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Effects of Hybridization and Triploidization on Gene Expression in Rainbow Trout (*Oncorhynchus mykiss*)♀ × Brook Trout (*Salvelinus fontinalis*)♂ Hybrids

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Abstract

The transcriptomic effects of hybridization and triploidization were investigated in the diploid and triploid rainbow trout, diploid brook trout and in the triploid hybrids of rainbow trout and brook trout. The examined fish were reared under identical conditions for about two and a half years after hatching. Expression of ten genes involved in cellular respiration (*Atp5bp*, *Slc25a5*), mitochondrial functioning (*Mrpl28*, *Micu2*), ribosome biogenesis (*Rpl24*, *Rps24*), proteasome-mediated protein turnover (*Derl1*, *Psmc2*) and protein chaperoning (*Hsp90B1*, *Pdia4*) were studied in liver and muscle tissues. Most of the analyzed genes displayed comparable expression levels across all the fish groups, with triploid hybrids showing expression patterns more similar to the purebred diploid trout. Compared to all other fish groups, purebred triploid rainbow trout exhibited significant upregulation of *Slc25a5*, *Derl1*, *Rps24* and *Rpl24* genes in liver and downregulation of *Micu2* gene muscles. In turn, triploid hybrids showed marked upregulation of *Atp5pb*, *Slc25a5*, *Rpl24*, *Rps24* and *Pdia4* genes in muscle and downregulation of *Hsp90B1* gene in both liver and muscle when compared to all the fish groups examined. Although protein synthesis- and energy-related genes were upregulated in the muscles of triploid hybrids, the recoded growth performance data did not indicate clear evidence of growth heterosis, suggesting that the potential benefits of increased heterozygosity in this cross may be counterbalanced by metabolic inefficiencies. Three- to fourfold downregulation of heat-shock protein genes was observed in both tissues of triploid hybrids compared with purebred diploid and triploid trout, indicating possible maladaptive genomic incompatibilities usually observed in salmonid fish hybrids that may reduce their heat tolerance under aquaculture conditions. The obtained results also revealed significant upregulation of genes linked to liver protein synthesis and energy production, along with muscle protein turnover in the triploid rainbow trout, supporting the hypothesis that the higher energy demands for maintaining proper protein concentrations in the larger cytoplasmic cell volume of triploid fishes may be met through enhanced protein metabolism.

Keywords: aquaculture; hybridization; triploidization; gene expression; salmonid fishes

1. Introduction

Distant hybridization is a genetic breeding technique widely used in aquaculture to produce fish with enhanced or new traits that are more suitable for commercial production and better aligned with the customer preferences [1–3]. The integration of genomes from different species results in rapid changes to both, genotype and phenotype, often triggering hybrid heterosis [4–6]. As a result, distant fish crosses may exhibit higher growth rates, enhanced resistance to pathogens, increased tolerance

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to unfavorable environmental conditions, improved meat quality and more attractive appearance [7–11]. There is, nevertheless, a negative relationship between hybrid fitness and the accumulation of neutral or adaptive genetic differences between the parental genomes, making the crossing of different species not only an opportunity for trait superiority but also a challenge, as it usually leads to the hybrid breakdown [12–14].

Induced triploidization is another reproductive biotechnique utilized in aquaculture to address the challenges of precocious sexual maturation in fish [15] and reduced viability of distant fish hybrids [16]. In aquaculture, triploidization is artificially induced by exposing fertilized eggs to chemical or physical shocks (*i.e.*, sub-lethal temperature or high hydrostatic pressure), which prevents the extrusion of the second polar body from the egg [17,18]. The nuclear genome of the resulting autotriploid embryos comprises three (3n) sets of chromosomes (two from the female and one from the male) [19]. The odd number of chromosomes in the triploid fish disturbs gonadal development and gamete production that usually result in the functional sterility what in turn eliminates the negative effects of sexual maturation on the growth rate, meat quality and overall fitness of the diploid individuals [20,21]. Production of the sterile triploids also allows for the mitigation of potential ecological risks posed by escaping of cultured fish from the farms or being introduced into natural watersheds for the recreational purposes [22,23].

Available studies indicate that in triploid hybrids, combination of two divergent genomes has generally a greater impact on the fish transcriptome than presence of additional set of chromosomes [24–27]. In the case of distant hybridization, one of the most pronounced outcomes observed in fish is the transcriptomic shock, characterized by a sudden and substantial shift in the genome-wide expression patterns [27–30]. Accumulating evidence indicates that the observed transcriptomic dysregulation in distant salmonid fish hybrids is mainly associated with significant under-expression of genes involved in energy metabolism, proteostasis and stress response [31–34]. On the other hand, the impact of triploidy on gene expression in fish is unexpectedly smaller than anticipated, as only a limited number of genes in the autotriploid fish exhibit differential expression compared to diploids [25,35–40]. Most of the upregulated genes in triploids are associated with energy metabolism, protein synthesis and stress response, likely as an adaptive mechanism counteracting their physiological disadvantages associated with limited aerobic energy budget and increased susceptibility to hypoxia and thermal stress [35–40]. In turn, the downregulated genes in autotriploid fish are linked with the cellular division processes, reflecting cell cycle disruptions caused by the presence of odd chromosome number in their nuclei [25,38].

The rainbow trout (*Oncorhynchus mykiss*) is the second most important salmonid species in aquaculture, with total production exceeding one million tons in 2022 [41]. Unfortunately, global production of the species has been significantly limited in recent decades due to its low resistance to viral haemorrhagic septicaemia (VHS) and infectious hematopoietic necrosis (IHN) [42]. Both diseases are currently widespread around the world, causing vast economic losses for the rainbow trout farms due to the lack of effective treatments [43–45]. Existing reports have demonstrated that certain charr species (*Salvelinus sp.*) exhibit resistance to the mentioned infections; however, their aquaculture production is more challenging and far less profitable than that of rainbow trout [46,47]. In this regard, triploid crosses between rainbow trout and brook trout (*Salvelinus fontinalis*) have lately garnered attention as a promising candidate for the commercial production in the fish farms affected by frequent outbreaks of VHS and IHN [48]. Preliminary studies indicated that mentioned 3n hybrids can inherit at least partial resistance to VHSV, showing delayed disease onset and lower cumulative mortality relative to pure rainbow trout [46]. However, it remains unclear how strongly this resistance is expressed in hybrids and whether it is consistently maintained.

The “Salmocross” project has been recently initiated in Poland to develop an efficient biotechnological method for production of triploid hybrids of rainbow trout and brook trout, along with optimizing their commercial rearing technology [49]. Despite the considerable potential of this hybrid for the aquaculture production, information on the influence of sub-genome reconciliation together with polyploidization on the transcriptome network and physiology is unavailable. The aim

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of this study was, therefore, to explore how distant hybridization and triploidization reshape the expression of genes central to energy metabolism, proteostasis and stress response in rainbow trout × brook trout crosses. For this purpose, the expression of ten genes involved in core metabolic and stress response processes previously identified in RNA-seq studies as among the most differentially expressed in salmonid hybrids was quantified using real-time PCR.

2. Results

2.1. Growth Performance of Examined Fish

After about 2 years and 6 months of rearing, the highest average body weight and length were observed in purebred rainbow trout, both triploid (3n RT, bw=689 ±77g, bl=38.3 ±1,7cm) and diploid (2n RT, bw=666 ±75g, bl=37 ±1,6cm). In contrast, the lowest growth parameters were recorded in purebred diploid brook trout (2n BT, bw=454 ±50g, bl=1.4cm) and triploid hybrids (3n RT×BT, bw=481, ±68g bl=35.2 ±1.5cm). Statistically significant differences (p<0.05) in both traits were found between the group of purebred rainbow trout (2n RT, 3n RT) and the group comprising diploid purebred brook trout together with triploid hybrids (2n BT, 3n RT×BT). No significant differences (p>0.05) were detected between diploid (2n RT) and triploid (3n RT) purebred rainbow trout, nor between purebred diploid brook trout (2n BT) and triploid hybrids (3n RT×BT) (Figure 1).

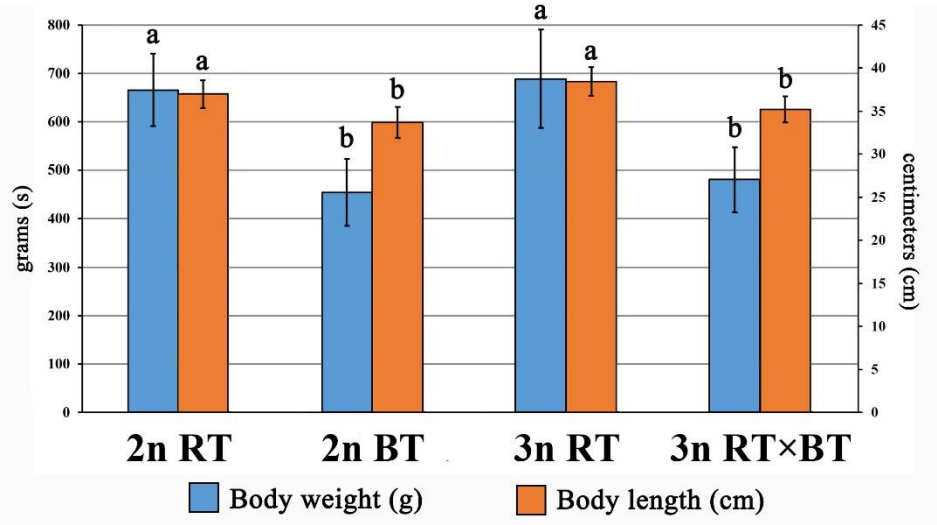

Figure 1. Average body weight and length recorded for diploid (2n RT) and triploid rainbow trout (3n RT), diploid brook trout (2n BT), as well as triploid hybrids between rainbow trout × brook trout (3n RT×BT) examined under the present study after about two years and six months of rearing under identical aquaculture conditions.

2.2 Ploidy and Hybridization Confirmation

Performed flow cytometry analysis confirmed ploidy level of diploids (2n RT and 2n BT) and triploids (3n RT and 3n RT×BT) as assumed (Figure 2a,b). The amplification of *OMM-1279* microsatellite DNA marker yielded products of length 200–300 base pair (bp) for rainbow trout and 550–1500 bp for brook trout (Figure 2c). The molecular screening conducted on all the sampled fish revealed genotypes originating from both parental species, confirming their hybrid status. Moreover, the carried-out PCR-based DNA genotyping affirmed the genetic sex of all the sampled fish to be females (Supplementary figure 1).

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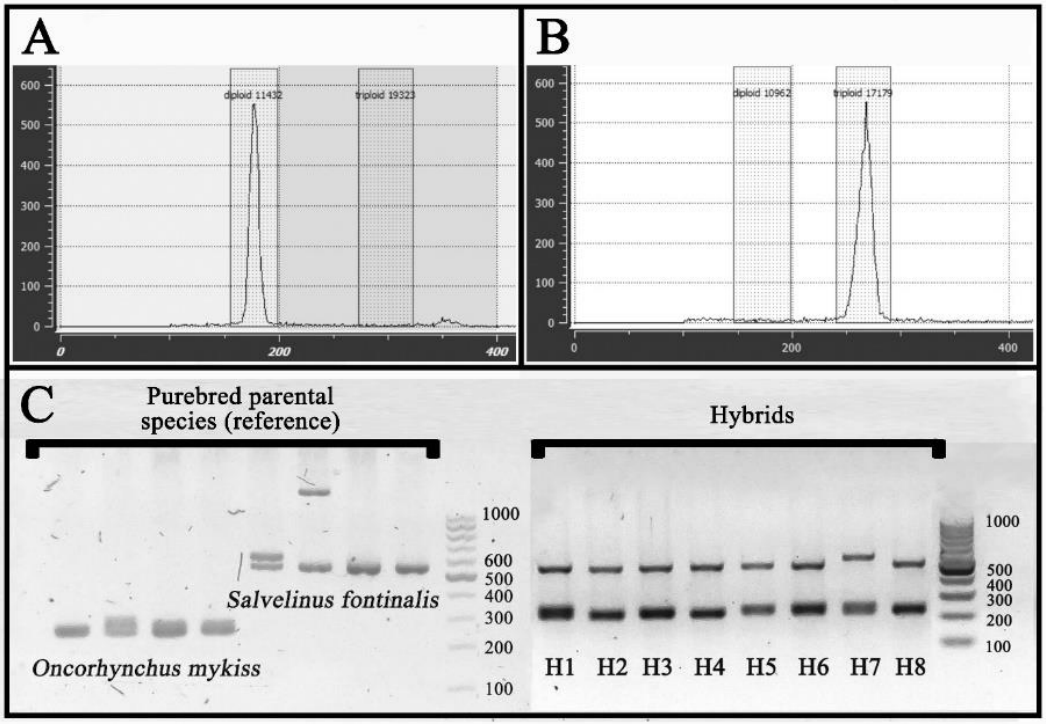

Figure 2. Results of ploidy verification and hybrid status confirmation of the fish used in the current study. Flow cytometry profiles of cellular DNA content of (A) the diploid (2n) and (B) the triploid (3n) individuals sampled. (C) genotyping results of the triploid rainbow trout × brook trout (*Salvelinus fontinalis*) hybrids and purebred parental species (reference) by PCR amplification of *OMM-1279* microsatellite DNA marker. DNA weight marker: 100 bp DNA ladder (A&A Biotechnology s.c., Poland).

2.2 Gene Expression Analysis

The mRNA expression for each investigated gene was detected in every tissue sampled from all fish (Figure 3 and 4). In the liver tissue, significantly (p<0.05) higher mRNA transcription levels of *Slc25a5*, *Derl1*, *Rps24* and *Rpl24* genes were recorded for triploid purebred rainbow trout when compared to diploid rainbow and brook trout, as well as triploid hybrids (Figure 3b,c,e,f). Insignificant (p>0.05) differences in the gene expression levels among all studied fish groups were observed for *Atp5pb*, *Psmc2*, *Mrpl28* and *Micu2* genes in the liver. For the *Pdia4* gene, significant (p<0.05) differences in transcription levels were observed only in diploid brook trout, where expression was approximately three times lower than in all other fish groups (Figure 3a, d and 4a, b, d).

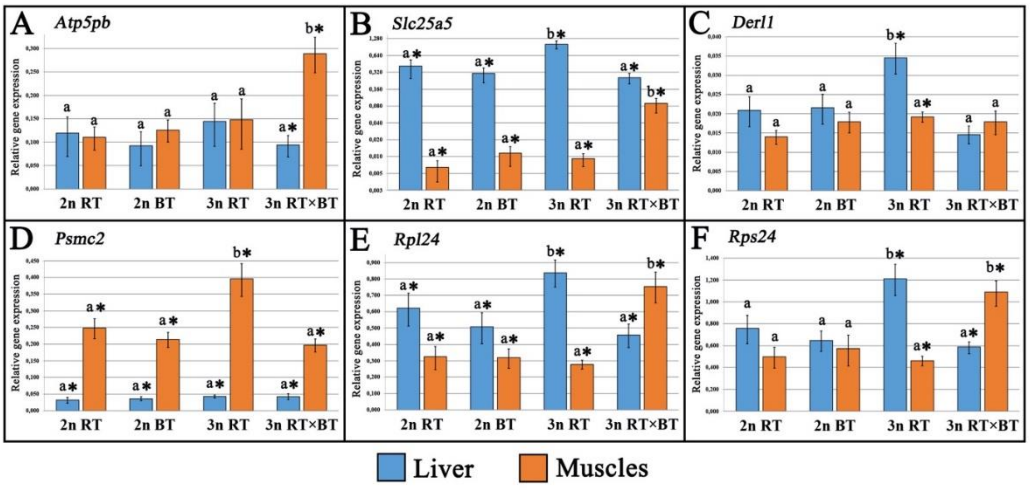

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Figure 3. Relative expression levels of (**a**) ATP synthase peripheral stalk-membrane subunit b (*Atp5pb*), (**b**) ADP/ATP translocase 2 (*Slc25a5*), (c) Derlin1 (*Derl1*), (**d**) 26S proteasome regulatory subunit 7 (*Psmc2*) (**e**) 60S Ribosomal Protein L24, (*Rpl24*) and (**f**) 40S ribosomal protein S24 (*Rps24*) in the liver and muscle tissues sampled from diploid (2n RT) and triploid rainbow trout (3n RT), diploid brook trout (2n BT), as well as triploid hybrids between rainbow trout × brook trout (3n RT×BT) examined under the present study. Distinctive letters indicate statistically significant (p<0.05) differences in the recorded gene expression levels between examined fish groups within each tissue type. Asterisks indicate statistically significant (p<0.05) differences in the gene expression levels between tissues within fish group. Values are presented as fold changes relative to the reference genes (*β-actin* and *elongation factor 1-alpha*). The measure of variation is derived from the respective SEM of the Ct values. Data is shown as the mean with standard deviation.

For muscle tissue, significant (p<0.05) upregulation in the mRNA transcription level was detected in triploid hybrids for majority of examined genes (*Atp5pb*, *Slc25a5*, *Rpl24*, *Rps24* and *Pdia4*) with slightly (p>0.05) higher expression in triploid rainbow trout when compared to their diploid rainbow trout and brook trout (Figure 3a, b, e, f and 3d). In the case of *Psmc2* gene, the recorded expression levels significantly higher (p<0.05) in triploid rainbow trout, when compared to all other fish groups (Figure 3d). Among all analyzed genes significant (p<0.05) downregulation of expression level was detected for *Micu2* gene in the triploid rainbow trout (Figure 4b). Lack of significant (p>0.05) differences in the gene expression levels between all studied fish groups were observed in *Derl1* and *Mrpl28* genes in the muscle tissue (Figure 3c and 4a).

Three- to four-fold lower (p<0.05) expression levels of *Hsp90B1* gene were detected in both tissues of triploid hybrids compared to diploid and triploid rainbow trout, as well as diploid brook trout. Compared to diploid rainbow trout and brook trout specimens slightly upregulated *Hsp90B1* transcription levels were recorded in triploid rainbow trout, however; the observed differences were insignificant (p>0.05) in both tissues examined (Figure 4c).

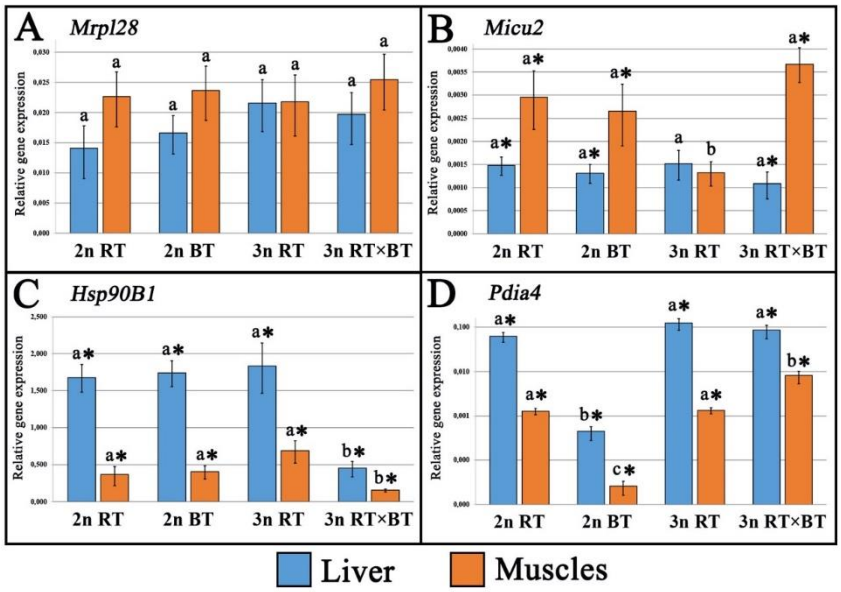

Figure 4. Relative expression levels of (**a**) Mitochondrial ribosomal protein L28 (*Mrpl28*), (**b**) Mitochondrial calcium uptake protein 2 (*Micu2*), (**c**) Heat shock protein 90 β family member 1 (*Hsp90B1*), (**d**) Protein disulfide-isomerase A4 (*Pdia4*) in the liver and muscle tissues sampled from diploid (2n RT) and triploid rainbow trout (3n RT), diploid brook trout (2n BT), as well as triploid hybrids between rainbow trout × brook trout (3n RT×BT) examined under the present study. Distinctive letters indicate statistically significant (p<0.05) differences in the recorded gene expression levels between examined fish groups within each tissue type. Asterisks indicate statistically significant (p<0.05) differences in the gene expression levels between tissues within fish group. Values are presented as fold changes relative to the reference genes (*β-actin* and *elongation factor 1-alpha*). The

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measure of variation is derived from the respective SEM of the Ct values. Data is shown as the mean with standard deviation.

3. Discussion

Interspecific hybridization and triploidization are recognized as an important reproductive biotechniques used in aquaculture [3,5,18,50]. Crossing different salmonid species enables the production of commercially desirable fish stocks, while induced triploidy improves their early survival and prevents precocious sexual maturation [16,51–53]. Along with the instant and severe genotype modification, associated with the loss and/or gain of novel genes and alleles, as well as establishment of new gene expression patterns, both techniques also lead to the phenotypic and physiological changes in the resulting fish progeny [54–57]. Nevertheless, interspecific hybridization generally has a stronger impact on the fish transcriptome than triploidization [24–27]. Interactions among divergent sets of genes, alleles, transcription factors and chromatin profiles originating from different sub-genomes result in fast and widespread transcriptomic dysregulation in distant fish crosses, with many genes showing substantial up- or down-regulation when compared to their purebred parental species [27,29,30,32,33,58]. Although triploidization leads to increased heterozygosity and gene dosage, its impact on the fish transcriptome is weaker, as only a limited number of genes in triploid fishes shows significant differences in expression level compared to diploids, which is attributed to the phenomenon of gene dosage compensation [35–38]. In the present study, the effects of hybridization and triploidization on expression patterns of ten selected genes involved in pathways related to cellular respiration and energy production, proteasome-mediated protein turnover, ribosome biogenesis, mitochondrial functioning, as well as protein chaperoning were examined in the liver and muscle tissues of triploid (3n) crosses between rainbow trout and brook trout that are promising for the commercial aquaculture production. Flow cytometry analysis and DNA genotyping confirmed that the examined fish were triploid hybrids between mentioned species (Figure 2a, b).

One of the key outcomes of the additional set of chromosomes in polyploids is the genome dosage effect, which involves an increase in gene expression proportionally to the number of its copies [59–61]. Unlike mammals, fishes adapt well to the genome alterations caused by ploidy changes because of their ability to functional re-diploidization [25,30,62–64]. The observed stabilization of genome expression in polyploid fishes towards a diploid state is achieved through gene dosage compensation, which relies on mechanisms of cell number reduction per tissue volume [65,66], chromatin remodeling [67], DNA methylation [63] and microRNA-mediated post-transcriptional regulations [68,69]. Available studies suggest that the dosage compensation in triploids involves proportional silencing of each gene copy, with all three remaining transcriptionally active in an additive pattern, typically resulting in genome-wide homeolog expression dominance (HED) and bias (HEB) towards the maternal genome [58,70–73]. In this study, most analyzed genes, *i.e.*, *Slc25a5*, *Derl1*, *Rpl24*, *Rps24* (in muscles), *Psmc2*, *Micu2* (in the liver), *Atp5bp*, *Mrpl28*, *Hsp90* and *Pdia4* (in both tissues), exhibited dosage compensation in the purebred triploid rainbow trout with expression levels comparable to their diploid counterparts (Figure 3 and 4). Among mentioned genes, the *Atp5pb*, *Micu2*, *Pdia4* (in the liver tissue), *Derl1* (in the muscles) as well as *Mrpl28* and *Psmc2* (in both liver and muscles) also displayed unchanged expression levels in the triploid rainbow × brook trout hybrids, when compared to the diploid rainbow trout and brook trout, likely indicating their essential role in maintaining key physiological functions necessary for the viability of the examined hybrids.

In polyploids, genome dosage compensation mechanisms do not always precisely match expression levels to that observed in diploids, as some genes exhibit significant upregulation or downregulation, which is referred to as positive and negative dosage compensation [63,74]. Intriguingly, all genes that displayed positive (*Slc25a5*, *Rpl24*, *Rps24*, *Derl1* and *Psmc2*) or negative (*Micu2*) dosage compensation in the tissues of purebred triploid rainbow trout relative to their diploid counterparts also exhibited comparable expression levels between the examined triploid

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hybrids and the purebred diploid parental species (Figure 3b–f and 4b). Given that diploid hybrids between rainbow and brook trout are inviable and die before yolk sac resorption [53], it can be speculated that the induction of triploidization in the examined hybrids may alleviate the severity of transcriptomic shock caused by merging genomes from different species. Indeed, accumulating number of studies indicate that whole-genome duplications following hybridization can weaken the post-zygotic reproductive constraints, enhancing viability and fitness of the allopolyploid fishes [75–79]. Available research indicates that the observed reduction in transcriptomic dysregulation in allopolyploid fishes primarily stems from increased gene expression flexibility, driven by variable dosage compensation, which involves the silencing of cis-regulatory elements and the enhancement of trans-regulatory genome mechanisms [26,27,63,80]. As triploid fish hybrids examined in this study inherit two chromosome sets from rainbow trout (2n) and one from brook trout (1n), their increased viability observed is most probably associated with the transcriptional dominance of the maternal sub-genome. Numerous studies have revealed that allotriploid fishes retain a larger portion of the maternal genome, including maternal-specific genes together with profiles of genome methylation and transposable element density, which reduces incompatibilities between nuclear and mitochondrial genomes in hybrid offspring, alleviating the severity of potential cytonuclear conflicts that could impair core cellular physiological processes [58,72,81–85].

The first generation of fish hybrids typically exhibits enhanced trait parameters, a phenomenon known as heterosis or transgressive inheritance [1,2,78]. The observed trait superiority in distal fish crosses over purebred parental species is usually linked with enhanced growth parameters [86–88], innate immune response [89,90] and resistance to unfavorable environmental conditions [91–93]. Available studies indicate that the heterosis effect in allopolyploid fishes is mainly attributed to their increased heterozygosity together with gene redundancy, incomplete dosage compensation and maternal/paternal dominance effects [24,63,80,94]. Multiple transcriptomic studies have linked the upregulation of genes associated with protein synthesis and energy metabolism to the growth heterosis in allopolyploid fishes [24,25,58,95]. Similarly, significant upregulation of genes involved in protein synthesis (*Rpl24*, *Rps24* and *Pdia4*) and energy-production (*Atp5pb*, *Slc25a5* and *Micu2*) was detected in the muscles of examined the triploid hybrids between rainbow and brook trout, potentially indicating heterosis effect linked to growth and metabolic efficiency (Figure 3a, b, e, f and 4b, d). However, recorded in this study morphological data did not show clear sign of growth heterosis in the examined triploid hybrids (Figure 1), suggesting that the potential benefits of increased heterozygosity in this cross may be counterbalanced by metabolic inefficiencies. The comparable growth performance of the examined triploid hybrids to diploid purebred brook trout might indicate that maternal rainbow trout's sub-genome dominance at the transcriptional level leads to their improved viability but not translates into enhanced growth. It is also important to note that extensive research on allotriploid and autotriploid salmonid fishes has highlighted the critical role of the maternal genome's origin in shaping traits complex among hybrid families [20,70,71,96–98]. Therefore, further research on elucidation how the maternal origin of the genome influences growth rate, resistance to environmental stress and pathogens, survivability, as well as deformity rates in the triploid hybrids of rainbow trout × brook trout examined here, offers viable opportunity to gain valuable information for the future improvement of their aquaculture production.

Allopolyploidization is known to impose significant stress on organisms by causing molecular, chromosomal and cellular changes that disrupt key processes essential for genomic stability, protein synthesis and metabolic regulation [21,24,30,99,100]. In the case of autotriploid fishes, their normal development is hypothesized to depend on the upregulation of stress-response genes, which likely act as a compensatory mechanism to mitigate physiological (*e.g.*, oxidative stress) and environmental (*e.g.*, hypoxia, hyperthermia) challenges arising from their limited aerobic energy budget and reduced capacity to store energy reserves [21,65,66,70,101,102]. The *Hsp90B1* and *Pdia4* genes analyzed in this study are central to cellular protein homeostasis, ensuring their proper folding, stabilization after synthesis, as well as repair during thermal and oxidative stress [103–105]. Consistent with previous research, a slight but statistically insignificant upregulation of both genes

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was also observed in the examined triploid (3n) purebred rainbow trout compared to diploid individuals (Figure 3c, d) [9,35,37–40]. Conversely, a three- to four-fold downregulation of *Hsp90B1* gene expression was observed in the liver and muscles of the examined triploid hybrids compared to purebred diploid and triploid rainbow trout (Figure 4c). These findings align with other studies on allopolyploid salmonid fishes that have reported significant downregulation of stress-response genes, particularly those involved in protein folding and structural maintenance. It is believed that this downregulation results from improper gene expression regulation or negative epistatic effects due to maladaptive genome incompatibilities between the parental species [28,32–34,106,107]. Nevertheless, the observed *Hsp90B1* suppression could also reflect tissue-specific developmental timing, with expression peaks occurring earlier or later than the sampled stages of ontogenetic development, or altered mRNA stability in hybrids. In the case of allotriploid fishes, it may represent an adaptive response to allotriploidization, that relies on reallocating energy from protein chaperoning to other essential functions such as osmoregulation, immune defense and cellular repair [103,108,109]. Despite the triploid hybrids examined in this study developed under optimal aquaculture conditions comparable to purebred diploid rainbow trout, the recorded suppression of *Hsp90B1* gene could potentially impair their tolerance to suboptimal high temperatures, particularly during early ontogenetic development. Nevertheless, no research has yet been performed to assessed the impact of the recorded underexpression of heat shock proteins on the viability of the examined triploid hybrids under suboptimal high temperatures, particularly during early ontogenetic development.

Despite the expectation that larger cells may have lower mass-specific metabolic rates [102,110,111], several studies revealed that autotriploid fishes often exhibit similar or even higher metabolic rates along with increased postprandial energy demands for protein processing when compared to diploids [21,65,112,113]. It is hypothesized that this phenomenon results from higher translation rates in polyploid cells, compensating for their increased cytoplasmic volume to maintain proper protein concentrations [114–118]. In this study, significant upregulation of genes involved in ribosome biogenesis (*Rpl24* and *Rps24*) and endoplasmic reticulum–associated protein degradation (*Derl1*) in the liver, together with increased expression of the protein turnover gene (*Psmc2*) in the muscles of the purebred triploid rainbow trout was recorded (Figures 3b, c, e, f, and 4b), likely indicating enhanced liver protein synthesis, along with muscle protein turnover [119–121]. Additionally, higher expression of the energy production gene *Slc25a5* in the liver, accompanied by a marked suppression of the mitochondrial function gene *Micu2* in muscles, suggests a shift in energy allocation toward liver metabolism in the examined purebred triploid rainbow trout [110,115,122–124]. However, the increased expression of the *Psmc2* gene may be also linked to an enhanced rate of hypertrophic muscle growth observed in triploid fish [21,65,125]. Previous research on triploid brook trout has revealed approximately one-third faster ammonia excretion and lower crude protein retention compared to diploids, suggesting that the increased liver energy demands for protein processing in triploids may be met through muscle protein metabolism [113]. These metabolic constraints, stemming from the elevated energy costs of protein synthesis and turnover, as well as ammonia detoxification, may be additional contributors to the reduced stress tolerance of autotriploid fishes under high temperatures, hypoxia, or exhaustive exercise. If true, adjusting triploid diets to include higher levels of lipids or carbohydrates might enhance energy storage and reduce protein waste, thereby improving their aquaculture efficiency. However, further studies are required to validate this very tentative hypothesis.

4. Materials and Methods

4.1. Ethics

This study was carried out in strict accordance with the recommendations in the Polish ACT of 15 January 2015 on the Protection of Animals Used for Scientific or Educational Purposes (Journal of Laws 2015, item 266). The experimental protocol used in this study was approved by the Local Ethical

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Committee for Experiments on Animals in Bydgoszcz, Poland (Permission no. 9/2022). This article does not contain any studies with human participants performed by any of the authors.

4.2. Fish Origin and Maintenance

The fish examined in this study were produced using rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) gamete donors from the broodstocks kept at the Department of Salmonid Research (DSR) of the Inland Fisheries Institute (IFI) in Olsztyn, Rutki (Poland) (54°19'51.1"N 18°20'15.1"E). Purebred diploid (2nRT) and triploid (3nRT) stocks of rainbow trout examined in the current study were obtained through the fertilization of the eggs with milt from the sex-reversed females, i.e., neo-males (XX). Diploid purebred brook trout (2nBT) specimens were obtained from the mixed-sex stock that was produced through routine species reproduction. In turn, triploid hybrids between rainbow trout and brook trout (3n RT×BT) were obtained through the controlled crossing of rainbow trout females and brook trout males.

Three- and four-year-old spawners used as gamete donors were reared in ponds with a capacity of 10–30 m^3 and a water flow 5 dm^3/s. The mean water temperature was 10°C and oxygenation level exceeded 80% of maximal oxygen concentration at a given temperature. The fish were fed with commercial broodstock feed (Aller Bronze 3 mm and Aller Silver 3 mm, Aller Aqua, Poland) three or four times daily in amounts ranging from 0.5 to 1.5% fish biomass depending on water temperature [126]. Fish feeding was stopped two weeks before the spawning period. Prior to the gamete stripping, selected spawners were anesthetized with MS-222 (50 mg/dm^3, Sigma-Aldrich, Spain). Eggs were individually collected from five random rainbow trout and brook trout females as well as inseminated with milt obtained from two rainbow trout neo-males and two brook trout males. Before fertilization, spermatozoa quality was assessed by directly observing milt activated with sperm activating medium (SAM) (1 mM $CaCl_2$, 20 mM Tris, 30 mM glycine, 125 mM NaCl pH 9.0) [127] under an optical microscope (Nikon Eclipse E 2000) at a total magnification of 100×.

The protocol for the triploidization of rainbow trout eggs fertilized with homologous and heterologous sperm involved a five minute high hydrostatic pressure (HHP) shock of 9500 psi that was applied 350 degree minutes (CTMs) after the egg activation [128]. The HHP shock was generated using the TRC-APV electric/hydraulic device (TRC Hydraulics Inc. in Dieppe, Canada) and an aqua pressure vessel constructed by technician staff at the Fish Farm "Pstrąg Tarnowo". All batches of eggs; purebred diploid (2n) and triploid (3n) rainbow trout, as well as triploid (3n) crosses of rainbow trout and brook trout were incubated at a temperature of 7.0 ±0.5°C, with oxygen levels maintained at 10.8 ±0.5 mg/L and the pH at 7.5 ±0.1, in the separate vertical incubators with egg trays.

Hatched fish were reared under identical conditions in tanks and ponds supplied with water from the Radunia River (a mean temperature of 10°C and oxygenation levels above 80%) for about two years and six months (82 weeks). After yolk-sac resorption, the fry was stocked into 0.3 m^3 plastic tanks with a water flow rate of 1 liter per second (l/s). When the fish attained a mean body weight of 5 and 100 g/indiv., they were transferred to 1 m^3 and 3 m^3 tanks, respectively, with a flow rate of 4 l/s. After reaching 300 g/indiv., they were reared in 10–30 m^3 concrete rectangular ponds with a flow rate of 6 l/s. The fish were fed commercial feed formulated for salmonids (Aller Aqua Company, Poland) three to four times daily in accordance with rainbow trout feeding recommendations at specific temperatures [129]. During the rearing phase, fish were fed manually until they attained a mean body weight of 10 g, after which automatic timer feeders (FIAP 1520, Fiap Company, Germany) were used. Feeding ceased during periods when the water temperature exceeded 20°C.

After about two years and six months of rearing, forty randomly selected fish from each experimental stocks were weighed by a Radwag C315.60.C2.M warehouse scale (±1 g) and measured by scale (±1 mm) for total body mass (bm) and length (bl). Then, eight individuals from each experimental group were chosen for transcriptomic analyses. All selected fish had a confirmed ploidy level and were molecularly verified as females to ensure the reliability of the study material and eliminate potential sex-biased variability in the expression of analyzed genes. Before sampling, the fish were humanely sacrificed by cutting spiral cord. Liver and muscle tissues (approximately 0.5

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cm^3) were immediately collected, submerged into RNAlater™ solution (Thermo Fisher Scientific, Waltham, MA, USA), incubated at 4°C for 24 hours and finally stored at -24°C until further analyses.

4.3. Ploidy, Genetic Sex and Hybridization Verification

The ploidy level of the examined fish was confirmed with CyFlow® Ploidy Analyzer (Sysmex Partec GmbH, Germany) equipped with a CyViewTM software v1.8.0.82. For this purpose, adipose fins were sampled from each fish and then immediately processed with a CyStainTM UV Precise T kit (Sysmex Partec GmbH, Germany). Before analysis, about 10 mg of fin material was minced, incubated for 5 min in Extraction Buffer solution, and then stained with DAPI Staining Buffer.

To verify the genetic sex and hybrid status of the of the examined fish, PCR-based DNA genotyping was carried out. For this purpose, small pelvic or pectoral fin clips were collected from each sampled fish, preserved in 96% ethanol and stored at a temperature of 4°C until genetic material extraction. Fin clips previously collected from other random four rainbow trout and brook trout individuals were used as a reference. Genomic DNA was isolated from collected fin clips using the standard Chelex-100 method [130]. The quality and amount of the isolated genomic DNA was checked by the NanoDrop™ One spectrophotometer (Thermo Scientific). The genetic sex of the sampled fish was verified by the PCR duplex amplification of the Y-chromosome linked DNA marker (*sdY*) and 18S rDNA as a positive control [131]. In turn, hybrid status of obtained progeny resulted from crossing rainbow trout and brook trout was verified by application *OMM-1279* microsatellite DNA marker [132]. The PCR amplifications were carried out using 10 ng of the isolated DNA template in a reaction mixture of 12.5 µl total volume composed by the 1×GoTaq® Hot Start Green Master Mix (Promega), as well as 0.4 µM of each *SdY* and 0.1 µM of each 18S rDNA primers (genetic sex screening) or 0.3 µM of each *OMM-1279* primers (hybrid status verification). PCR amplifications were performed with a Mastercycler® X50s (Eppendorf, Germany, Hamburg) under the following conditions: an initial denaturation at 96°C for 4 min, followed by 35 cycles at 94°C for 30 s, annealing at 60°C for 45 s, elongation at 72°C for 45 s and a final elongation step at 72°C for 10 min. The resulting products of the PCR amplifications were separated in a 2.0% agarose gel (Sigma-Aldrich, St. Louis, USA) stained with ethidium bromide (0.05 mg/ml) and then visualized by a UV trans-illuminator (Vilber Laurmat ECX-20.M, Eberhardzell, Germany).

4.4. RNA Extraction, cDNA Synthesis, and Real-Time PCR Analysis

Total RNA from preserved tissues was extracted using the Bead-Beat Total RNA Mini kit (A&A Biotechnology, Gdańsk, Poland), following the manufacturer's instructions. Residual DNA in the extracted RNA samples was removed using the Clean-Up Concentrator kit (A&A Biotechnology, Gdańsk, Poland). RNA concentration and purity were measured with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was assessed by 1% agarose gel electrophoresis. The obtained RNA samples were immediately processed further.

Purified total RNA samples of satisfactory quality were used to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The reaction mixtures were prepared in a total volume of 20 µL, composed of 1X Reaction Buffer, 5 µM of Random Hexamer primer, 1 µM of dNTP Mix, 20U of RiboLock RNase Inhibitor, 20U of RevertAid M-MuLV RT reverse transcriptase and 1 µg of RNA sample. The reverse transcription reactions were carried out on a Mastercycler® X50a (Epppendorf, Germany). The samples were incubated for 5 min at 25°C, followed by 60 min at 42°C, and terminated by heating at 70°C for 5 min. The obtained cDNA samples were diluted 1:10 in DEPC-treated water and stored at -20°C for further processing.

Transcriptomic analyses included ten target genes involved in five critical biological processes important for viability and fitness of salmonid fish hybrids, namely: (1) cellular respiration and energy production (*ATP synthase peripheral stalk-membrane subunit b* and *ADP/ATP translocase 2*), (2) proteasome-mediated protein turnover (*derlin1* and *26S proteasome regulatory subunit 7*), (3) ribosome biogenesis (*60S Ribosomal Protein L24* and *40S ribosomal protein S24*), (4) mitochondrial functions (*mitochondrial ribosomal protein L28* and *mitochondrial calcium uptake protein 2*) and (5) protein

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chaperoning (*heat shock protein 90 β family member 1* and *protein disulfide-isomerase A4*). The primer sequences for the analyzed genes were designed based on genetic information deposited for rainbow trout in the GenBank (release 101) and Ensembl (release 109) databases or derived from the available literature (Table 1). All available isoforms and splice variants of the analyzed genes were taken into consideration before primer construction. To eliminate, inter-specific sequence variation as a source for differential estimates of gene expression the conserved gene regions between rainbow trout and brook trout were identified and taken into consideration. The primers were designed with default parameters, including the primer location on the exon-exon junction and a qPCR product length of approximately 100–200 bp and checked for complementarity with genomes of both species using the Primer Blast Designing Tool online software (NCBI). All the primers were also amplified in rainbow trout and brook trout, sequenced using a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) and subjected to BLAST analysis against the GenBank nucleotide data to confirm that all primers amplify the correct gene transcript targets.

Table 1. Forward (F) and reverse (R) primer sequences used for the real-time PCR analysis of ten selected genes in the present study.

Gene	Primer Sequences	Amplicon size (bp)	References
ATP synthase peripheral stalk-membrane subunit b (Atp5pb)	F: AAGAAGGAGCAGTGGAGGGC R: CACCATGTGGACCCTCTCCC	108	ENSOMYG00000009141, XM_021616957.2
ADP/ATP translocase 2 (Slc25a5)	F: GGATTCTCCGTGTCGGTCCA R: GGACGGTATCGAAGGGGTAGG	178	ENSOMYG00000025595, XM_021561140.2
Derlin1 (Derl1)	F: AACTGATTGGGAACCTGGTGGG R: GTGGCGCTCCAAACCCAGAC	153	ENSOMYG00000057377, XM_036935651.1
26S proteasome regulatory subunit 7 (Psmc2)	F: ATCAGGGTCATCGGCTCAGA R: GCCCCTCCAATAGCGTCAAT	137	[124]
60S Ribosomal Protein L24 (Rpl24)	F: CAAGAAGGGCCAGTCTGAAG R: CAGGCTTCTGGTTCCTCTTG	119	[133]
40S ribosomal protein S24 (Rps24)	F: AAACCGGCTGCTTCAGAGGAA R: GCCACCACCAAACTGTGTCC	160	ENSOMYG00000047442, XM_036986845.1
Mitochondrial ribosomal protein L28 (Mrpl28)	F: CCAGGATGGCCTATGGGGAG R: GTCTAGTGCGCGAGCCGTTA	175	ENSOMYG00000021960, XM_021587405.2
Mitochondrial calcium uptake protein 2 (Micu2)	F: GACAGTGCCTAAGGAAGGTATCA R: ACCATTCCTGCCAAAGAAGAAGG	97	ENSOMYG00000009310, XM_021617766.2
Heat shock protein 90 β family member 1 (Hsp90B1)	F: TTGCGTGGAACTAAGGTGA R: CCAATGAACTGAGAGTGCT	104	[134]
Protein disulfide-isomerase A4 (Pdia4)	F: ATGAGAAAGCTTCACACACGCT R: CACCAGTGGCAGGATGTGTTTC	92	ENSOMYG00000007216, XM_021620393.2

Real-Time PCR analysis was carried out using designed primers for the target genes, choosing β-actin (*Actb*) (F: GCCGGCCGCGACCTCACAGACTAC, R: CGGCCGTGGTGGTGAAGCTGTAGC) and elongation factor 1-alpha (*Elf1a*) (F: TTAAGCAACCATGGGAAAGG, R: TACCTGCCGGTCTCAAACTT) as the house-keeping genes due to their proven stable expression level across different tissues [39,135–137]. Moreover, numerous studies indicate that the mentioned reference genes show a 1:1 dosage effect between triploid and diploid fishes and maintain stable expression levels in hybrids between rainbow trout and other salmonids, making them suitable as

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internal controls for relative mRNA transcription analyses in the fish examined in this study [87,95,138–140]. The qPCR was performed by a Cielo 6 Real-Time PCR System (Azure Biosystems, Italy) using the PowerTrack SYBR Green Master Mix (Applied Biosystems, California, USA). Reaction efficiencies were estimated from the slopes of the standard curves made of 10-fold serial cDNA dilutions starting from about 20 ng/µL. The optimal reaction conditions for both assays displayed an efficiency between 90 and 110%. The qPCR reaction mixtures were prepared in a total volume of 10 µL, consisting of 1X PowerTrack SYBR Green Master Mix, 0.5–0.8 µM (target genes) and 0.15 µM (housekeeping gene) of each primer, as well as 5 ng of cDNA. The Real-Time PCRs were run in triplicates with the following thermal cycling conditions: an initial polymerase activation step at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s (denaturation), 20 s at 60°C (primer annealing) and 72°C for 15 s (elongation). During each run, negative controls using pure water and non-transcribed RNA were used to exclude reagents and samples contamination. The analysis of the melting curve (60–95°C) at the end of each run concluded the protocol. Fluorescence data were collected after the elongation step and in 0.1°C steps on the melting curve. The relative expression was calculated based on the difference between Ct values for reference and target genes using the Livak and Schmittgen's [141] equation. The expression levels (Ct values) of the reference genes were normalized using the geNorm software [142].

4.5. Statistical Analysis

Statistical analyses of the fish body weight and length together with obtained fold change expression values for target genes relative to the normalized expression levels of reference genes (*ß-actin* and *Elf1a*) were carried out using Statistica software v.10.0 (StatSoft Inc., Tulsa, USA). Prior to analysis, the normality of data distribution and homogeneity of variances were assessed using the Shapiro–Wilk test and Levene's test, respectively. A two-way ANOVA was performed, with fish group (2nRT, 2nBT, 3nRT and 3nRT×BT) and tissue (liver and muscles) as fixed factors. Tukey's HSD post hoc test was used to determine significant differences ($p<0.05$) between fish groups and tissues.

5. Conclusions

This study provides preliminary insights into the transcriptomic effects of hybridization and triploidization in the triploid hybrids between rainbow and brook trout. Most of the analyzed here genes displayed dosage compensation in tissues of the triploid rainbow trout. In the triploid hybrids, stabilization of both positively and negatively compensated genes was recorded what suggests that combination of hybridization and triploidization might alleviate transcriptomic shock in the examined fish cross. Nevertheless, the recorded results did not indicate that the examined triploid hybrids confer a growth advantage over rainbow or brook trout, suggesting that any potential benefits of increased heterozygosity in this cross may be offset by metabolic inefficiencies. Genes encoding heat shock proteins were reduced by three- to four-fold in both tissues of the examined triploid crosses compared to diploid and triploid rainbow trout, possibly diminishing their thermal resilience under aquaculture conditions. Moreover, upregulation of genes linked to the hepatic protein synthesis and energy metabolism, along with enhanced muscle protein turnover in the triploid rainbow trout, reinforcing the idea that increased protein metabolism may compensate for the elevated energy demands required to sustain adequate protein levels in the enlarged cytoplasmic volume of the triploid cells. Further research elucidating how the maternal genome influences growth rate, resistance to environmental stress and pathogens, survivability and deformity rates in triploid hybrids of rainbow trout × brook trout, may provide valuable insights for enhancing their future aquaculture production.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors upon request.

Conflicts of Interest: The authors declare no conflicts of interest.

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