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ORIGINAL PAPER

Hybridization barriers between the congeneric antarctic notothenioid fish *Notothenia coriiceps* and *Notothenia rossii*

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Abstract

Hybridization between species and the establishment of hybridization barriers can influence the diversification of species. Antarctic notothenioid fishes represent a prime example of marine adaptive radiation that diversified in the icy waters of Antarctica from an ancestral population that innovated antifreeze glycoproteins. The processes by which Antarctic notothenioid species evolved, however, remain elusive, and interspecific hybridization or the establishment of hybridization barriers between lineages may have influenced species diversification. To evaluate the current hybridization potential of notothenioids, we performed an experimental in vitro fertilization cross between two sympatric and congeneric notothen species using oocytes from the bullhead notothen *Notothenia coriiceps* and sperm from the marbled notothen *N. rossii*. Resulting embryos developed to late gastrula/early neurula stages and then suddenly died. Genetic analyses of embryos and parents demonstrated that the embryos lacked detectable paternal DNA and were thus gynogenetic. While premating barriers are likely to exist between the two species, this experiment suggests a strong postmating, prezygotic reproductive barrier preventing hybridization between the sister species due to gametic incompatibility in this directional cross. Our study provides novel information on mechanisms that may have contributed to the divergence and maintenance of these two ecologically important congeneric species.

Keywords Cryonotothenioid · Nototheniidae · Postmating prezygotic barrier · Gametic incompatibility · Speciation

Introduction

Hybridization between species and the establishment of barriers to prevent hybridization can influence the diversification of species (Seehausen 2004; Mallet 2007; Genner and Turner 2012; Abbott et al. 2013; Taylor and Larson 2019; Marques et al. 2019; Gillespie et al. 2020; Pfennig 2021). Antarctic notothenioid fishes, also known as cryonotothenioids, constitute a remarkable example of marine adaptive radiation that diversified during the past 10.7 million years (Bista et al. 2023) from a single ancestor to more than a hundred species that now inhabit the frigid waters of the Southern Ocean (Eastman and Eakin 2021). While geographic isolation likely explains the divergence of several cryonotothenioid lineages and species by allopatric speciation (e.g., Hüne et al. 2015; La Mesa et al. 2017; Desvignes et al. 2020), a few population genetics studies revealed putative hybrids between parapatric sister species (Marino et al. 2013; Dornburg et al. 2016a, b; Schiavon et al. 2021), which suggests

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incomplete reproductive isolation between some closely related species. Furthermore, an in vitro cross between a blackfin icefish female *Chaenocephalus aceratus* and a male ocellated icefish *Chionodraco rastrospinosus* produced intergeneric hybrids that survived past hatching to swimming larvae; unfortunately, further study of hybrid survival and development was precluded by logistic constraints of work in Antarctica (Desvignes et al. 2019). These examples of possible natural hybridization between related cryonotothenioid species motivate the analysis of the roles of interspecific hybridization and of hybridization barriers in the diversification of this iconic clade. Here, we evaluated the hybridization potential between two sympatric and congeneric notothenioid species: the bullhead notothen *Notothenia coriiceps* and the marbled notothen *N. rossii* (Eastman et al. 2011; Duhamel et al. 2014; Caccavo et al. 2021) by performing in vitro fertilization using *N. coriiceps* oocytes and *N. rossii* sperm. Results revealed gynogenetic embryos activated by, but with no genetic contribution from, the sperm and embryos that died uniformly well before hatching, consistent with a strong postmating, prezygotic barrier to hybridization.

Methods

Collection of adult specimens

The female bullhead notothen *Notothenia coriiceps* specimen was collected during the night of April 20–21, 2018, using an 18-ft otter trawl equipped with rockhopper gear deployed from the *ARSV Laurence M. Gould* at a depth of ~ 150 m in the Antarctic Specially Protected Area (ASPA) 152 (Western Bransfield Strait) located southwest of Low Island (63°29'S 62°41'W), as previously described (Desvignes et al. 2022). The male marbled notothen *Notothenia rossii* specimen was captured during the night of June 5–6, 2018, in the ASPA 153 (Eastern Dallmann Bay) (63°55'S 62°47'W), about 45 km distant, using the same rockhopper otter trawl at a depth of ~ 180 m.

Upon collection, live fish were transferred immediately from the trawl net to the ship aquaria (1-m^3 Xactics™, Cornwall, Ontario, Canada), which were supplied with flow-through seawater and enhanced aeration. Live fish were transported within 1–2 days to aquaria facilities at Palmer Station, Antarctica, where they were maintained in 2.5-m^3 circular flow-through seawater tanks at ambient temperatures of − 1 to 0 °C following previously described husbandry guidelines (Le François et al. 2017).

All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Oregon (#13-27RRAA) and of Northeastern University (#18-0103R), and access to ASPAs 152 and 153 was authorized by Antarctic Conservation Act Permit ACA 2016–025.

Gametes and in vitro fertilization

On June 7, 2018, mature oocytes were obtained from the gravid *N. coriiceps* female (2072 g total weight, 42.4 cm total length, estimated gonadosomatic index 34%) by applying gentle pressure to its abdomen after sedation with MS-222 at 50 mg•L^{-1}. Stripped oocytes were collected in a stainless-steel bowl and kept chilled in ovarian fluid on ice to prevent oocyte activation.

After euthanasia with a lethal dose of MS-222 at 100 mg•L^{-1}, testes of the mature *N. rossii* male (1021 g total weight, 43.4 cm total length, estimated gonadosomatic index 0.53%) were dissected and rapidly cut into small fragments in a petri dish kept on ice to let semen exude and maximize semen volume for oocyte fertilization. A sample of exuded semen was diluted in filtered sea water and immediately observed under a microscope to verify sperm capacitation (Le François et al. 2020).

For in vitro fertilization, ~ 1 mL of semen exudate from the *N. rossii* male was added to *N. coriiceps* oocytes in the stainless bowl and ~ 2.5 L of filtered, UV-treated seawater at ~ 0 °C was gradually added to activate the sperm. The mixture of oocytes and sperm was gently agitated for 5 min and rinsed three times over half an hour with filtered and UV-sterilized seawater to minimize egg adhesion to one another.

Observations of developing embryos

Positively buoyant embryos were initially incubated at ~ 0 °C with gentle aeration in four 1-L beakers containing ~ 1500 embryos each. Every other day, 75% of the water was renewed with preconditioned filtered and UV-sterilized seawater. Dead embryos were removed daily. After 10 days, embryos were transferred to a tray of a vertical incubation system (MariSource Inc., WA) with constant flow-through of filtered, aerated, and UV-sterilized seawater at -1 to 0 °C. Several embryos were randomly collected every few days for developmental and morphological observations and were imaged using a dissecting microscope. Developmental stages corresponded to those previously described for *N. coriiceps* (Postlethwait et al. 2016) and medaka (Iwamatsu 2004). At 21 days post fertilization (dpf), embryonic development stalled, and five of the remaining embryos were sampled and preserved in 70% ethanol for subsequent DNA analyses. During incubation, the water temperature remained stable around 0 °C and embryos from several other pure *N. coriiceps* crosses raised in other trays of the same vertical incubator did not experience any mortality event, ruling out

technical issues that could have affected the experimental embryos. All surviving embryos produced, from the hybrid cross and from the pure *N. coriiceps* crosses, were humanely euthanized with a lethal dose of MS-222 at 100 mg•L^{-1}.

Genetic Analysis

To test whether *N. coriiceps* x *N. rossii* embryos were true F1 hybrids, we amplified segments of two polymorphic nuclear genes, *myh6* (*myosin, heavy chain 6, cardiac muscle, alpha*) and *rho* (*rhodopsin*), and the polymorphic mitochondrial gene *mt-co1* (*cytochrome c oxidase I, mitochondrial*), from five embryos and from both parents. PCR reactions were performed using primers as previously described (Desvignes et al. 2019), and amplicons were sequenced by Sanger sequencing (GENEWIZ, Cambridge, MA, USA). Results were compared to sequences in NCBI GenBank and the Barcode of Life Data System (BOLD System)(Ratnasingham and Hebert 2007) to identify species-specific alleles. Sequencing results are provided in Online Resources 1–3.

Results

Experimental embryos initiated development but died before neurulation

Hardening of the egg chorion was observed within a few hours post fertilization, indicating successful hydration and cortical reaction. Hydrated eggs measured on average 4.4 mm in diameter, with a wet weight of 49.5 ± 1.3 mg and a dry weight of 4.2 ± 0.3 mg (~ 8.5% of wet weight). At 1 dpf, embryos were at the 2-cell stage (Fig. 1A), and at 2 dpf, they had advanced to the 4-cell stage. On average, embryos reached the 64-cell stage by 3 dpf, the 256-cell stage by 5 dpf, and the 1 k-cell stage by 7 dpf. At 8 dpf, the embryonic dome of cells flattened to reach a late blastula stage with the formation of a rudimentary germ ring and embryonic shield (Fig. 1B). At 9 dpf, embryos transitioned to an early gastrula stage with a defined germ ring and embryonic shield (Fig. 1C). Between 10 and 12 dpf, embryos progressed towards the mid-gastrula stage (Fig. 1D-E) and by 14 dpf, embryos had reached ~ 30% epiboly (Fig. 1F). At that age, embryonic chorions became abnormally weak and occasionally ruptured, even with careful handling. Between 14 and 21 dpf, most embryos had stopped developing or had died. By 21 dpf, several embryos were arrested at late-gastrula to early neural stages with an established embryonic

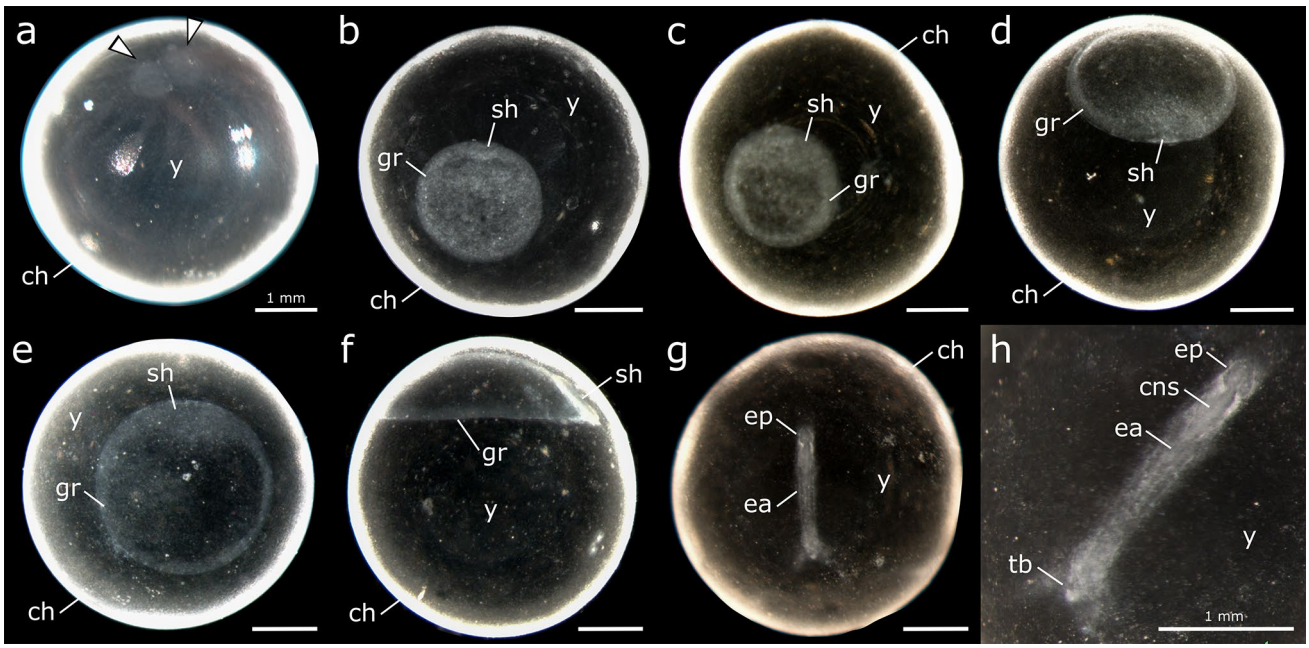

Fig. 1 Development of N. coriiceps x N. rossii embryos. **A** 2-cell stage, 1 dpf. Arrowheads point at the two cells. **B** Late blastula, 8 dpf. **C** Early gastrula, 9 dpf. **D** Early gastrula, 10 dpf. **E** Early gastrula, 12 dpf. **F** ~ 30% epiboly, 14 dpf. **G,H** Late gastrula/early neurula, 21 dpf. **H** Embryonic axis at higher magnification. Abbreviations: ch, chorion; cns, central nervous system anlagen; ea, embryonic axis; ep, eye primordium; gr, germ ring; sh, embryonic shield; tb, tail bud; y, yolk

axis and the anlagen of a central nervous system posterior to eye primordia (Fig. 1F). None of the experimental embryos developed distinct somites or a Kupffer's vesicle. Embryos did not survive past this stage.

Experimental embryos were gynogenetic

The *N. coriiceps* x *N. rossii* embryos could be true hybrids or, alternatively, might have derived by gynogenesis. To evaluate these two hypotheses, we amplified and sequenced fragments of one mitochondrial and two nuclear genes from both parents and five different experimental embryos.

The sequencing of the mitochondrial marker *mt-co1* from the five experimental embryos confirmed the presence of maternal *N. coriiceps* mitochondrial DNA and the absence of paternal *N. rossii* mitochondrial DNA (Fig. 2A, Supplementary Table 1), as expected given the maternal inheritance of mitochondria.

PCR amplification of the two nuclear genes *myh6* and *rho*, however, revealed the presence of only maternal alleles in the embryos and failed to amplify paternal sequences, although the same primers efficiently amplified DNA from the *N. rossii* father (Fig. 2B). At all nucleotide sites differentiating the two parental alleles, nucleotides in the progeny were resolved unambiguously as maternal variants (three sites in *myh6* and 11 sites in *rho*, Supplementary Table 1) with no paternal variants detected. Thus, experimental embryos appeared to be devoid of paternal DNA and were, therefore, gynogenetic. Whether the embryos were gynogenetic haploids or gynogenetic diploids could not be assayed and would have required cytological analyses of embryos, or the identification of heterozygous loci in each embryo that could have been derived from meiotic recombination events associated with maternal polymorphisms if the gynogenetic embryos had been derived by the production of half-tetrads (Streisinger et al. 1981).

Discussion

Development of experimental embryos

Embryos resulting from the in vitro fertilization of a female *N. coriiceps* by a male *N. rossii* developed at the same rate as embryos resulting from a pure *N. coriiceps* cross raised in similar thermal conditions hovering around the freezing temperature of seawater (Postlethwait et al. 2016) and reached the onset of neurulation around 20 dpf. The *N. coriiceps* x *N. rossii* embryos, however, developed more slowly than pure *N. rossii* embryos raised in the Kerguelen Islands at temperatures ranging from 2 to 4.5 °C (Camus and Duhamel 1985), which reached neurulation around 14 dpf. The difference in incubation temperature likely explains the difference in developmental rates. No *N. coriiceps* x *N. rossii* embryos, however, survived past 21 dpf or developed past an early neurula stage.

Experimental embryos are likely gynogenetic haploids

Genetic analyses revealed the absence of detectable paternal DNA in the hybrid embryos but confirmed the presence of maternal mitochondrial and nuclear alleles, demonstrating that experimental embryos were gynogenetic. Gynogenetic embryos are generally of three main types: haploids, diploid half tetrads, or diploid doubled haploids (Streisinger et al. 1981). Haploid embryos of different fish species generally display the "haploid syndrome", which encompasses a variety of phenotypes ranging from early embryonic death to embryos with short and stocky bodies, small eyes, uninflated swim bladder, and apoptosis in the brain, and they usually die before hatching (Purdom 1969; Streisinger et al. 1981; Luo and Li 2003; Delomas and Dabrowski 2016; Zhou et al. 2022). The *N. coriiceps* x *N. rossii* embryos thus presented phenotypes compatible with the haploid syndrome but they did not develop somites, well-formed eyes or brain, and appeared to die at an earlier developmental stage than typical haploid fish do.

Although our data are consistent with experimental embryos being haploid, we cannot rule out the alternative hypothesis that the embryos were gynogenetic diploids. If the experimental embryos were doubled haploids, then the observed mortality could be caused by homozygosity of lethal alleles of two different linked complementary genes for which the *N. coriiceps* mother was heterozygous, in which case half of the embryos would be homozygous for one of the lethal alleles and the other half homozygous for the other lethal mutation. Alternatively, many embryonic-lethal alleles might have been heterozygous in the genome of the *N. coriiceps* mother so that no embryos would be free of lethal alleles, in which case, the population of embryos would have shown a number of different lethal syndromes, which we did not see. We therefore conclude that the experimental embryos were likely gynogenetic haploids.

Barriers to hybridization between *N. coriiceps* and *N. rossii*

Although barriers to hybridization may be somewhat permissive between some closely-related cryonotothenioid species (Marino et al. 2013; Dornburg et al. 2016a, b; Desvignes et al. 2019; Schiavon et al. 2021), results presented here suggest that directional hybridization between congeneric notothen *N. coriiceps* females and *N. rossii* males may be impossible and that reproductive isolation in this direction at least may be complete. The

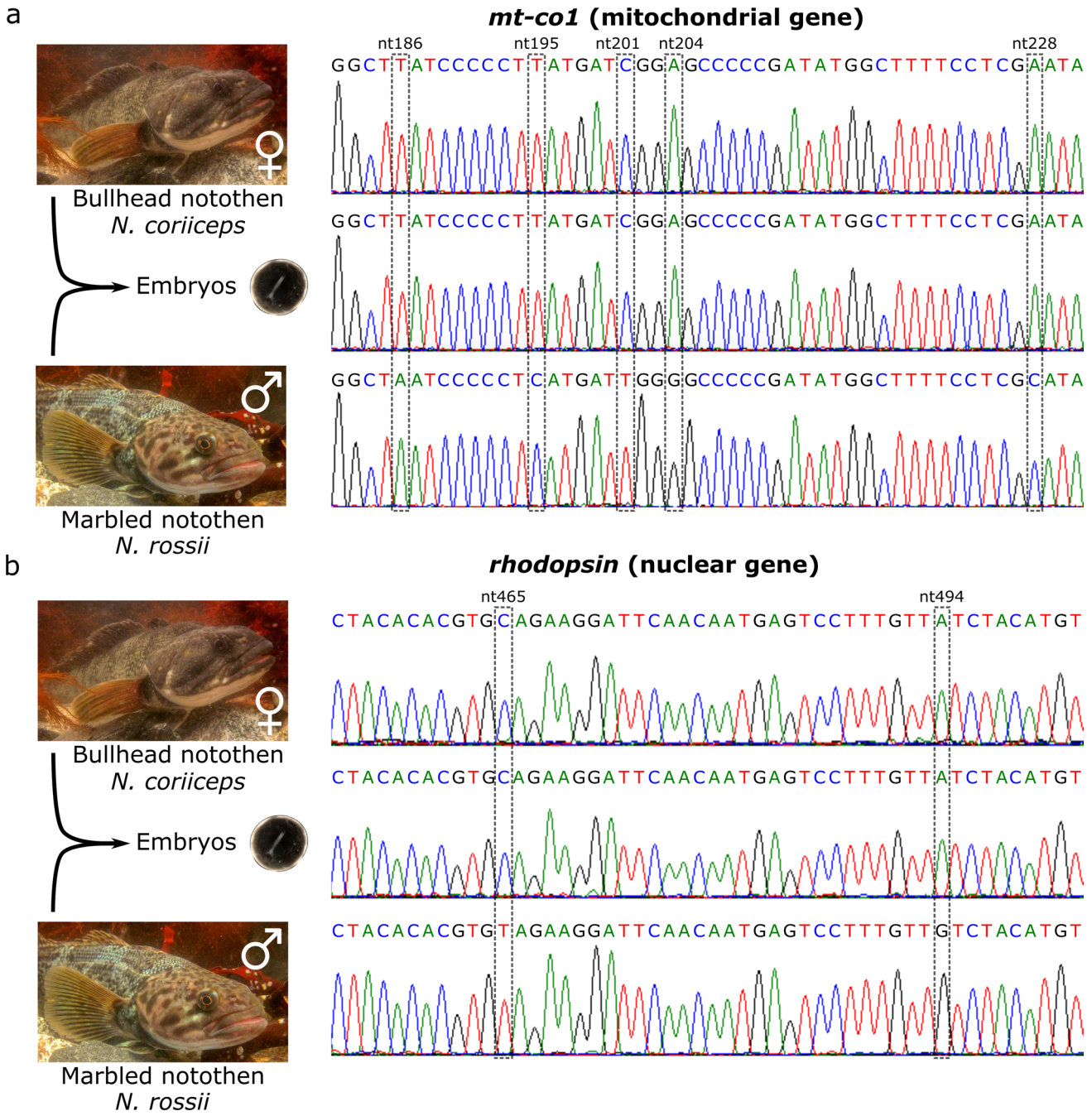

Fig. 2 Genetic analyses demonstrated that the embryos were gynogenetic. **A** Sanger sequencing for the mitochondrial gene *mt-co1* identified only the maternal allele in all five experimental embryos. **B** Sanger sequencing for the nuclear gene *rhodopsin* identified only the maternal allele in all five experimental embryos. This result demonstrated the absence of the paternal allele, and thus, the gynogenetic status of the embryos. The positions of single nucleotide polymorphisms (SNP) in the sequences are given with respect to the maternal alleles. Images illustrate adult specimens of the parental species, not the exact specimens used for the experimental cross

embryonic lethality observed in this interspecific cross raises questions concerning barriers to hybridization between the two congeneric species.

Among premating hybridization barriers, temporal isolation and ecological isolation seem unlikely in this case because *N. coriiceps* and *N. rossii* have overlapping distributions (Duhamel et al. 2014; Ferreira et al. 2017; Calì et al. 2017) and reproductive seasons (Kock and Kellermann 1991; Ferreira et al. 2017), consistent with our experience of simultaneously and regularly collecting mature adults of both species in the same trawl or baited trap. We cannot exclude, however, that *N. coriiceps* and *N. rossii* reproduce

at different times of day, in different spawning grounds, or both, which would constitute temporal and ecological barriers to hybridization. Furthermore, although both species are broadcast spawners whose females release large, positively buoyant eggs in potentially large spawning aggregates (White et al. 1996; Sapota 1999; Calì et al. 2017), species-specific reproductive behavior via assortative mating and courtship may nevertheless prevent hybridization. Therefore, premating hybridization barriers may contribute to the reproductive isolation of the two species, although the present study does not have the data to test this hypothesis.

Our data are, however, consistent with the hypothesis that postmating barriers likely play a major role in preventing interspecific hybridization between *N. coriiceps* females and *N. rossii* males. Indeed, our genetic analyses failed to reveal the presence of paternal DNA in the embryonic genome. Therefore, either the *N. rossii* sperm pronucleus entered the egg cell but was unable to fuse with the *N. coriiceps* oocyte pronucleus to generate a diploid zygote nucleus (karyogamy), or the *N. rossii* sperm was able to activate the *N. coriiceps* oocytes but the sperm pronucleus failed to enter the oocyte.

Karyotypic differences between the two species ($2n = 22$ in *N. coriiceps* and $2n = 24$ in *N. rossii* (Prirodina and Neyelov 1984; Doussau de Bazignan and Ozouf-Costaz 1985; Van et al. 1987; Ozouf-Costaz et al. 1991; Tomaszkiewicz et al. 2011)) are unlikely to have generated any problems in chromatids separating during anaphase of mitosis. Hybrids of species with different karyotypes (i.e., chromosomal hybrids) are not uncommon, often viable, and have intermediate number of chromosomes compared to the two parents or may be triploid or tetraploid (e.g., in fish LeGrande et al. 1984; Harvey et al. 2002; Ostberg et al. 2013; Suzuki et al. 2017; Vasil'ev et al. 2021) and other metazoans, including the mule (Benirschke et al. 1962; Medarde et al. 2012; Lukhtanov et al. 2020; Galindo et al. 2021; Noronha et al. 2022)). The impact of a karyotype with unpaired chromosomes usually becomes apparent only during meiosis, where mispairing of chromosomes leads to aneuploid gametes and inviability of the aneuploid offspring of hybrid parents. Even in cases where chromosome number is the same, individuals of the heterogametic sex are often sterile in an interspecific cross, a situation known as Haldane's rule (Haldane 1922; Coyne and Orr 2004; Coyne 2018). Given these considerations, it would be surprising, if the *N. rossii* sperm pronucleus had fused with *N. coriiceps* oocyte pronucleus, that not a single embryo would have survived longer than neurulation, and that none of the tested embryos had detectable paternal DNA.

The most likely explanation for the gynogenetic genome of the hybrid embryos is thus that *N. rossii* sperm were incompatible with *N. coriiceps* oocytes while still able to activate embryonic development. The micropyle, a narrow canal in the chorion of fish oocytes that prevents polyspermy by limiting entry to only one sperm, has diverse morphologies and sizes adapted to the sperm characteristics of each species (Riehl and Kock 1989; Yanagimachi et al. 2013, 2017) and may contribute to preventing interspecific crosses (Kinsey et al. 2007). The micropyle in *N. coriiceps* oocytes has a narrow pit of 16 µm and a canal diameter of 4.5 µm, whereas that of *N. rossii* oocytes lacks a pit and has a diameter of 6–7 µm (Riehl and Kock 1989; White et al. 1996). Therefore, *N. rossii* sperm may, for example, be too large to pass through the micropyles of *N. coriiceps* oocytes, consistent with the observation that differences in micropyle size influence fertilization rates between medaka species (Iwamatsu et al. 1997). Furthermore, the micropyle secretes a sperm attractant that helps guide the sperm to and through the micropyle (Yanagimachi et al. 2013, 2017). Perhaps the micropylar sperm attractant of *N. coriiceps* oocytes does not effectively guide *N. rossii* sperm all the way through the micropyle. More broadly, the many reproductive proteins necessary for the fusion of gametes, the decompaction of the sperm pronucleus, and the fusion of pronuclei may be other sources of incompatibility between the two species (Swanson and Vacquier 2002; Kinsey et al. 2007). We can nonetheless conclude that *N. rossii* sperm were able to activate *N. coriiceps* eggs and to trigger embryonic development, although gamete fusion may not have happened and incorporation of paternal chromosomes into the zygote nucleus did not occur. Therefore, gametic incompatibility appears to explain the absence of paternal DNA that we observed in developing embryos and the failure to obtain true *N. coriiceps* x *N. rossii* diploid hybrids.

Thorough characterization of sperm and oocyte morphologies for each species are needed to assess whether morphological features of *N. coriiceps* and *N. rossii* gametes contribute to their incompatibility. In addition, to decisively conclude about the presence or absence of post-mating, prezygotic reproductive isolation between the two sister notothen species, our experiment would need to be replicated, the reciprocal cross (*N. rossii* oocytes x *N. coriiceps* sperm) would need to be analyzed, and additional controls would have to be added to the experimental design (i.e., fertilization of *N. coriiceps* oocytes by *N. coriiceps* sperm to verify oocyte viability, fertilization of *N. rossii* oocytes by *N. rossii* sperm to verify sperm quality). Such a complete experimental design is unfortunately exceedingly hard to implement in the field in Antarctica where capture of reproductively active and gravid specimens is never guaranteed and installations and time to raise embryos are limited. Nonetheless, the experimental design we were able to perform still provides opportunities to draw some conclusions despite logistical shortcomings. Here, our analysis of this directional experimental hybrid cross revealed a likely strong postmating, prezygotic barrier to

hybridization between *N. coriiceps* females and *N. rossii* males, which could contribute to the divergence and maintenance of these two ecologically important Antarctic congeners. This strong barrier to hybridization might have been erected if premating barriers to hybridization were permissive, which is not excluded given that *N. coriiceps* and *N. rossii* have mostly sympatric distributions, largely overlapping reproductive seasons, similar reproductive strategies, and are both broadcast spawners reproducing in potentially large aggregates. This gamete incompatibility might also result simply from the two species being too divergent after ~ 3–6 million years of evolution (Parker et al. 2022; Bista et al. 2023). In African cichlids, which underwent an adaptive radiation in the same timeframe as cryonotothenioids (Ronco et al. 2020), the study of interspecific fertilization rates in 26 heterospecific pairs revealed that the strength of gamete incompatibility increases linearly with divergence time, with complete gamete incompatibility achieved after around 4 million years (Stelkens et al. 2010). The case of a strong postmating, prezygotic hybridization barrier between the congeneric *N. coriiceps* females and *N. rossii* males therefore contrasts with that of icefishes in which reproductive season and nesting behavior may represent the main hybridization barriers (Desvignes et al. 2019). The diversification of cryonotothenioid species has thus likely been influenced by the establishment of various types of hybridization barriers that have potentially been influenced by the mode of reproduction of each species.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00300-023-03216-7.

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Declarations

Competing interests The authors declare no competing interests.

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