

Langan and Brooks:

# Exploratory Analysis of the Application of Animal Reduction Approaches in Proteomics: How Much Is Enough?

## Supplementary Data

**Tab. S1: Proteomic studies limited to early life stages of *Danio rerio***

Not reported is denoted by “-” symbol. The term “physiological processes” covers studies related to cellular organization, metabolism, reproduction etc. Studies are denoted as embryo (<120 hpf, orange) and larvae at (>120 hpf, blue). For fathead, only one study was identified, with approximately 3-4 larval fish used per replicate (Moreton et al., 2020). The column titled “standardized” specifically refers to the use of the fish embryo acute toxicity (FET; OECD 236) test (or its precursor), which provides guidelines intended to determine the acute or lethal toxicity of chemicals or perturbations on embryonic stages of zebrafish; “no” denotes no standardized method; “yes” denotes the use of a standardized methodology. “Total proteins” refers to the total number of protein group identifications at a 5% FDR. Abbreviations: polycyclic aromatic hydrocarbons, PAH; Two-dimensional gel electrophoresis, 2DE; one-dimensional gel electrophoresis, 1D shotgun; data dependent acquisition, DDA; data independent acquisition, DIA; not reported in the study, NR.

Year	Study area	Age range	Pool number	Experimental n	Standardized	Protein (µg)	Method	Total proteins	References
2019	Method development	Embryo	20-40 (yolked); 225-575 (deyolked)	-	No	30	DDA	504-2129	Purushothaman et al., 2019
2019	Method development	Embryo (deyolked)	500	3	No	Not reported	DIA	NR	Lin et al., 2019
2019	Organophosphate	Larvae	300	3	No	150	DDA	NR	Shi et al., 2019
2019	Method development	Embryo	1	3	No	~1	DDA	86-426	Lombard-Banek et al., 2019
2018	Nanomaterials & PAH	Embryos (deyolked)	90	3	No	200	DDA	NR	Della Torre et al., 2018
2018	Nanomaterials	Larvae	600	3	No	NR	DDA	NR	Zou et al., 2018
2018	Plastic & plasticizer	Larvae	200	2-3	No	200	DDA	4466	Dong et al., 2018
2018	Stimulant/ recreational drug	Larvae	170-180	1	No	60	DDA	NR	Parolini et al., 2018
2017	Physiological processes	Embryos	200	2	No	NR	DDA	4387	Wu et al., 2017
2017	Nanomaterials & PAH	Embryos (deyolked)	30	3	No	200	DDA	NR	Binelli et al., 2017
2016	Physiological processes	Larvae	NR	4	No	1000	DDA	189	Kwon et al., 2016
2016	Harmful algal bloom	Embryos	30-40	3	No	30	DDA	2440	Frøyset et al., 2016
2016	Physiological processes	Embryos	NR	3-4	No	500	DDA	2166	Kwon et al., 2016
2015	Deyolking	Embryos	300	2	No	500	DDA	159	Rahlouni et al., 2015
2015	Pesticide	Embryos	250	3	No	350	DDA	NR	Liu et al., 2015

2014	Physiological processes	Embryos (deyolked)	1000	2	No	500	DDA	8363	Alli Shaik et al., 2014
2013	Biomedical	Embryos	16	3	No	NR	DDA	2493	Schmid et al., 2013, p. 20
2012	Method development	Embryos (deyolked)	200	NR	No	76	DDA	3464	Lößner et al., 2012
2012	PAH	Embryos	54	2	No	100-1500	DDA	<20	Gündel et al., 2012
2011	Method development	Embryos	30 mg of tissue	3	No	800	DDA	227	Baycin-Hizal et al., 2011
2011	Physiological processes	Larvae	12	3	No	400	DDA	<20	Gómez-Requeni et al., 2011
2011	Harmful algal bloom	Embryos	20	3	No	120-600	DDA	40	Li et al., 2011
2010	Physiological processes	Larvae	15	4	No	400	DDA	20	Gómez-Requeni et al., 2010
2010	Pesticide & drug	Larvae	40	3	Yes	50	DDA	<20	Hanisch et al., 2010
2009	Perfluorinated chemicals	Larvae	200	3	No	350-1000	DDA	<20	Shi et al., 2009
2008	Physiological processes	Embryos (deyolked)	30	NR	No	100	DDA	1400	Lemeer et al., 2008
2007	Method development	Embryos	20-30	5	No	NR	DDA	797	Price et al., 2007
2007	Physiological processes	Embryos (deyolked)	1500	3	No	10,000-25,000	DDA	800	Lemeer et al., 2007
2006	Physiological processes	Embryos	NR	NR	No	NR	DDA	NR	Guérardel et al., 2006
2006	Physiological processes	Embryos (deyolked)	100	NR	No	50	DDA	35	Link, 2006
2006	Physiological processes	Embryos (deyolked)	600-800 (<18 hpf); 200-300 (>24 hpf)	2-3	No	NR	DDA	108	Tay et al., 2006
2003	Endocrine disrupting chemicals	Embryos	20-35	5-6	No	10	DDA	NR	Shrader et al., 2003

**Tab. S2: Summary of search parameters**

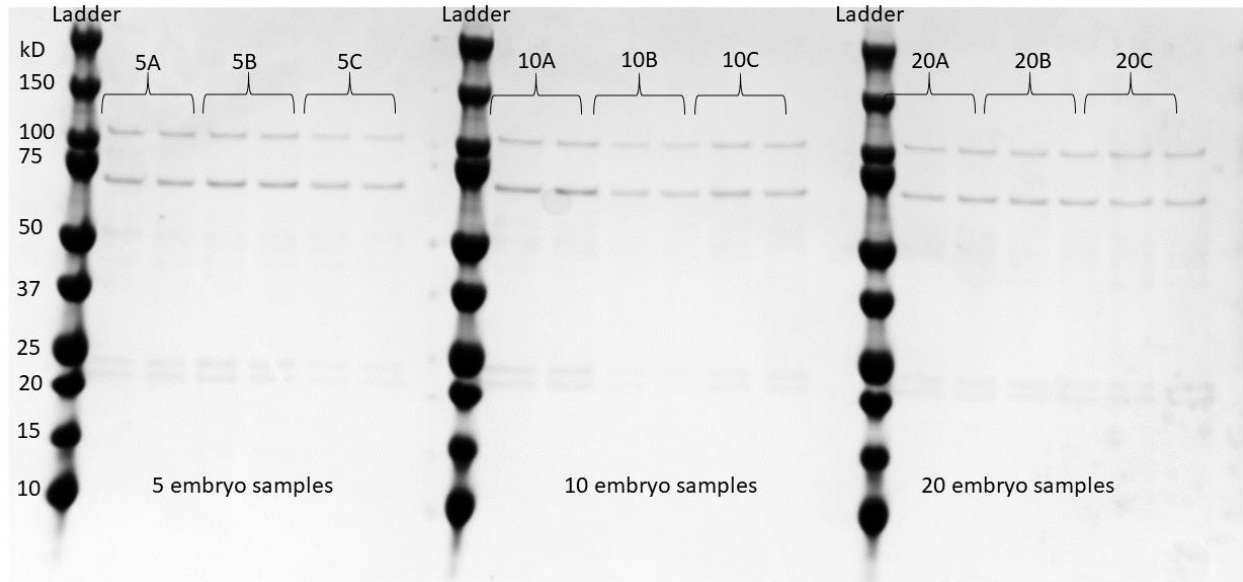
It should be noted that protein identifications were run using a precursor tolerance of 20 and 50 ppm.

<b>MS parameters</b>		
Equipment	Waters Synapt GS2	
MS type	Data independent acquisition (MS <sup>e</sup> ) (window size; 1500 m/z)	
Fragmentation	Collision induced disassociation (CID)	
<b>Search parameters</b>	<b>Zebrafish</b>	<b>Fathead minnow</b>
<b>Database</b>	<i>Danio rerio</i> (UNIPROT)	<i>Pimephales promelas</i> (NCBI;GCF_016745375.1)
<b>DB version</b>	UP000000437	EPA_FHM_2
<b>Database sequences</b>	46,849	47,578
<b>Enzyme</b>	Trypsin	Trypsin
<b>Fixed modification</b>	Carbamidomethyl [C]	Carbamidomethyl [C]
<b>Variable modifications</b>	Oxidation [M], Acetyl (Protein N-term)	Oxidation [M], Acetyl (Protein N-term)
<b>Precursor tolerance</b>	20 ppm + 50 ppm	20 ppm + 50 ppm
<b>Fragment tolerance</b>	0.5 Da	0.5 Da

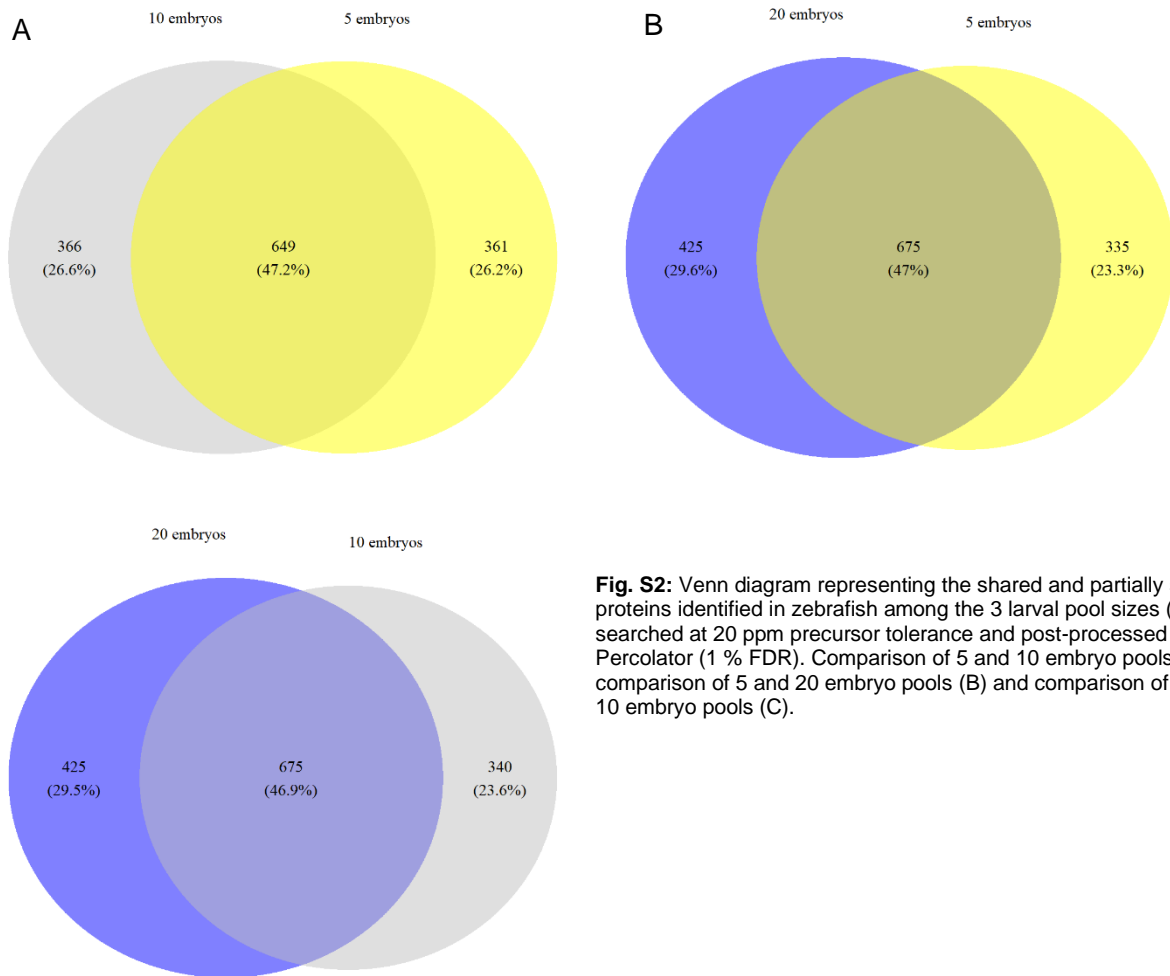
**Tab. S3: Subset of gene ontology classification for zebrafish based on unique proteins identified in each larval pool (see Fig. 3)**

Order is based on the top 3 ranking of gene counts per pool size, although this is extended for 20 embryos to allow for comparisons among the pool sizes.

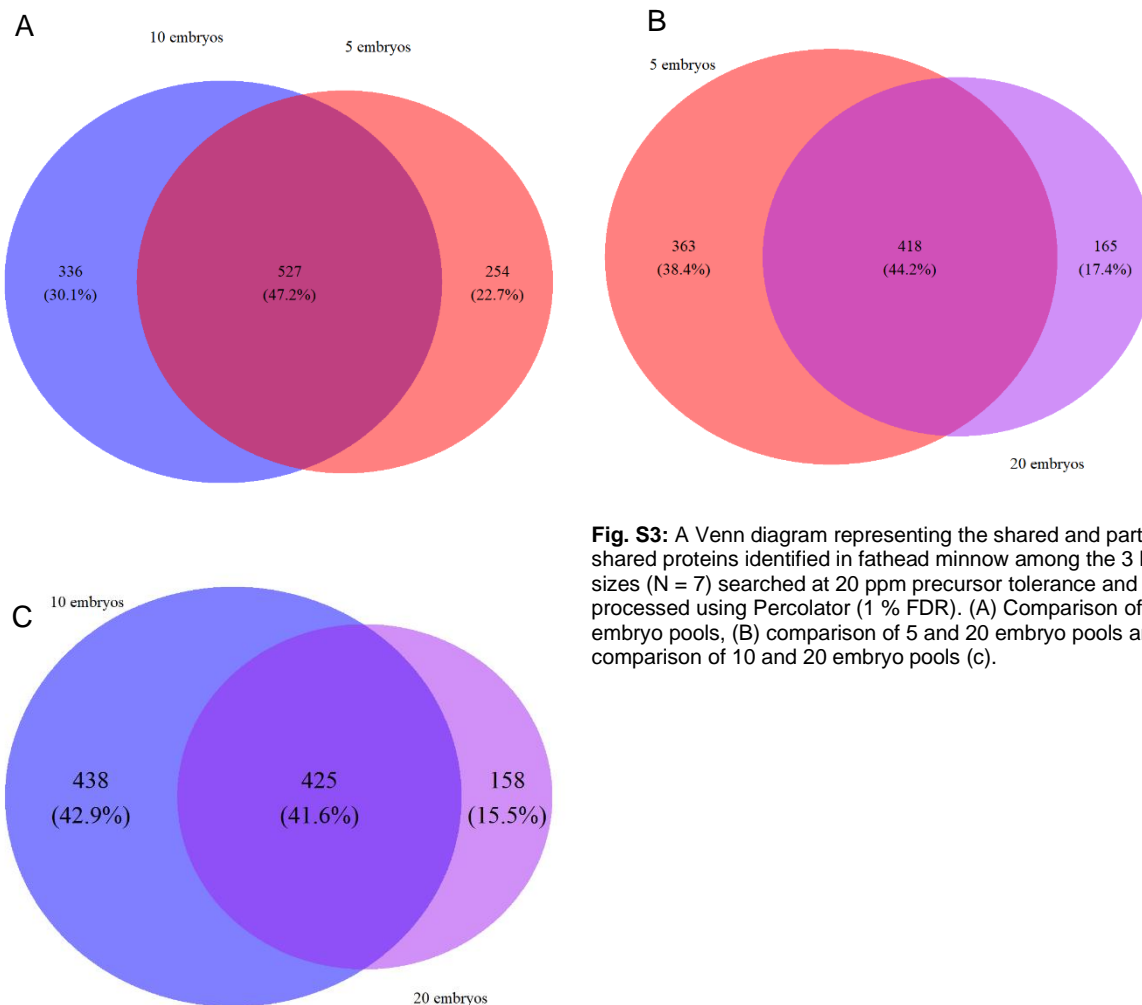
Organism	Organism pool size	Ontology	GO-ID	GO-ID name	Gene count	% Unique proteins
Zebrafish	5	Biological processes (BP)	GO:0071704	Organic substance metabolic process	51	25
			GO:0044238	Primary metabolic process	48	24
			GO:0044237	Cellular metabolic process	47	23
	10		GO:0044237	Cellular metabolic process	35	17
			GO:0071704	Organic substance metabolic process	34	16
			GO:0044238	Primary metabolic process	33	16
	20		GO:0050789	Regulation of biological processes	62	23
			GO:0050794	Regulation of cellular processes	57	21
			GO:0071704	Organic substance metabolic process	54	20
			GO:0044238	Cellular metabolic processes	50	19
Zebrafish	5	Molecular function (MF)	GO:0097159	Organic cyclic compound binding	29	14
			GO:1901363	Heterocyclic compound binding	28	14
			GO:0043167	Ion binding	27	13
	10		GO:0097159	Organic cyclic compound binding	24	12
			GO:1901363	Heterocyclic compound binding	23	11
			GO:0043167	Ion binding	19	9
	20		GO:0043167	Ion binding	46	17
			GO:0097159	Organic cyclic compound binding	44	17
			GO:1901363	Heterocyclic compound binding	43	16



**Fig. S1:** Equal amounts of three of the seven zebrafish embryo protein samples ( $n = 3$ ) per pool size were SDS-PAGE fractionated with technical replicates indicated by brackets. No difference in the protein amounts are apparent.



**Fig. S2:** Venn diagram representing the shared and partially shared proteins identified in zebrafish among the 3 larval pool sizes ( $N = 7$ ) searched at 20 ppm precursor tolerance and post-processed using Percolator (1 % FDR). Comparison of 5 and 10 embryo pools (A), comparison of 5 and 20 embryo pools (B) and comparison of 20 and 10 embryo pools (C).



**Fig. S3:** A Venn diagram representing the shared and partially shared proteins identified in fathead minnow among the 3 larval pool sizes (N = 7) searched at 20 ppm precursor tolerance and post-processed using Percolator (1 % FDR). (A) Comparison of 5 and 10 embryo pools, (B) comparison of 5 and 20 embryo pools and (C) comparison of 10 and 20 embryo pools (c).

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