Concerning an Article by Ehl et al.: False Premise Leads to False Conclusions

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In their recent paper, entitled "With or without W? Molecular and cytogenetic markers are not sufficient for identification of environmentally-induced sex reversal in the bearded dragon", Ehl et al. [2021] demonstrate that a published PCR sex test developed for the bearded dragon (F4-F1 marker) [Quinn et al., 2010] is only partially diagnostic for sex because the sex-linked sequence upon which the test is based is subject to recombination with sex and therefore inappropriate for studies of sex reversal. The authors use the deficiencies of this now superseded PCR F1-F4 sex test developed by Quinn et al. [2010] to call into question the more recent and robust sex reversal work of Holleley et al. [2015] and subsequent work on this system in *Pogona vitticeps* [Castelli et al., 2021].

This criticism is completely misplaced, because none of the sex reversal work criticised by Ehl et al. [2021] used the original Quinn F4-F1 PCR sex test.

Indeed, the limitation of the Quinn F4-F1 test was recognised early by our group, because some phenotypic males were genotyped as ZW individuals using the Quinn F4-F1 sex test. To address this limitation, different sex-linked sequences (using primers H2 and F of Quinn et al. [2010]) were characterised and developed as an improved PCR sex test (H2-F) [Holleley et al., 2015], which correctly assigned the individuals misassigned by the Quinn

F4-F1 test. The robust Holleley H2-F PCR sex test has been used in all subsequent studies of sex reversal in the dragon lizard [e.g., Castelli et al., 2021; Whiteley et al., 2021] in which no phenotypic males scoring as ZW genotypes were reported.

We therefore completely reject the claims of Ehl et al. [2021] that the sex tests used in our sex reversal work on *P. vitticeps* were in any way inadequate.

The factual error by Ehl et al. [2021] evidently arose because of the failure of the authors to recognise the difference between these 2 molecular sex tests. The 2 tests use different PCR primers to amplify sequences from different members of a family of highly repetitive elements [Quinn et al., 2010], so it was unreasonable to assume that the 2 tests targeted the same region of the dragon W chromosome (as subsequently confirmed). The authors were evidently confused by the derivation of the 2 sets of sex-linked sequence upon which the tests were based from the same family of repetitive sequences that lie on the Z and W chromosome in high copy numbers relative to autosomes [Quinn et al., 2010]. The Quinn F4-F1 test is based on a sequence within this repetitive series with 4 W-specific SNPs detectable by PCR. The Holleley H2-F test is based on a different copy of the repetitive sequence at a different location on the W chromosome and is distinguished by the presence of 2 considerable W-specific deletions (150 bp and 14 bp). The 2 sequences are amplified by PCR using different primers. The sex specificity of the Quinn F4-F1 test is derived from one sex-specific SNP in

the sequence to which one of the primers anneals; the sex specificity of the Holleley H2-F test uses amplicon length variation arising from the 2 W-specific indels in the amplified sequence. Thus, the 2 PCR tests have a fundamentally different basis.

The Quinn F4-F1 sequence is likely to be distant from the sex-determining locus on the W chromosome. Collating the results of our studies, we find that approximately 15% of phenotypic males score as having the Quinn W-specific sequence ($n = 36$ “ZW” males of a total of 229 phenotypic males; total number of tests = 517 individuals), validating the recombination inferred by Ehl et al. [2021] to have occurred in their familial lineage. In contrast, the Holleley H2-F sex-specific sequence must be very close to the sex-determining locus, since no recombinants have been detected among more than 900 individuals collated across our multiple studies, including those of the Castelli et al. [2021] study. Directly comparing the 2 tests, 339 individuals whose phenotypic sex was reliably known were tested with both PCR tests. The Quinn F1-F4 test yielded 11 individuals that scored as ZW but presented a male phenotype; in contrast, all 11 were correctly identified as ZZ using the Holleley H2-F test.

For these reasons, it is incorrect for Ehl et al. [2021] to equate the 2 loci and extend their criticism of the Quinn F4-F1 sex test to studies using the Holleley H2-F test. The 2 underlying tests are not equivalent; they target *different* sequences at *different* loci on the W chromosome. They reside at *different* distances from the sex-determining locus and indeed are subject to very *different* rates of recombination with sex, resulting in a detectable misidentification rate for the Quinn F1-F4 test and zero for the Holleley H2-F test. It is false to claim that the 2 tests differ only in the primer pairs used to isolate them.

Ehl et al. [2021] did not have access to the detailed data we have accumulated to demonstrate the different behaviours of the 2 PCR tests with respect to recombination. However, we believe sufficient information was readily available to Ehl et al. [2021] at the time of their experiments to indicate the 2 PCR tests could not be claimed to be equivalent and should not have been used as a premise in the absence of direct supporting evidence. The 2 tests had already been described in detail in published papers [Quinn et al., 2007, 2010; Holleley et al., 2015]. Details of the method for the Holleley H2-F PCR test were described in by Holleley et al. [2015], including the PCR conditions necessary to amplify the W-specific sequence and the internal positive control. Moreover, the distinction between the 2 tests would have been immediately evident had Ehl

et al. [2021] compared the amplicons they generated with those reported from the Holleley H2-F test, which were published at the time (GenBank accession numbers EU938138.1 and KM508988) or that could have been generated by Ehl et al. [2021] with a simple PCR and Sanger sequencing.

Thus, Ehl et al. [2021] claimed equivalency of the 2 PCR tests without demonstrating equivalency when, in our opinion, there were ample indications at the time of publishing that this is not a reasonable premise, and it is the omission of this essential step that has led to false conclusions. Had Ehl et al. [2021] applied both PCR tests, the difference in misassignment rates would have become evident. Had Ehl et al. [2021] compared the sequence they amplified with the publicly available data, the difference in sequence identity would have become evident. But, from our understanding, they did neither.

We therefore completely reject the claims of Ehl et al. [2021] and Erratum that the validity of the Holleley sex test was unknown at the time of their experiments.

In summary, although the general call for caution in Ehl et al. [2021] in the use of unvetted sex-linked sequences to infer sex reversal is legitimate, the authors were quite mistaken in using inadequacies of one PCR test (F1-F4 of Quinn et al. [2010]) to call into question other studies that did not use that test [e.g., Holleley et al., 2015; Castelli et al., 2021]. Their unfounded criticism of the application of the PCR sex test used by Holleley et al. [2015] and Castelli et al. [2021], and their uncalled-for suggestion that Castelli et al. [2021] were swayed by the allure of a good story in the face of opposing facts, are without foundation.

Conflict of Interest Statement

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Author Contributions

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