was only affected by photoperiod (lighting: $F_{1,9}$=5.4, P=0.04; heat-shock: $F_{2,9}$=0.61, P=0.56). Vaterite prevalence was overall 15% higher in the LL than in the LD 12:12 treatment. However, 72% of males within the LD 12:12 treatment became sexually mature. The lower vaterite prevalence in the LD 12:12 treatment was due to the maturation of males (maturation: $F_{2,14}$=6.9, P=0.008). Vaterite prevalence in LD 12:12 mature males was 15% lower than that in LD 12:12 immature fish, and 26% lower than that in the LL group, which were all immature (Fig. 1B).

Experiment 3 – effects of temperature and light regime (growth rate) on vaterite prevalence

Vaterite otoliths were 3 times more prevalent in the fastest-growing fish than in the slowest (Fig. 1C). Mean fish length and weight varied depending on both light and temperature treatments

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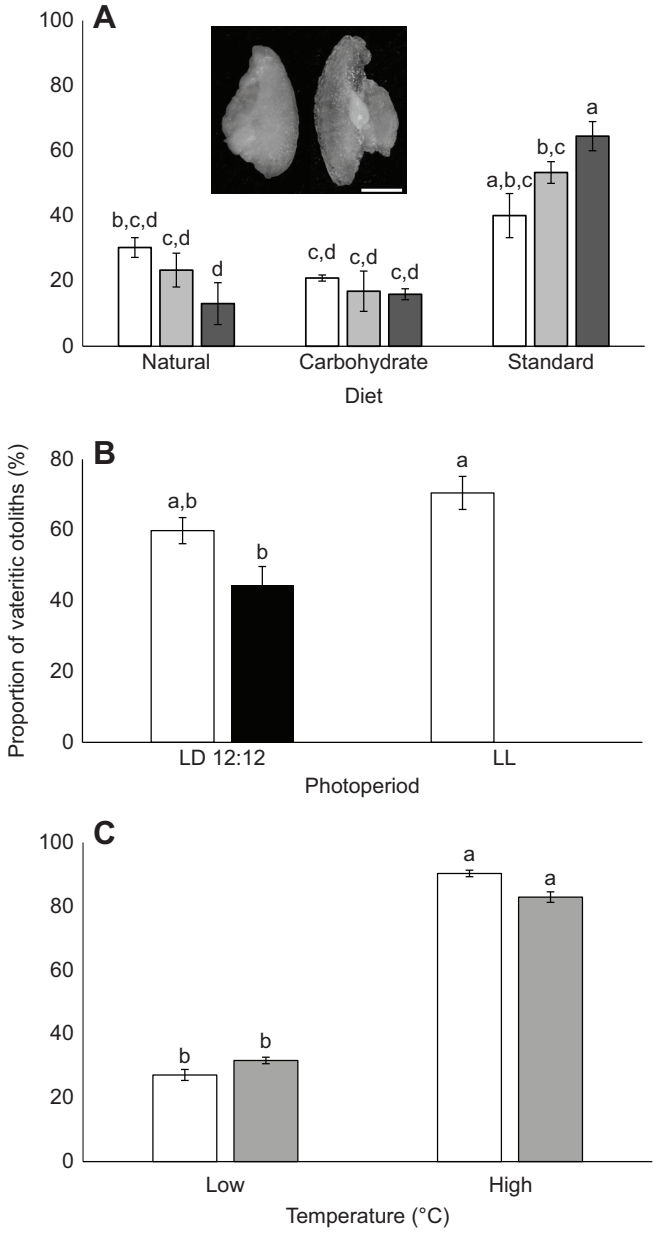

Fig. 1. Prevalence of vaterite otoliths. (A) Genetically wild (white, *n*=6 tanks), hybrid (grey, *n*=6) and farmed (black, *n*=6) Atlantic salmon fed different diets (experiment 1; *n*=810 fish assessed in total). (B) All immature (white, *n*=5 tanks) and mature males (black, *n*=4) from all heat-shock treatments combined reared under different photoperiods (LD 12:12, 12 h light:12 h dark; LL, continuous light; experiment 2; *n*=560 fish assessed in total). (C) Fish reared under continuous light (LL, white, *n*=6 tanks) or LD 18:6 (grey, *n*=6) at different temperatures (experiment 3; *n*=480 fish assessed in total). Bars show raw means±s.e.m., while letters show significant groupings (P<0.05) as determined by *post hoc* Tukey HSD tests (which are based on pairwise comparisons of least squares means and a pooled s.e.m.). Inset: sagittal otoliths from a juvenile Atlantic salmon. Scale bar, 1 mm. The left otolith is entirely aragonite, while the right otolith is approximately 90% vaterite by planar area.

(light×temperature interaction: length $F_{1,8}$=39, P<0.001, weight $F_{1,8}$=63, P<0.001). Length and weight differed between temperature treatments but were greatest in the LL (high temperature: 16.0±0.2 cm, 60±1.8 g; low temperature: 5.1±0.06 cm, 1.5±0.03 g) versus LD 18:6 (high temperature: 14.8±0.2 cm, 46±0.3 g, low temperature: 5.1±0.06 cm, 1.5±0.03 g) treatments (P<0.05 in all cases). Vaterite prevalence varied depending on the interaction between lighting and temperature, and with temperature alone; there was no main effect of lighting (light×temperature interaction: $F_{1,8}$=11, P=0.011, temperature: $F_{1,8}$=992, P<0.001; lighting: $F_{1,8}$=0.6, P=0.45). *Post hoc* pairwise comparisons showed that vaterite prevalence in the high temperature treatment was 63% higher than that in the low temperature treatment under LL conditions, but only 51% higher under LD 18:6 conditions (Fig. 1C).

Vaterite prevalence increased with growth rate in experiments 1 and 3 (Fig. 2, R^2=0.89, P<0.001).

DISCUSSION

Despite long-standing awareness of the occurrence of abnormal vaterite replacement in sagittal otoliths, few studies have investigated why vaterite forms. By rearing fish of wild, hybrid and domesticated origin, and manipulating diet and the rearing environment, we show that fast-growing fish are 3 times more likely to have vateritic otoliths than slow-growing fish (Fig. 2), providing the first compelling evidence that abnormally fast growth is likely to be the universal underlying cause. By optimising conditions to maximise growth rates, we successfully induced vaterite formation in 90% of otoliths.

Vaterite incidence in the slowest-growing fish (29%) was not as low as in wild populations (13%; Reimer et al., 2016), which could indicate that there are multiple causes of vaterite formation, fish with vateritic otoliths have higher mortality rates in the wild, or sub-optimal hatchery conditions still produce, on average, faster-growing fish than wild conditions.

Sweeting et al. (2004) hypothesised that metabolic rate may influence vaterite formation. They also examined premature maturation (‘jacking’) in salmon, expecting that the increased metabolic rate would increase vaterite prevalence, but they found no evidence to support this. They used a coarser method of vaterite classification (a scale of 1–4 based on vaterite area) and measured prevalence at the organism level rather than as the percentage of affected otoliths, so it is possible that their sample size was not large enough to detect the effect. To date, vaterite prevalence with respect to maturation has only been investigated in males. A similar investigation into females may further clarify the connection between growth rate, maturation and vaterite prevalence.

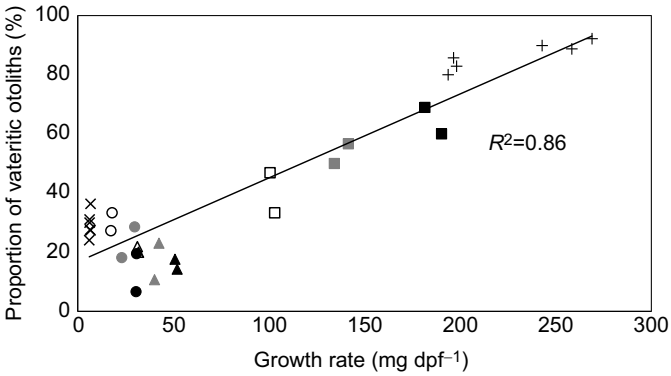

Fig. 2. Proportion of vaterite otoliths from experiments 1 and 3 in response to growth rate. The relationship between vaterite prevalence and growth rate was determined by a linear regression. Experiment 1 is divided by temperature treatment: high temperature (+, *n*=6) and low temperature (×, *n*=6). Experiment 3 (*n*=18) shows each genetic origin×diet treatment: farmed (black), hybrid (grey) and wild (white) origin by standard (squares), carbohydrate-rich (triangles) and natural diet (circles).

How fast growth leads to abnormal vaterite formation is unclear, although once normal aragonite deposition is disrupted, vaterite formation appears permanent. There are two possible mechanisms leading to disruption of aragonite.

(1) The organic matrix is a protein aggregate lattice, the composition of which influences the crystal polymorph (Mann, 2001; Falini et al., 2005), and aragonite is only deposited when all components are present. Fast growth might change the composition of otolith matrix proteins in a way that favours deposition of vaterite over aragonite. Using *in vitro* crystallisation experiments, Tohse et al. (2009) found that otolith matrix macromolecule- 64 (OMM-64) favours the formation of vaterite, whereas the presence of OMM-64 in combination with otolin-1 favours the formation of aragonite. If fast growth results in a decrease in the otolin-1/OMM-64 ratio within the protein aggregate, this could cause the switch to abnormal vaterite otoliths.

(2) A high $[Ca^{2+}]/[CO_3^{2-}]$ ratio in the endolymph promotes aragonite formation over other calcium carbonate polymorphs (Chen and Xiang, 2009). Inorganic carbon (HCO_3^-) is transported to the endolymph across the saccular membrane by energy-dependent mechanisms involving HCO_3^--ATPase and Cl^-/HCO_3^- exchangers (Tohse and Mugiya, 2001). Presumably, faster-growing fish have more energy, which may lead to a greater rate of transport of HCO_3^- relative to Ca^{2+} into the endolymph, resulting in a lower $[Ca^{2+}]/[CO_3^{2-}]$ ratio and favourable conditions for vaterite formation.

Identifying which of these mechanisms contributes to faster growing fish being more likely to have abnormal vateritic otoliths requires further research.

Our findings have potential implications for the food production industry, as well as supportive breeding or restocking programmes in the wild. Aquaculture industries promote increasing individual growth (Thodesen and Gjedrem, 2006; Asche and Bjørndal, 2011) as it increases feed conversion efficiency, which in turn increases sustainability and economic efficiency (Cook et al., 2000). Rapid growth has previously been shown to increase the incidence of cataracts (Ersdal et al., 2001), but the present study is the first to show that it permanently deforms otoliths. If fish welfare is negatively impacted through impaired hearing (Reimer et al., 2016), the industry could reduce growth rates to prevent abnormal vaterite formation. Alternatively, commercial farms may need to investigate methods of reducing vaterite prevalence while striving to maintain or increase growth rate.

Reducing vaterite prevalence may also be important for wild fish conservation and stock enhancement, as restocking (or supportive breeding) programmes rely on the survival of hatchery-reared juveniles (Sweeting et al., 2003). Rearing environments are typically optimised to maximise juvenile size at release, with the aim of reducing mortality (Cross et al., 2009). However, despite these efforts, return rates of hatchery-reared juveniles remain low (Araki et al., 2008; Beamish et al., 2012). As hearing loss may affect post-release survival through compromised predator evasion (Sand and Karlsen, 2000) and navigation (Gagliano et al., 2008), our results suggest that reducing vaterite prevalence by limiting growth rate could benefit fish restocking efforts. Vaterite prevalence in wild or stocked salmon returning from the sea has never been investigated, but it may reveal the relationship between early growth rate, vaterite formation and marine survival.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: T.R., T.D., S.E.S.; Methodology: T.R., T.D., A.W., P.F., T.H., K.A.G., M.F.S., S.E.S.; Validation: T.R., T.D., K.A.G., M.F.S., S.E.S.; Formal analysis: T.R., A.W., P.F., K.A.G., M.F.S., S.E.S.; Investigation: T.R., T.H., K.A.G., M.F.S.; Resources: T.R., T.H., K.A.G., M.F.S., S.E.S.; Data curation: T.R., A.W., P.F., K.A.G., M.F.S.; Writing - original draft: T.R.; Writing - review & editing: T.R., T.D., K.A.G., M.F.S., S.E.S.; Visualization: T.R., K.A.G., M.F.S., S.E.S.; Supervision: T.H., K.A.G., S.E.S.; Project administration: T.R., T.H., K.A.G., M.F.S., S.E.S.; Funding acquisition: T.D., P.F., T.H., K.A.G., S.E.S.

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