Langan and Brooks:

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

Supplementary Data

Table S1: Proteomic studies limited to early life stages (< 120 hpf) 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) and larvae at (>120 hpf). For fathead, only one study was identified, with approximately 3-4 larval fish used per replicate (Moreton et al., 2020). In respect to the column titled standardized testing, we specifically refer 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. The use of standardized toxicology testing guidelines is outlined in Standardized regulatory method, where "no" denotes no standardized method, and "Yes" denotes the use of a standardized methodology. Total protein specifically refers to the total number of protein group identification 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)

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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		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)

Table S2: Summary of search parameters
It should be noted that protein identifications were run using a precursor tolerance of 20 and 50 ppm.

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

Table S3: Subset of gene ontology classification for zebrafish based on unique proteins identified in each larval pool (see Fig. 4)

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	Larval 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

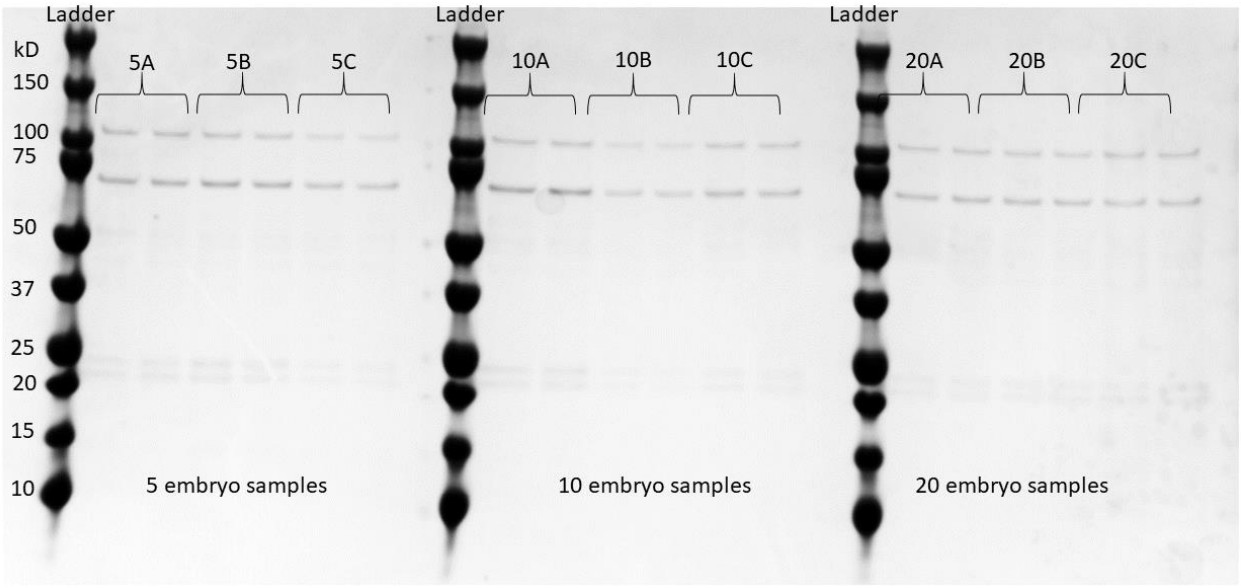

Figure S1: SDS-PAGE (zebrafish) Equal amounts of three of the seven zebrafish embryo protein samples (n=3) per pool size were SDS-PAGE fractionated with technical replicates indicated by brackets. No difference in the protein pro les are apparent.

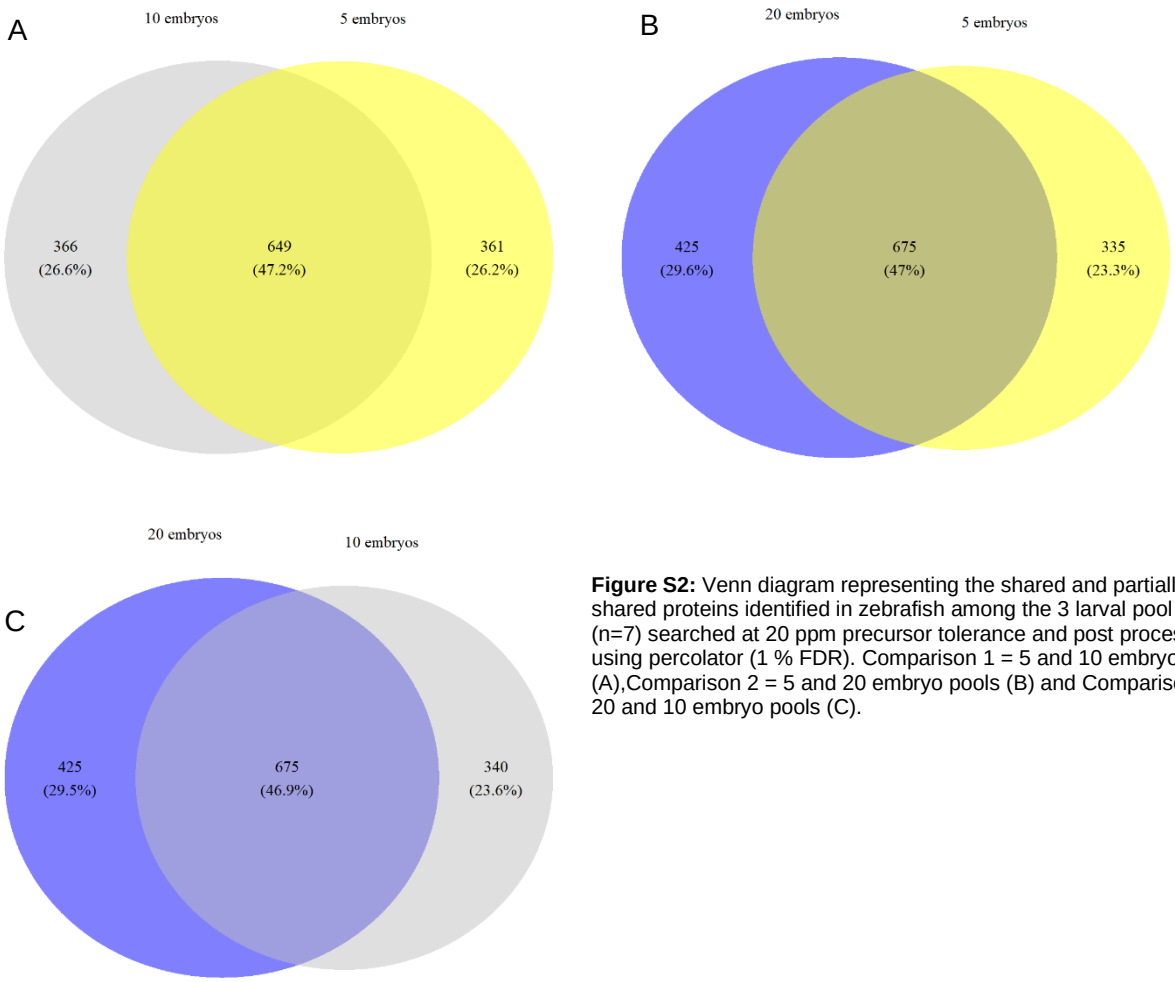

Figure S2: Venn diagram representing the shared and partially shared proteins identified in zebrafish among the 3 larval pool sizes (n=7) searched at 20 ppm precursor tolerance and post processed using percolator (1 % FDR). Comparison 1 = 5 and 10 embryo pools (A),Comparison 2 = 5 and 20 embryo pools (B) and Comparison 3 = 20 and 10 embryo pools (C).

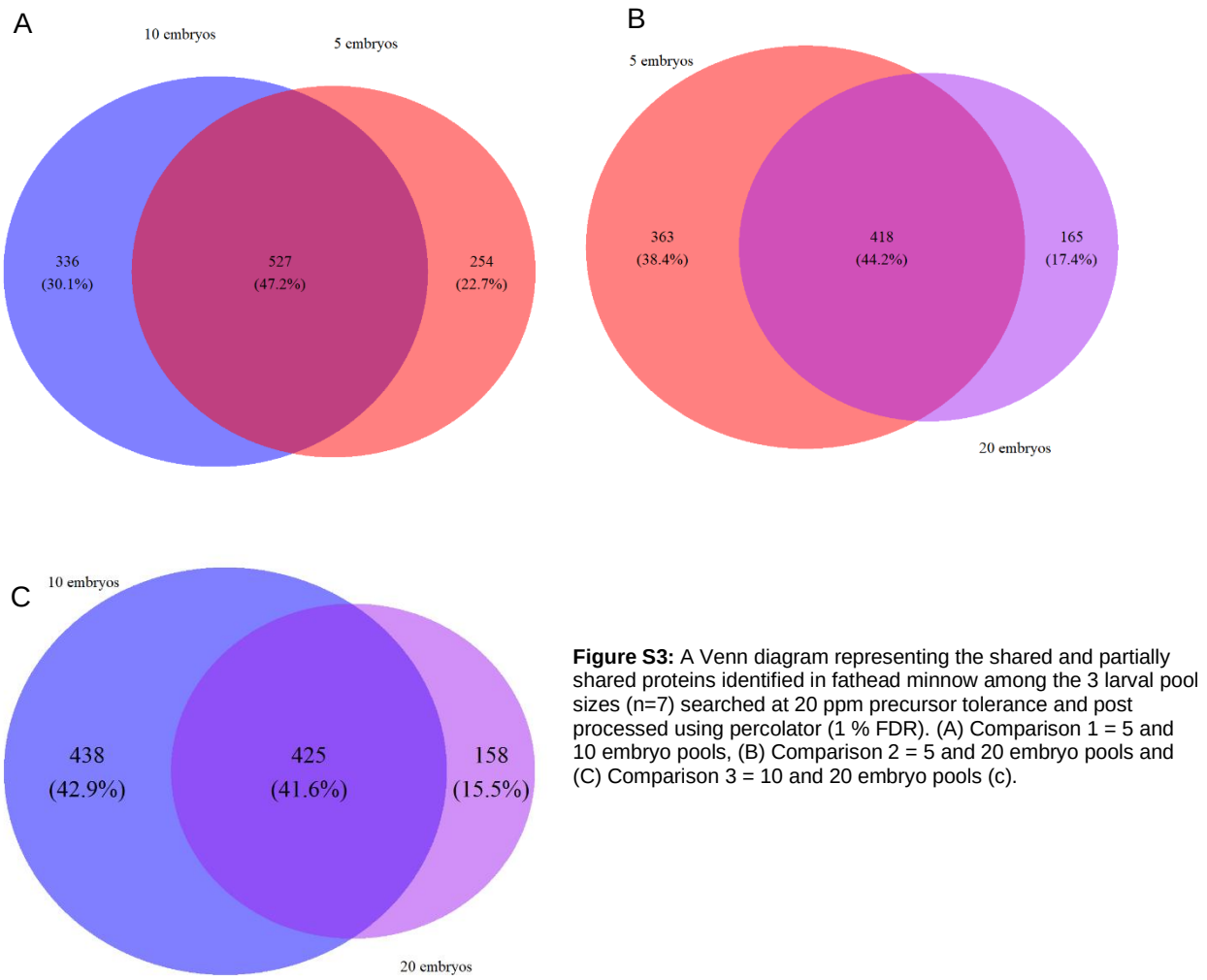

Figure S3: A Venn diagram representing the shared and partially shared proteins identified in fathead minnow among the 3 larval pool sizes (n=7) searched at 20 ppm precursor tolerance and post processed using percolator (1 % FDR). (A) Comparison 1 = 5 and 10 embryo pools, (B) Comparison 2 = 5 and 20 embryo pools and (C) Comparison 3 = 10 and 20 embryo pools (c).

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