Investigating Bouncer's role in species-specific fertilization

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Fertilization is central to the perpetuation of all sexually reproducing species. An important aspect of this process, particularly for externally fertilizing organisms, is that the sperm of one species fertilizes only the eggs of the same species, thereby maintaining species specificity. How this is achieved on the molecular level remains unclear. Recently, we uncovered Bouncer, a protein essential for fertilization in zebrafish (1). Females mutant for this small, oocyte-expressed, and GPI-anchored protein are infertile, while mutant males retain normal fertility. Though Bouncer is conserved among vertebrates, the high level of sequence dissimilarity among homologs suggested that Bouncer may contribute to species specificity in fertilization. By substituting zebrafish Bouncer with Bouncer from medaka in the zebrafish egg, we found that changing this single factor allowed cross-fertilization between these two evolutionarily distant fish species. Thus, Bouncer mediates species specificity in sperm-egg interactions in the case of medaka and zebrafish. We are currently exploring whether Bouncer's species-specific function is conserved, and when, phylogenetically, the cross-fertilization barrier mediated by Bouncer arose between reproductively isolated species. In addition, we are using Bouncer's zebrafish/medaka specificity to determine the protein region and key amino acids that mediate Bouncer's function. Moreover, unlike zebrafish, medaka and many other fish species express a second, secreted Bouncer isoform in the egg whose function is still unknown and is currently under investigation. This work gives insight into species-specific mechanisms in vertebrate fertilization and opens the door to understanding the process of fertilization as a whole.

Bouncer is an egg membrane protein required for fertilization

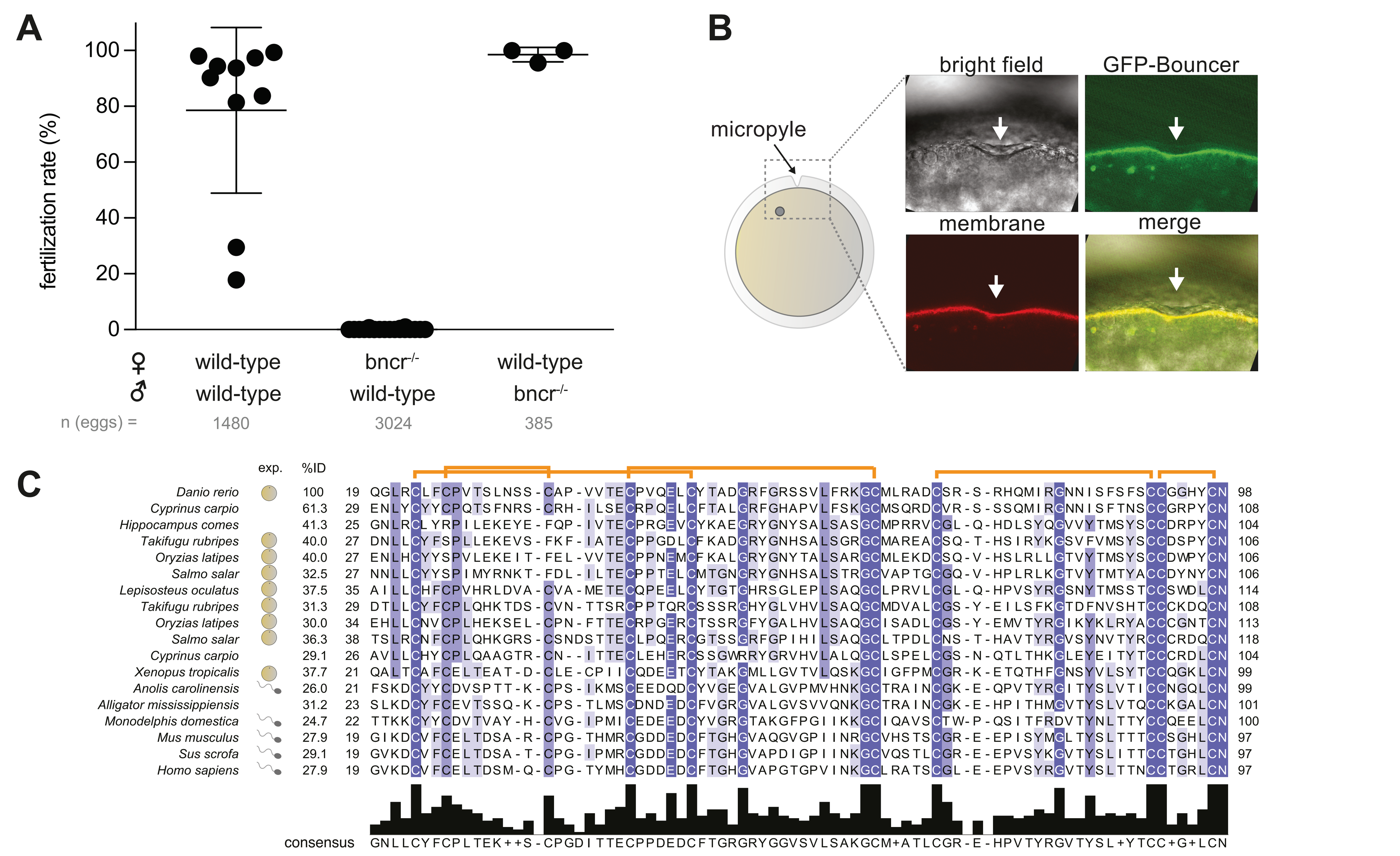

Figure 1. Bouncer is a short, conserved, membrane protein. (**A**) Bouncer is required for female fertility in zebrafish, while Bouncer mutant males retain normal fertility. (**B**) GFP-Bouncer (green) localizes to the egg membrane (red, lyn-tomato) and to vesicles. (**C**) Apart from the well-conserved cysteines, Bouncer shows high amino acid divergence among different species (%ID, percent sequence identity with the mature domain of zebrafish Bouncer). Bouncer is restricted to either the male (sperm symbol) or the female (egg symbol) germline.

Can Bouncer from other species rescue fertilization in zebrafish?

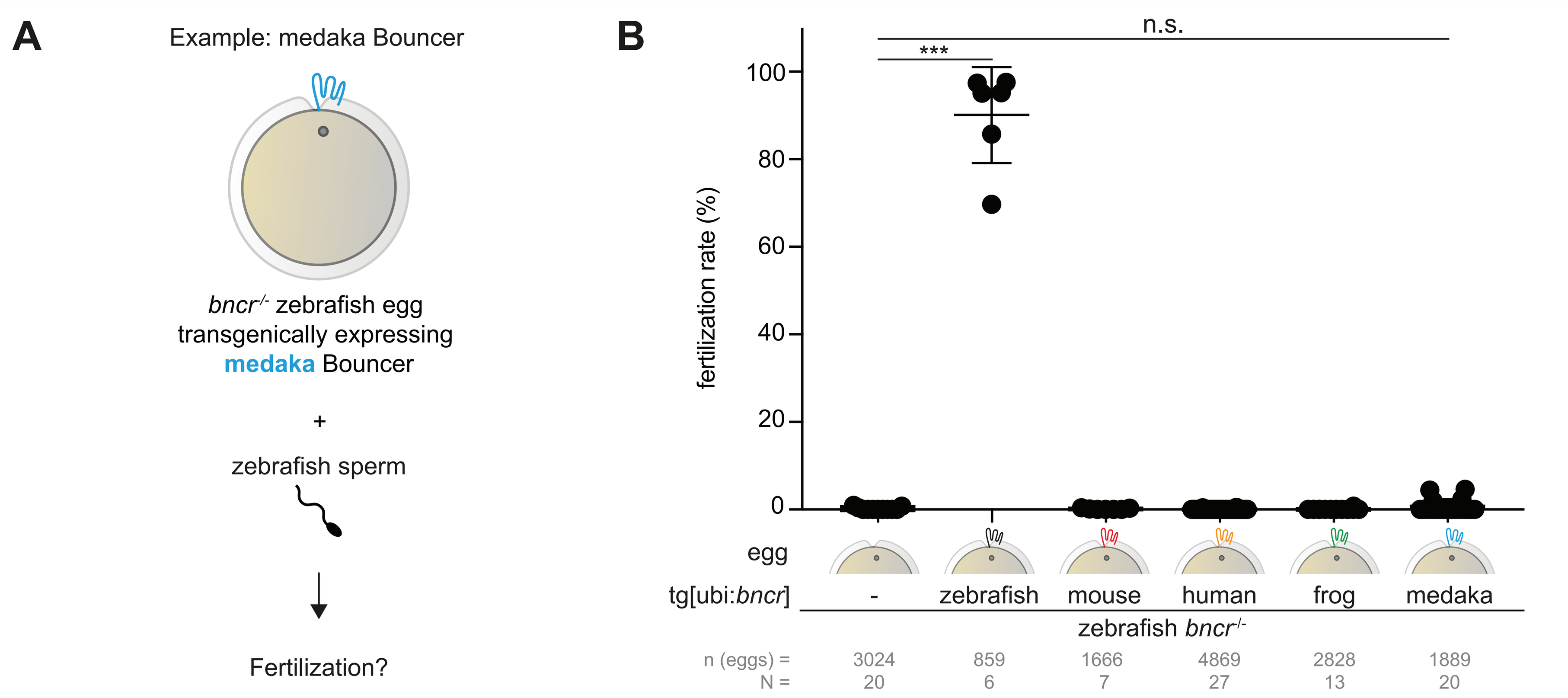

Figure 2. Bouncer homologs from other species are not sufficiently conserved to rescue fertilization in zebrafish. (**A**) Fertilization assay in which eggs from transgenic females were tested for fertilization with zebrafish sperm. (**B**) Transgenic zebrafish lines, each expressing Bouncer from a different species (mouse, human, frog (*Xenopus laevis*), and medaka (*Oryzias latipes*)), were generated in the zebrafish *bncr*-/- background and the females were tested for fertility with wild-type zebrafish males. None of the transgenic constructs rescued fertilization efficiently in the zebrafish *bncr* mutant background, while expressing zebrafish *bncr* as a transgene in the same manner fully rescued fertility. Means ± SD are indicated. (Kruskal-Wallis test with Dunn's multiple comparisons test. ***P < 0.0002, n = number of eggs, N = number of biological replicates).

Is medaka Bouncer sufficient to allow entry of conspecific sperm?

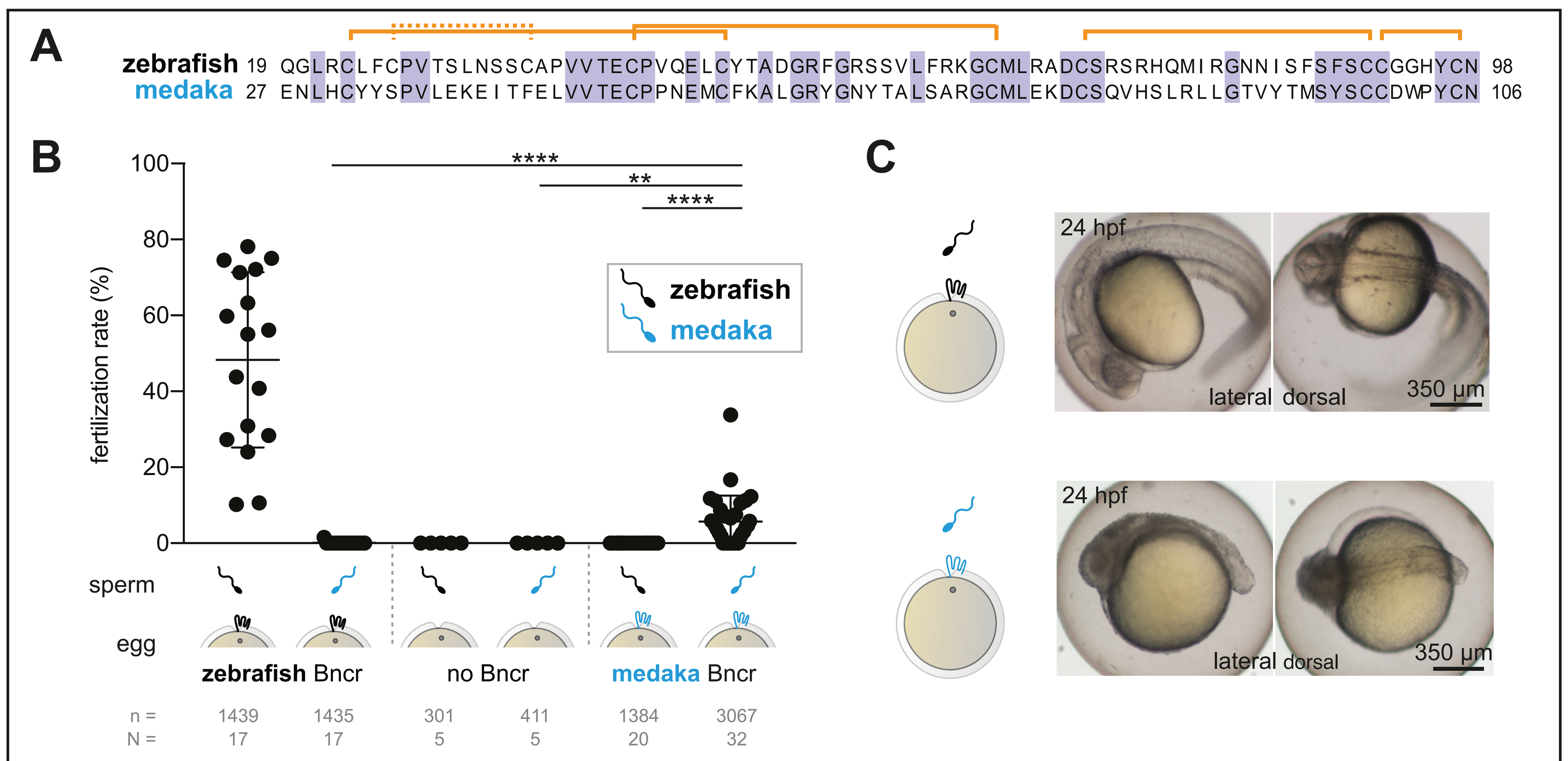

Figure 3. Bouncer mediates species-specific fertilization. (**A**) Zebrafish and medaka Bouncer share only 40% amino acid sequence identity. Orange brackets denote predicted disulfide bonds. (**B**) Medaka Bouncer is sufficient to allow entry of medaka sperm into zebrafish eggs (5.7% average fertilization rate in IVF experiments). Means ± SD are indicated. (Kruskal-Wallis test with Dunn's multiple comparisons test. ****P < 0.0001; **P = 0.0052; n = number of eggs; N = number of biological replicates). (**C**) Fertilization of zebrafish eggs expressing only medaka Bouncer with medaka sperm yields medaka-zebrafish hybrid embryos.

References and Acknowledgements

References: 1. Herberg et al. 2018, *Science*

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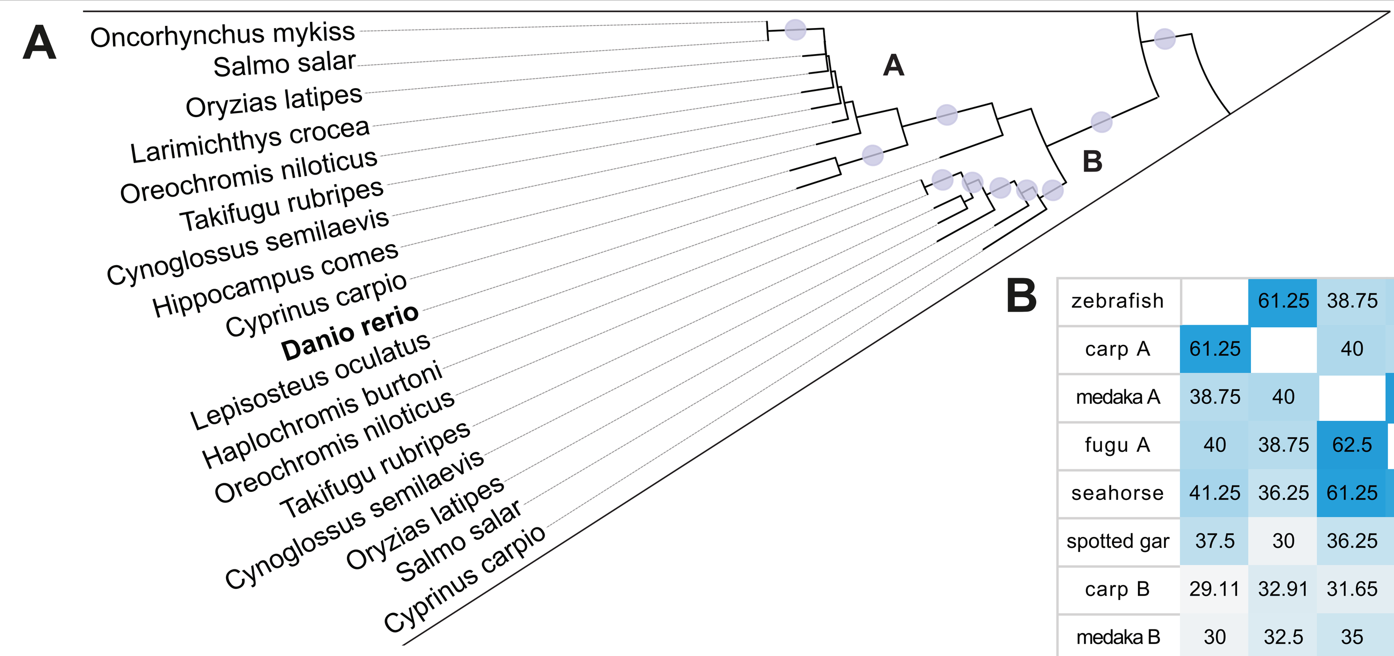
FWF Der Wissenschaftsfonds. HFSP Vienna BioCenter

Does Bouncer phylogeny correlate with cross-fertility?



B

zebrafish		61.25	38.75	40	41.25	37.5	29.11	30	31.25
carp A	61.25		40	38.75	36.25	30	32.91	32.5	30
medaka A	38.75	40		62.5	58.75	36.25	31.65	35	33.75
fugu A	40	38.75	62.5		58.75	36.25	31.65	35	38.75
seahorse	41.25	36.25	61.25	58.75		41.25	34.18	36.25	32.5
spotted gar	37.5	30	36.25	36.25	41.25		39.24	41.25	38.75
carp B	29.11	32.91	31.65	31.65	34.18	39.24		48.1	48.1
medaka B	30	32.5	35	35	36.25	41.25	48.1		50
fugu B	31.25	30	33.75	38.75	32.5	38.75	48.1	50	

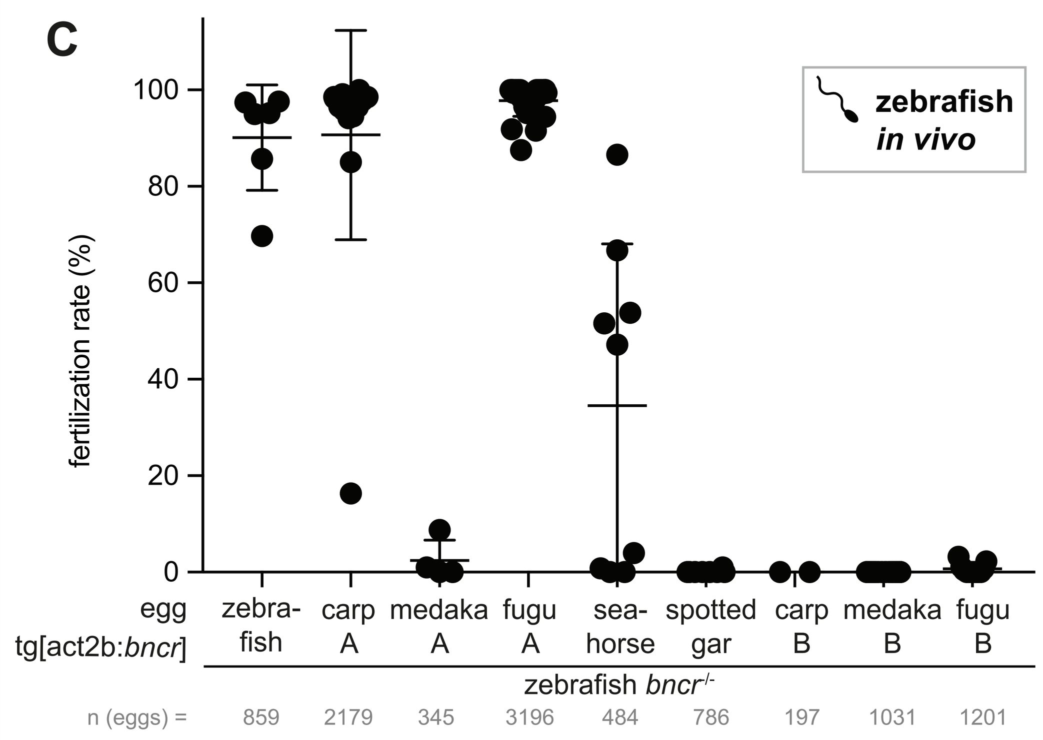
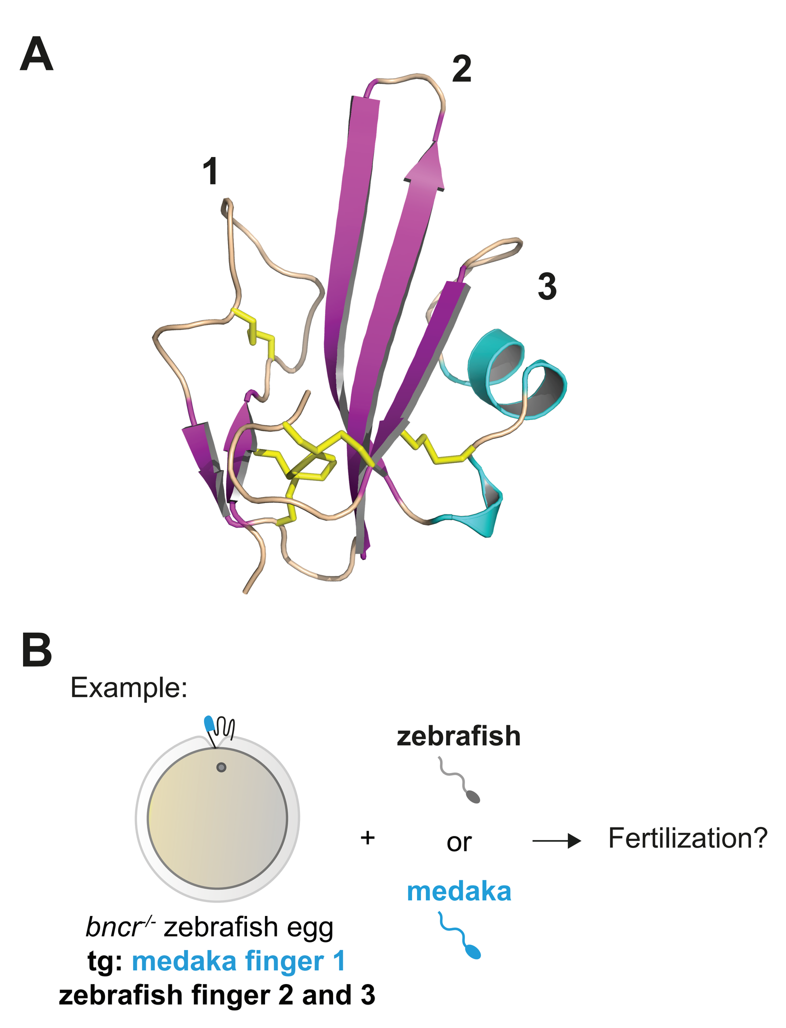


Figure 4. Phlyogenetic distance among Bouncer proteins does not correlate with cross-fertility. (**A**) Phylogenetic tree depicting the evolutionary relationships among Bouncer proteins from a selected set of fish species. The upper clade comprises Bouncer A proteins (including zebrafish Bouncer), while the lower clade, the Bouncer B proteins, are absent in zebrafish and their function is unknown. (**B**) Percent identity matrix of the mature domains of Bouncer A and B proteins from the fish species tested. (**C**) Transgenic zebrafish lines expressing a different fish Bouncer in the zebrafish *bncr*-/- background were crossed to wild-type zebrafish males *in vivo* to test rescue ability. Unexpectedly, fugu Bouncer A (40% ID with zebrafish Bouncer) and seahorse Bouncer (41.25% ID with zebrafish Bouncer) rescue fertility with zebrafish sperm, while spotted gar Bouncer and the Bouncer B proteins do not.

What region(s) of Bouncer mediate species specificity?



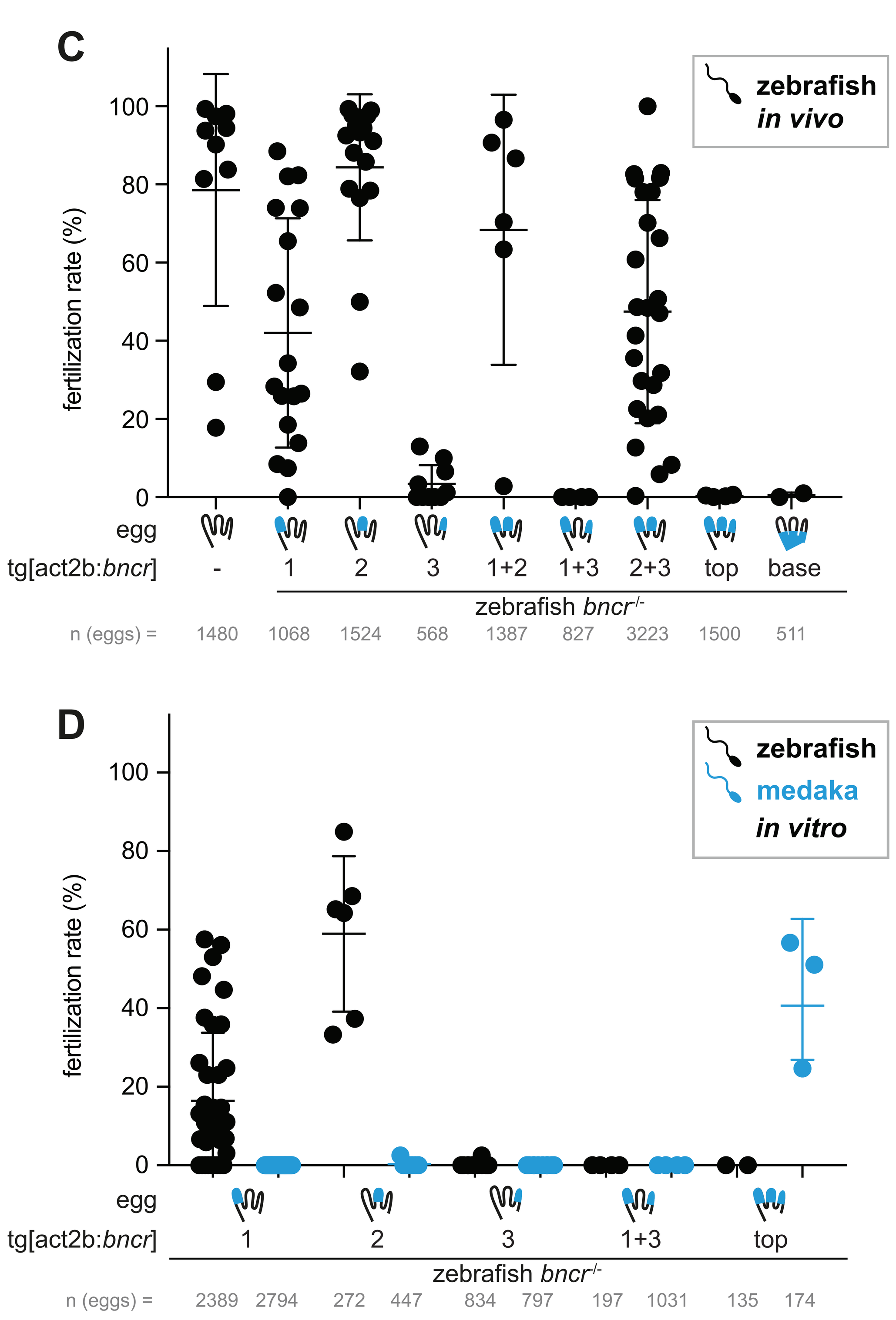
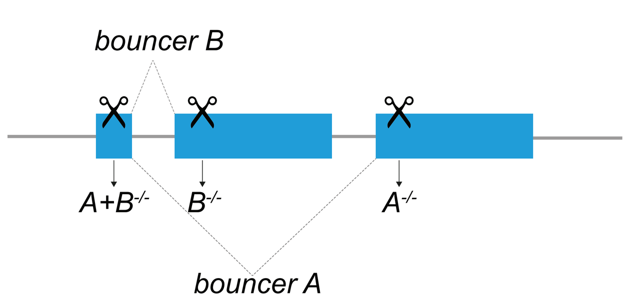


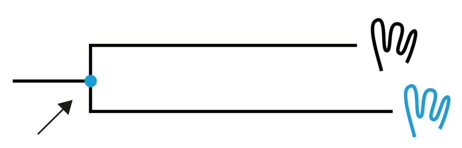
Figure 5. Zebrafish/medaka Bouncer chimeras suggest involvement of finger 3 in species specificity. (**A**) Structural model of Bouncer based on the related Ly6/uPAR protein CD59. Disulfide bonds (yellow) form the basis of the three-finger structure. (**B**) Strategy to test which of Bouncer's fingers mediate(s) species specificity. (**C**) Fertility of zebrafish/medaka Bouncer chimera lines *in vivo* with wild-type zebrafish sperm. Constructs with finger 3, 1+3, the top or base of medaka Bouncer result in the most reduced fertility, whereas constructs with medaka finger 1, 1+2, or 2+3 are able to rescue fertility. (**D**) None of the chimeric Bouncer constructs tested thus far rescue fertility with medaka sperm *in vitro*, while *in vitro* fertilization rates with zebrafish sperm recapitulate those *in vivo*.

Current Questions and Outlook

1. What are the roles of Bouncer A and B in medaka?

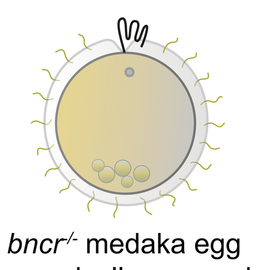


3. When did the Bouncer-mediated zebrafish/medaka cross-fertilization barrier arise?



Test predicted ancestral Bouncer states for fertilization rescue with zebrafish and medaka sperm

2. Can reciprocal (zebrafish-medaka) hybrids be formed?



bncr-/- medaka egg transgenically expressing **zebrafish** Bouncer + zebrafish sperm ⟶ Fertilization?

4. Medaka-zebrafish hybrid characterization