



<http://www.diva-portal.org>

Postprint

This is the accepted version of a paper published in *Journal of Fish Biology*. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the original published paper (version of record):

Nissling, A., Larsson, R. (2018)  
Population specific sperm production in European flounder *Platichthys flesus*:  
Adaptation to salinity at spawning  
*Journal of Fish Biology*, 93(1): 47-52  
<https://doi.org/10.1111/jfb.13667>

Access to the published version may require subscription.

N.B. When citing this work, cite the original published paper.

Permanent link to this version:

<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-364060>

1 **Population specific sperm production in flounder *Platichthys flesus* - adaptation to salinity at**  
2 **spawning**

3  
4 A. Nissling<sup>a\*</sup>, R. Larsson<sup>b</sup>

5  
6 <sup>a</sup>Ar Research Station, Department of Ecology and Genetics, Uppsala University, SE-621 67 Visby,  
7 Sweden, <sup>b</sup>Institute of Marine Research, SLU Aqua, SE-453 21 Lysekil, Sweden

8  
9 \*Author responsible for correspondence: Anders Nissling. Tel.: +46 (0)498 224630; e-mail:

10 [anders.nissling@ebc.uu.se](mailto:anders.nissling@ebc.uu.se)

11  
12 Running headline: ADAPTATION OF SPERM PRODUCTION IN *P. FLESUS*

---

13  
14 Pre-printed manuscript of article published in Journal of Fish Biology

15 [wileyonlinelibrary.com/journal/jfb](http://wileyonlinelibrary.com/journal/jfb)

16 Please cite this article as: Nissling, A. & Larsson, R. (2018). Population specific sperm production in  
17 flounder *Platichthys flesus* - adaptation to salinity at spawning. Journal of Fish Biology 93, 47-52. DOI:  
18 10.1111/jfb.13667 (Accepted: 19 April 2018).

20

## ABSTRACT

21

22 Marine teleosts inhabiting the brackish water Baltic Sea have adapted to the less saline water with  
23 activation of spermatozoa at low salinity hypo-osmotic conditions but with shorter longevity and  
24 lower swimming speed that affect the fertilization capacity. Aiming to elucidate if the fertilization  
25 capacity may be maintained by increasing the number of spermatozoa produced, testis size for the  
26 euryhaline flounder *Platichthys flesus* with external fertilization was assessed along a salinity gradient;  
27 with spawning at a salinity of  $\approx 7$ , 10-18 and 30-35. Fulton's condition factor ( $100 \text{ somatic fish mass}$   
28  $\text{total fish length}^{-3}$ ) averaged  $0.881 \pm 0.085$  (mean  $\pm$  S.D.),  $0.833 \pm 0.096$  and  $0.851 \pm 0.086$ , for fish  
29 spawning at  $\approx 7$ , 10-18 and 30-35, respectively, with no difference between areas, *i.e.* analysed fish  
30 were in similar nutritional condition. A general linear model, with testes dry mass as dependent  
31 variable and somatic mass as covariate resulted in a significant difference between areas/populations  
32 with larger testes for *P. flesus* spawning at a salinity of  $\approx 7$  but no difference between fish spawning at  
33 a salinity of 10-18 and 30-35. The result suggests that adaptation by increasing the number of  
34 spermatozoa produced may be a key mechanism for marine teleosts spawning in areas with low  
35 salinities to sustain the fertilization capacity as shown here for the euryhaline *P. flesus*.

36

37 Key words: flounder, salinity, adaptation, sperm production, testes size, population specific

40 A prerequisite for fishes to inhabit an area is the potential to reproduce, i.e. the distribution is restricted  
41 by the ability to cope with environmental conditions at spawning. Hence, adaptations for reproduction  
42 have evolved and continue to evolve in response to local environmental conditions. Accordingly,  
43 preferences for, or tolerances to, environmental parameters such as salinity vary amongst species, and  
44 amongst populations within species given the wide range of habitats utilized by fishes (*e.g.* Wootton,  
45 1990).

46 To accomplish successful reproduction activation of the spermatozoa is crucial for fertilization. The  
47 spermatozoa remain quiescent in the testes and seminal plasma at isotonic conditions ( $\approx 300 \text{ mOsm kg}^{-1}$ ;  
48 corresponding to a salinity of  $\approx 10$ ) prior to the release into the water when motility is triggered by  
49 the hypo- or hyperosmotic conditions in freshwater- and marine species, respectively (*e.g.* Morisawa  
50 & Suzuki, 1980; Stoss, 1983; Billard, 1986; Alavi & Cosson, 2006). For marine fishes, spermatozoa  
51 activation occurs at discharge in high saline water, 30-35, triggered (as shown for at least some  
52 species) by an efflux of water from the spermatozoa to the hyperosmotic environment which results in  
53 an elevated internal concentration of calcium ions ( $\text{Ca}^{2+}$ ), and initiation of motility (*e.g.* Cosson *et al.*,  
54 2008; Boj *et al.*, 2015). Thus, salinity/osmolality and/or concentrations of specific ions are  
55 determinant factors for spermatozoa activation and fertilization capacity in fishes. Some marine fishes  
56 inhabit and reproduce also in brackish water with adaptation of spermatozoa motility to low salinities  
57 (see below). Although the mechanism is yet not fully understood this represents an interesting  
58 challenge concerning the evolution of coping with low osmotic differences between the internal and  
59 external osmolality and adaptation of spermatozoa motility. Spermatozoa motility is additionally under  
60 the influence of maternal factors (egg/ovarian fluid) enhancing both the swimming speed and the  
61 proportion of motile sperm as shown for the marine Atlantic cod, *Gadus morhua* L. (Litvak & Trippel,  
62 1998). However, the effect ceased at low salinities (salinity of 15; see Litvak & Trippel, 1998),  
63 corresponding to the salinity of spermatozoa activation in cod in marine waters (15-16; Nissling &

64 Westin, 1997). This suggests that the initiation of spermatozoa motility is still governed by  
65 salinity/osmolality and/or concentrations of specific ions.

66 The capacity for fertilization is a question of spermatozoa longevity and speed and direction of  
67 swimming as well as the number of spermatozoa activated. Aiming to assess the relative importance of  
68 spermatozoa motility vs the number of spermatozoa produced in relation to intensity of sperm  
69 competition (*i.e.* in relation to spawning behaviour as internal/external fertilization,  
70 polygamy/monogamy, communal spawning/distinct pairing) Stockley *et al.* (1997) found an increase  
71 in both spermatozoa numbers and relative testes mass (*cf* gonado-somatic index) with intensity of  
72 sperm competition across species, *i.e.* as an adaptation to increase the fertilization capacity. Similarly,  
73 Awata *et al.* (2008) found an increase in the relative testes mass as an adjustment to sperm competition  
74 risk in the cichlid *Julidochromis transcriptus* (Matthes 1959) with polyandrous males producing larger  
75 testis compared to monogamous males. Stockley *et al.* (1997) ranked intensity of sperm competition  
76 for several teleost species based on spawning behaviour. Flounder *Platichthys flesus* L. was given a low  
77 rank, *i.e.* low sperm competition, as spawning in pairs (Table I in Stockley *et al.*, 1997).

78 For marine fishes to reproduce at low salinities, adaptations are required as both fertilization and egg  
79 development is affected at low salinities as shown for teleosts inhabiting the brackish water Baltic Sea  
80 (Solemdal, 1970; Westin & Nissling, 1991; Nissling *et al.*, 2002; 2006), with salinities ranging from 3  
81 to 9 in the surface water and between 10-20 in the deep basins below the halocline (see Voipio, 1981).  
82 Only a few fish species of marine origin have succeeded in colonizing the Baltic Sea, with adaptations  
83 of spermatozoa motility and egg characteristics that are considered population specific (Solemdal,  
84 1973; Thorsen *et al.*, 1996; Nissling & Westin, 1997).

85 *Platichthys flesus* inhabit both marine and brackish waters in coastal waters of the Eastern Atlantic from  
86 the White Sea to the Mediterranean and the brackish water Black Sea and Baltic Sea (FishBase, 2017)  
87 with spawning occurring at salinities ranging from 6-7 to 30-35. In the Baltic Sea two genetically  
88 distinct *P. flesus* ecotypes (Hemmer-Hansen *et al.*, 2007; Florin & Höglund, 2008) with different  
89 spawning strategies occur; spawning pelagic eggs in offshore deep basins below the permanent

90 halocline at a salinity of 10-20 and demersal eggs in coastal areas and on offshore banks at 6-9  
91 (Nissling *et al.*, 2002; 2015; Ustup *et al.*, 2013). For a more thorough description of salinity  
92 conditions in the Baltic Sea as well as spawning areas of *P. flesus* see e.g. Voipio (1981) and Nissling  
93 *et al.* (2002). In *P. flesus* spermatozoa activation occur at a salinity of  $\geq 9-10$  for the ecotype spawning  
94 in the deep basins and at  $\leq 3$  for the type spawning in coastal areas and on offshore banks vs at a  
95 salinity of  $\approx 11$  for *P. flesus* at marine conditions off Bergen, Norway in the North Sea (Solemdal,  
96 1970; Nissling *et al.*, 2002). However, spermatozoa motility is affected at low salinities with  
97 significantly shorter longevity and lower swimming speed (for methodology see studies on turbot  
98 *Scophthalmus maximus* L.; Nissling *et al.*, 2006) at a salinity of  $\leq 11-12$  and  $\leq 7$  for the deep basin- and  
99 the coastal spawning *P. flesus* ecotype respectively, with a decrease in fertilization (Nissling *et al.*,  
100 2002; observations in the present study). A more thorough overview of variability in salinity  
101 conditions and effects on the reproductive success of *P. flesus* in the Baltic Sea, with implications for  
102 spatial distribution and variability in stock abundance is given in Nissling *et al.* (2002) and in Ustup  
103 *et al.* (2013).

104 As given above both spermatozoa longevity and swimming speed decrease significantly with  
105 decreasing salinity affecting the fertilization rate in *P. flesus*. Hence, to compensate for decreased  
106 motility in marine fishes when spawning at low salinities, the fertilization rate may be maintained by  
107 increasing the number of spermatozoa produced. Aiming to elucidate the effects of salinity on adaptive  
108 reproductive strategy we assessed testes size in the euryhaline *P. flesus* with external fertilization,  
109 along a salinity gradient; spawning occurring in marine water at 30-35, at brackish water at 15-18, at  
110 10-12 and at  $\approx 7$ , *i.e.* potential adaptation of spermatozoa production in relation to salinity at spawning.

111

112

## MATERIAL AND METHODS

113

114 Fish were caught during spawning, by trawling at Lilla Middlegrund and Morups Bank in Kattegat  
115 (ICES SD 21) at 40-50 m depth in March 2015 and 2016, and in the Baltic Sea using gill-nets with

116 different mesh sizes in the Hanö Bight (SD 25) at  $\approx 70$  m depth in April 2015, and off Gotland (SD 28)  
117 at both 50-70 m and 10-20 m depth in April 2015 and 2016 using gill-nets with different mesh sizes  
118 (Fig. 1). Individuals in stage 3 (ICES, 2010), *i.e.* in running condition (milt flows at light pressure)  
119 were sampled and frozen. For fish sampled in SD 25 and SD 28, where both *P. flesus* ecotypes occur  
120 (Nissling *et al.*, 2015), determination of ecotype based on spermatozoa motility in different salinities  
121 (see Nissling *et al.*, 2002) was performed before being frozen. Spermatozoa motility was assessed at  
122 250 x magnifications under a microscope at a salinity of 15, 10.5, 7.5 and 6 respectively, at 7 °C.  
123 Individuals with swimming spermatozoa at both 10.5-15 and at 6-7.5 were considered coastal  
124 spawners (ecotype with demersal eggs) as opposed to individuals displaying swimming spermatozoa  
125 at 10.5-15 only considered deep basin spawners (ecotype with pelagic eggs; see Nissling *et al.*, 2002).  
126 For each determination semen was sampled directly from the male using a dry Pasteur-pipette, and for  
127 each salinity tested a droplet of semen was diluted in approximately 1 ml water, prepared from Baltic  
128 Sea water (salinity  $\approx 6.5-7$ ) and synthetic seasalt or by dilution with distilled water, to induce  
129 spermatozoa activation. The time from mixing to observation was approximately 5 s. Additionally, the  
130 initial motility of swimming spermatozoa was judged at a relative scale; swimming slow, at  
131 intermediate speed or fast, to obtain an idea of potential differences in spermatozoa motility between  
132 salinities.

133 The fish were thawed, and total length ( $\pm$ mm;  $L_T$ ), somatic mass [ $\pm 1$  g (intestine and testis removed);  
134  $W_S$ ] and testis wet mass ( $\pm 0.001$  g;  $W_{TW}$ ) measured. Stage of spawning was assessed based on colour  
135 and shape of testes, and photographed. Only individuals with testes judged as in early stage 3 were  
136 included in analysis, *i.e.* whitish bulbous testis as opposed to testis with a reddish-greyish cast and  
137 with partly flaccid lobes (see images shown as Supporting Information for examples). Dry mass ( $W_{TD}$ )  
138 of sampled testis was measured ( $\pm 0.0001$  g) after incubation at 60°C for 24 h. The relative testes mass  
139 (*cf* gonado-somatic index;  $I_G$ ) was calculated as  $100 W_{TD} W_S^{-1}$  and fish condition ( $C_F$ ) assessed as  
140 Fulton's condition factor ( $100 W_S L_T^{-3}$ ); considered representative based on similarity of the exponent  
141 in the length-mass relationship for *P. flesus* males in the Baltic Sea, 2.83, and the North Sea, 2.75  
142 (Fishbase, 2017).

143 Analysis included potential differences between fish according to area and *P. flesus* ecotype; Kattegat  
144 (KA), deep basin spawners in the Baltic Sea (BSDS) and coastal spawners in the Baltic Sea (BSCS).  
145 For fish sampled in SD 25 and SD 28 (both ecotypes occur) only individuals fulfilling both the  
146 criterion: determined according to ecotype based on spermatozoa motility, and caught at expected  
147 depth, 50-70 m and 10-20 m, for deep basin- and coastal-spawners respectively, were included in  
148 analysis (overlap in distribution of the ecotypes during spawning may occur with potentially  
149 hybridisation; see Nissling *et al.*, 2015). Potential differences in  $L_T$  and  $C_F$  were examined using  
150 ANOVA. Differences in  $W_{TD}$  were analysed by a General Linear Model (IBM SPSS Statistics  
151 software, version 22; www.ibm.com), with  $\log W_{TD}$  as dependent variable, area/ecotype as fixed factor  
152 and  $\log W_S$  as covariate, including the interaction between  $W_S$  and area/ecotype. The model was run  
153 stepwise with subsequent removal of non-significant variables ( $P>0.05$ ; starting with the highest) until  
154 only significant predictors remained.

155 Positions of sampling sites, dates of sampling and depth range and salinity are given in Table I  
156 together with the number of fish analysed.

157

158

## RESULTS

159

160 Total  $L_T$  of analysed *P. flesus* averaged 27.3 cm (range 22.8-33.2), 24.9 cm (19.7-33.6) and 25.1 cm  
161 (19.8-29.4) for fish from KA, BSDS and BSCS respectively with KA fish being significantly larger  
162 than fish from BSDS and BSCS ( $F_{2,101}=10.2$ ,  $P<0.001$ ) potentially reflecting the sampling methods  
163 (trawling vs caught with gill-nets with different mesh sizes). No difference in fish condition occurred  
164 ( $F_{2,101}=2.07$ ,  $P=0.131$ );  $C_F$  averaged  $0.851\pm 0.086$  (mean $\pm$ S.D.),  $0.833\pm 0.096$  and  $0.881\pm 0.085$  for *P.*  
165 *flesus* from KA, BSDS and BSCS respectively, indicating that the fish analysed were in similar  
166 nutritional condition.



167 Assessed initial spermatozoa motility at different salinities of the coastal- and deep-basin spawning *P.*  
168 *flesus* ecotype, respectively in the Baltic Sea is shown in Fig. 2. Spermatozoa swimming speed  
169 decreased with decreasing salinity, e.g. lower initial motility at 6-7.5 vs at 10.5-15 for BSCS fish and  
170 somewhat lower motility at 10.5 vs at 15 with no motility at 6-7.5 for BSDS fish, i.e. concomitant to  
171 assessment of duration of spermatozoa motility at different salinities as reported in Nissling et al.  
172 (2002), spermatozoa motility of *P. flesus* decreases with salinity and differs between the ecotypes.

173 Testes size, assessed as dry mass, differed significantly between the ecotypes ( $P < 0.001$ ; Table II), with  
174 larger testes for BSCS fish compared to KA and BSDS fish, and with no difference between BSDS  
175 and KA fish ( $P > 0.05$ ; pairwise comparisons). Testes dry mass, evaluated at a somatic mass of 0.149  
176 kg (Table II), averaged 1.37 (1.20-1.56) g (mean and 95% C.L.) for BSCS, and 0.51 (0.43-0.60) and  
177 0.46 (0.41-0.51) g for BSDS and KA fish respectively. Hence, the outcome yielded significantly  
178 larger testes for *P. flesus* spawning in coastal areas in the Baltic Sea at a salinity of 7-7.5 compared to  
179 *P. flesus* spawning offshore at salinity of 10-18 in the Baltic Sea deep basins and at 30-35 in the  
180 Kattegat (Fig. 3). Accordingly, the relative testes mass ( $I_G$ ) varied between  $0.82 \pm 0.34$  (mean  $\pm$  S.D.) for  
181 BSCS and  $0.31 \pm 0.14$  and  $0.31 \pm 0.13$  for BSDS and KA fish respectively, with corresponding values  
182 for the relative testes mass calculated using testes wet mass ( $W_{TW}$ ) of  $5.7 \pm 1.4$ ,  $2.4 \pm 1.2$  and  $2.0 \pm 0.6$ ,  
183 respectively.

184 Images of testes size for a 26 cm *P. flesus* from KA, BSDS and BSCS respectively is shown as  
185 Supporting Information.

186

187

## DISCUSSION

188

189 Analysis of testes investment of *P. flesus* along a salinity gradient revealed a significant difference in  
190 testes dry mass between areas/populations, with larger testis for *P. flesus* spawning in the Baltic Sea at  
191 a salinity of  $\approx 7$  compared to fish spawning in the Baltic Sea at a salinity of 10-18 and fish spawning in

192 the Kattegat at 30-35. The coefficient of determination in regressions varied between 0.38-0.65. This  
193 may be considered reasonable given that the testes included in analysis represent fish in early stage 3,  
194 *i.e.* intra-area/ecotype variability should be expected as individuals have commenced spawning.

195 The present study suggests, concomitant to Bagenal's theory about higher fecundity due to harsher  
196 conditions affecting reproduction at the borders of a species distribution compared to in the centre  
197 (Bagenal, 1966), a similar pattern concerning sperm production. For *P. flesus* in the Baltic Sea, living  
198 at the border of its distribution with respect to salinity, the reproductive success is limited by effects on  
199 both fertilization rate and egg development with implications for variability in the spatial distribution  
200 and in stock abundance (Nissling *et al.*, 2002; Ustupis *et al.*, 2013). For the ecotype spawning in  
201 coastal areas at a salinity of 6-9, the reproductive success is sustained by higher sperm production  
202 (present study) and higher fecundity (Nissling & Dahlman, 2010) compared to the ecotype spawning  
203 in the deep basins at a salinity of 10-20 as well as populations in marine waters. The coastal spawning  
204 *P. flesus* ecotype displayed significantly larger testes, 1.38 g (mean) dry mass vs 0.51 g and 0.46 g for  
205 the deep basin spawning ecotype and in marine water respectively for a 0.149 kg (somatic mass) fish  
206 (present study), and higher fecundity, 1726000 for coastal spawners vs 1097000 for deep basin  
207 spawners similar to fish in the North Sea in marine water with estimated fecundity of 920000 -  
208 1245000, for a 35 cm *P. flesus* (Nissling & Dahlman, 2010). Hence, to cope with lower fertilization  
209 rates and egg survival when spawning at low salinities, adaptations involve higher allocation of energy  
210 to gonad production with implications for the trade-off between somatic and gonad growth, as  
211 evaluated for female *S. maximus* from populations in environments with different salinities at  
212 spawning. For Baltic Sea *S. maximus*, spawning at a salinity of 6-7, the yearly energy allocation to  
213 gonad production in females amounted to  $\approx 19\%$  compared to  $\approx 7\%$  in marine conditions, adversely  
214 affecting the somatic growth at low salinities (Wallin & Nissling, unpublished). Similarly, for female  
215 *P. flesus* in the Baltic Sea, somatic growth of the coastal spawning ecotype is poorer compared to the  
216 deep basin spawning ecotype due to higher egg production (Nissling & Dahlman, 2010). For males  
217 however, there is no difference in somatic growth between the coastal- and deep-basin spawning *P.*  
218 *flesus* ecotype in the Baltic Sea ( $F=0.72$ , d.f.=1,  $P>0.05$ ) based on *P. flesus* caught off Gotland (SD

219 28) (Florin & Nissling, unpublished data). This is however not surprising given the difference in  
220 energy allocation to gonad production between male and female *P. flesus*. The gonado-somatic index  
221 (calculated as 100 gonad dry mass somatic wet mass<sup>-1</sup>) for females average 7.8% and 12.3% for deep-  
222 basin spawning and coastal spawning Baltic Sea *P. flesus*, respectively (data from Nissling &  
223 Dahlman, 2010), compared to 0,3% and 0,8% respectively for males (data from the present study), *i.e.*  
224 considerably lower energy allocation to gonad production for *P. flesus* males.

225 For males, fertilization capacity may be regarded as a question of spermatozoa motility in terms of  
226 duration, speed and direction or the in total distance covered, as well as the number of activated  
227 spermatozoa. Spawning at low salinities implies effects on both duration, speed and number of  
228 activated spermatozoa with effects on the fertilization rate as shown for flatfishes in the Baltic Sea; *S.*  
229 *maximus*, plaice *Platessa platessa* L. and *P. flesus* (Solemdal, 1973; Nissling *et al.*, 2002; 2006). Data  
230 for *P. flesus* in ICES SD 25 and SD 28, *i.e.* the same areas in the Baltic Sea as in the present study  
231 (Table IX and X in Nissling *et al.*, 2002), suggest similar duration of spermatozoa motility at a salinity  
232 of 7-15 for the coastal spawning *P. flesus* ecotype as for the deep basin spawning ecotype at 12-30, but  
233 with considerably shorter duration at lower salinities affecting the fertilization rate at  $\leq 6.5$  but also at 7  
234 (Fig. 5 in Nissling *et al.*, 2002). Similar to as for *S. maximus* in the Baltic Sea (see Fig. 3 and Table II  
235 in Nissling *et al.*, 2006), this suggests that also spermatozoa speed is crucial for successful fertilization  
236 of *P. flesus* as observations of initial swimming speed conducted to distinguish between the respective  
237 ecotype in the present study suggest (Fig. 2). However, neither of these studies was designed for  
238 evaluation of the relative importance of swimming speed, duration, direction and number of activated  
239 spermatozoa for the fertilization capacity. For an appropriate analysis of this, a more thorough  
240 methodology is required, *i.e.* in future studies *e.g.* a Computer-Assisted Sperm Analysis (CASA)  
241 systems with Assisted Sperm Morphology Analysis (ASMA) software enabling the estimation of a  
242 number of sperm motion parameters (see Mylonas *et al.*, 2016) should be used to address adaptations  
243 according to low salinities.

244

245 Studies of reproduction in marine fishes adapted to spawning at low salinities represents an interesting  
246 field not only for evaluation of the relative importance of spermatozoa swimming performances for the  
247 fertilization capacity of fishes in general, but also for mechanisms triggering the initiation of  
248 spermatozoa activity as motility occur at also hypo-osmotic conditions (at a salinity of <10) as  
249 opposed to at hyper-osmotic conditions in a fully marine environment. In this respect the role of  
250 aquaporins shown to be involved in both spermatozoa activation and motion pattern (Boj *et al.*, 2015)  
251 should be considered. As pointed out by Alavi & Cosson (2006), there is a wide range of variability in  
252 the ionic composition of seminal plasma/spermatozoa in fishes, both between species and among  
253 populations within species, allowing for adjustments in mechanisms concerning spermatozoa motility,  
254 *i.e.* probably in response to varying salinity in accordance to the wide range of spawning habitats  
255 utilized by fishes. The present study suggests that in fishes of marine origin, the number of  
256 spermatozoa produced may be a key mechanism to sustain the fertilization capacity and thus the  
257 ability to reproduce in areas with low salinities.

258

259

#### ACKNOWLEDGEMENTS

260

261 Many thanks to I. Wallin and M. Ovegård for helping hands during sampling. The study received  
262 partly funding from BONUS INSPIRE, the joint Baltic Sea research and development programme (Art  
263 185), funded jointly by the European Union's Seventh Programme for research, technological  
264 development and demonstration and the Swedish Research Council Formas.

265

266

267

268

## REFERENCES

269

270 Alavi, S.M.H. & Cosson, J. (2006). Sperm motility in fishes. (II) Effects of ions and osmolality: A  
271 review. *Cell Biology International* **30**, 1-14.

272 Awata, S., Takeyama, T., Makino, Y., Kitamura, Y. & Kohda, M. (2008). Cooperatively breeding  
273 cichlid fish adjust their testis size but not sperm traits in relation to sperm competition risk. *Behavioral*  
274 *Ecology Sociobiology* **62**, 1701-1710.

275 Bagenal, T.B. (1966). The ecological and geographical aspects of the fecundity of plaice. *Journal of*  
276 *the Marine Biology Association UK* **46**, 161-186.

277 Billard, R. (1986). Spermatogenesis and spermatology of some teleost fish species. *Reproduction*  
278 *Nutrition Development* **2**, 877-920.

279 Boj, M., Chauvigné, F. & Cerdà, J. (2015). Coordinated action of aquaporins regulates sperm motility  
280 in a marine teleost. *Biology of Reproduction* **93**(2), 40:1-11.

281 Cosson, J., Groison, A.L., Suquet, M., Fauvel, C., Dreanno, C. & Billard, R. (2008). Marine fish  
282 spermatozoa: racing ephemeral swimmers. *Reproduction* **136**, 277-294.

283 Florin, A.-B., Höglund, J. (2008). Population structure of flounder (*Platichthys flesus*) in the Baltic  
284 Sea: differences among demersal and pelagic spawners. *Heredity* **101**, 27-38.

285 Hemmer-Hansen, J., Nielsen, E.E., Grønkjær, P. & Loeschcke, V. (2007). Evolutionary mechanisms  
286 shaping the genetic population structure of marine fishes; lessons from European flounder (*Platichthys*  
287 *flesus* L.). *Molecular Ecology* **16**, 3104-3118.

288 Litvak, M.K. & Trippel, E.A. (1998). Sperm motility of Atlantic cod (*Gadus morhua*) in relation to  
289 salinity: effects of ovarian fluid and egg presence. *Canadian Journal of Fisheries and Aquatic*  
290 *Sciences* **55**, 1871-1877.

291 Morisawa, M. & Suzuki, K. (1980). Osmolality and potassium Ion: their role in initiation of sperm  
292 motility in teleosts. *Science* **210**, 1145-1146.

293 Mylonas, C.C., Duncan, N.J. & Asturiano, J.F. (2016). Hormonal manipulations for the enhancement  
294 of sperm production in cultured fish and evaluation of sperm quality. *Aquaculture*.  
295 [http://www.diversifyfish.eu/uploads/1/4/2/0/14206280/mylonas\\_2016\\_aqua\\_p1\\_online.pdf](http://www.diversifyfish.eu/uploads/1/4/2/0/14206280/mylonas_2016_aqua_p1_online.pdf)

296 Nissling, A., Thorsen, A. & da Silva, F.F.G. (2015). Fecundity regulation in relation to habitat  
297 utilisation of two sympatric flounder (*Platichthys flesus*) populations in the brackish water Baltic Sea.  
298 *Journal of Sea Research* **95**, 188-195.

299 Nissling, A. & Dahlman, G. (2010). Fecundity of flounder, *Pleuronectes flesus*, in the Baltic Sea –  
300 Reproductive strategies in two sympatric populations. *Journal of Sea Research* **64**, 190-198.

301 Nissling, A. & Westin, L. (1997). Salinity requirements for successful spawning of Baltic and Belt Sea  
302 cod and the potential for cod stock interactions in the Baltic Sea. *Marine Ecology Progress Series* **152**,  
303 261-271.

304 Nissling, A., Johansson, U. & Jacobsson, M. (2006). Effects of salinity and temperature conditions on  
305 the reproductive success of turbot (*Scophthalmus maximus*) in the Baltic Sea. *Fisheries Research* **80**,  
306 230-238.

307 Nissling, A., Westin, L. & Hjerne, O. (2002). Spawning success in relation to salinity of three flatfish  
308 species, Dab (*Pleuronectes limanda*), Plaice (*Pleuronectes platessa*) and Flounder (*Pleuronectes*  
309 *flesus*), in the brackish water Baltic Sea. *ICES Journal of Marine Science* **59**, 93-108.

310 Solemdal, P. (1970). The reproductive adaptation of marine teleosts to water of low salinity. *ICES*  
311 *International Council for the Exploration of the Sea*, Committee Meeting F:30.

312 Solemdal, P. (1973). Transfer of Baltic flatfish to a marine environment and the long term effects on  
313 reproduction. *Oikos* (suppl.) **15**, 268-276.

- 314 Stockley, P., Gage, M.J.G., Parker, G.A. & Møller, A.P. (1997). Sperm competition in fishes: the  
315 evolution of testis size and ejaculate characteristics. *The American Naturalist* **149**, 933-954.
- 316 Stoss, J. (1983). Fish gamete preservation and spermatozoan physiology. In *Fish physiology*, Vol IXB  
317 (Hoar, W.S., Randall, D.J. & Donaldson, E.M., eds), pp. 305-350. New York: Academic Press.
- 318 Thorsen, A., Kjesbu, O.S., Fyhn, H.J. & Solemdal, P. (1996). Physiological mechanisms of egg  
319 buoyancy in brackish water cod (*Gadus morhua* L.). *Journal of Fish Biology* **48**, 457-477.
- 320 Ustup, D., Müller-Karulis, B., Bergström, U., Makarchouk, A. & Sics, I. (2013). The influence of  
321 environmental conditions on early life stages of flounder (*Platichthys flesus*) in the central Baltic Sea.  
322 *Journal of Sea Research* **75**, 77-84.
- 323 Westin, L. & Nissling, A. (1991). Effects of salinity on spermatozoa motility, percentage of fertilized  
324 eggs and egg development of Baltic cod (*Gadus morhua* L.), and implications for cod stock  
325 fluctuations in the Baltic. *Marine Biology* **108**, 5-9.
- 326 Voipio, A. (1981). *The Baltic Sea*. Elsevier Oceanographic Series no. 30. Amsterdam: Elsevier  
327 Scientific Company.
- 328 Wootton, R.J. (1990). *Ecology of teleost fishes*. Fish and Fisheries series 1. UK: Chapman & Hall.

#### 329 **ELECTRONIC REFERENCES**

- 330
- 331 FishBase. Froese, R. & Pauly, D. (Eds) (2017). FishBase. World Wide Web electronic publication.  
332 [www.fishbase.org](http://www.fishbase.org) (06/2017). Available at  
333 <http://www.fishbase.org/Summary/SpeciesSummary.php?ID=1341&AT=flounder> (accessed 3 August  
334 2017).
- 335 ICES. (2010). Report of the Workshop on Sexual Maturity Staging of sole, plaice, dab and flounder  
336 (WKMSSPDF), 22-26 February 2010, Ijmuiden, The Netherlands. ICES CM 2010/ACOM:50.  
337 Available at [www.vliz.be/imisdocs/publications/223184.pdf](http://www.vliz.be/imisdocs/publications/223184.pdf)

338

## TABLES

339 Table I. Sampling locations, depth range and salinity, and date of catches of spawning *Platichthys flesus*  
 340 in the present study. P and D refer to the ecotype with pelagic and demersal eggs, respectively, and  
 341 numbers to fish used in analysis. *N.B.* fish of the deep basin spawning *P. flesus* ecotype in the Baltic  
 342 Sea (BSDS) from SD 25 and SD 28 were pooled in analysis.

ICES SD	Acronym	Date	Position	Depth (m)	Salinity	Number
SD 21 P	KA	March 10	N 56°50' E 12°10'	≈40	30-35	49
		March 17	N 57°02' E 11°51'	≈50		
SD 25 P	BSDS	April 8	N 55°59' E 14°79'	≈70	15-18	17
SD 28 P	BSDS	April 23	N 57°37' E 19°05'	≈50-70	10-12	4
			N 57°33' E 19°12'			
SD 28 D	BSCS	April 22	N 57°43' E 18°92'	≈10-20	7-7.5	32
			N 57°42' E 19°00'			

343

344 Table II. Results of the GLM analysis of gonad dry mass (log) in relation to somatic mass (log) for  
 345 *Platichthys flesus*, according to the respective sampling area and ecotype; P and D refer to the *P. flesus*  
 346 ecotype with pelagic and demersal eggs, respectively. Covariate appearing in the model was evaluated  
 347 at the somatic weight 0.149 kg.

Area (ICES subdivision)	Mean	Std. error	95% Confidence interval	
			Lower bound	Upper bound
KA SD 21 (P)	-0.341	0.024	-0.389	-0.293
BSDS SD 25 and 28 (P)	-0.294	0.036	-0.367	-0.222
BSCS SD 28 (D)	0.136	0.029	0.079	0.194

Between subject effects	d.f.	<i>F</i>	<i>P</i>
Area x somatic mass	2,101	1.745	0.180*
Somatic mass	1,101	106.3	<0.001
Area	2,101	86.95	<0.001

348 \*interaction omitted in final analysis

349



350

## FIGURE CAPTIONS

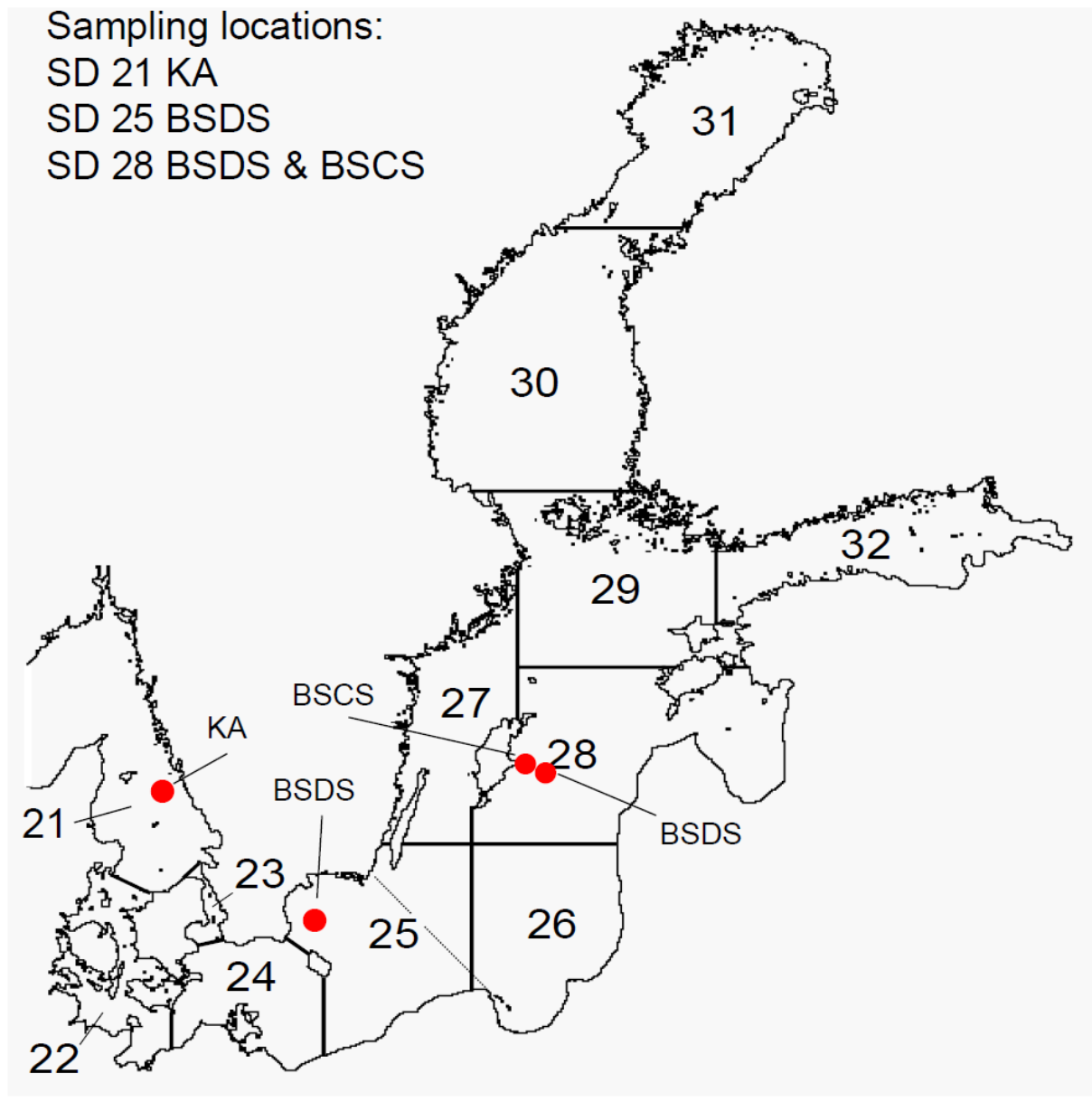
351 Fig. 1. The Baltic Sea and Kattegat with ICES subdivisions (SD) and positions of sampling locations  
352 for *Platichthys flesus*; KA, BSDS (two sites) and BSCS (Table I). Kattegat SD 21 and Baltic Sea SD  
353 22-32 (ICES statistical area 3; FAO fishing area 27, the Northeast Atlantic).

354 Fig. 2. Initial spermatozoa motility at different salinities of a) coastal- and b) deep-basin spawning  
355 *Platichthys flesus* sampled in ICES subdivision 25 and SD 28 in the Baltic Sea; shown as the share of  
356 males displaying spermatozoa motility as swimming slow, at intermediate speed or fast. n shows the  
357 number of fish assessed. *N.B.* for the deep-basin spawning ecotype, no motility occurred at salinities  
358 of 6 and 7.5.

359 Fig. 3. Relationship between testis dry mass (log g) and somatic mass (log kg) for *Platichthys flesus*  
360 according to area and ecotype; KA (Kattegat), BSDS (Baltic Sea deep-basin spawners) and BSCS  
361 (Baltic Sea coastal spawners). BSDS fish sampled in ICES subdivision (SD) 25 and SD 28 pooled in  
362 analysis.

363

364

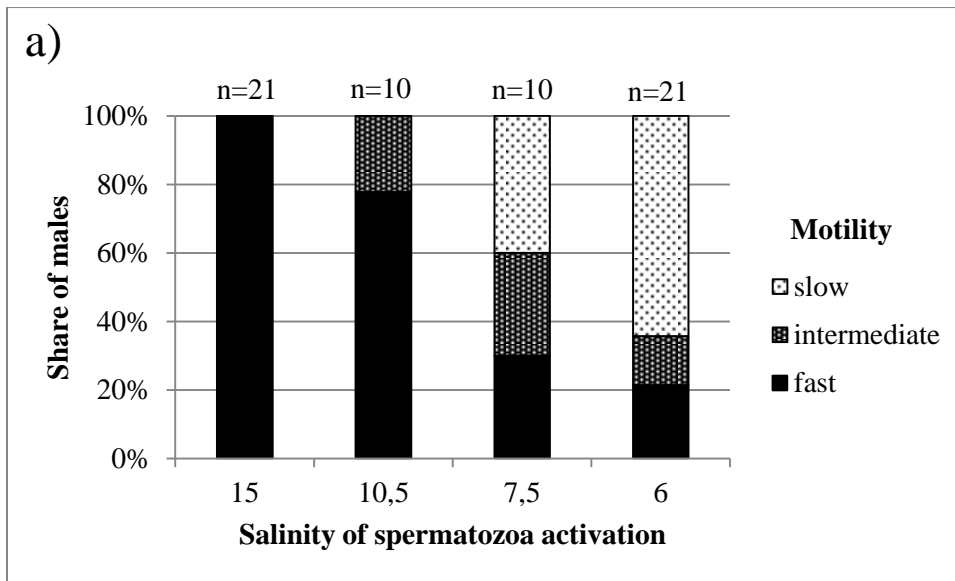


366

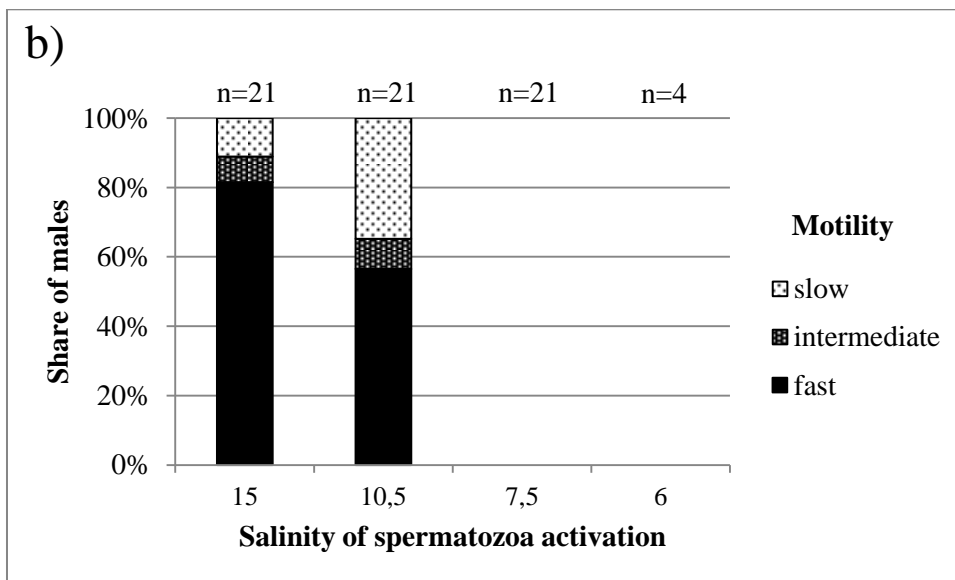
367

368 Figure 2.

369



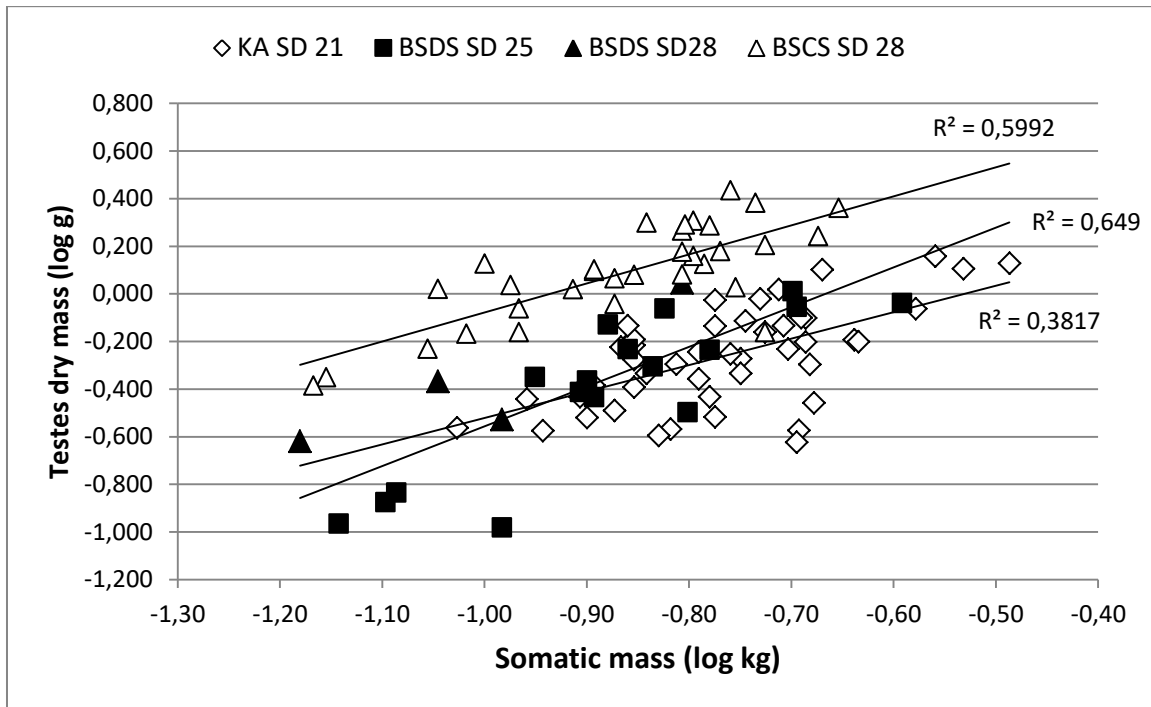
370



371

372

373 Figure 3.



374

375

376 **Population specific sperm production in flounder *Platichthys flesus* - adaptation to salinity at**  
377 **spawning**

378 **A. Nissling, R. Larsson**

379 e-mail: anders.nissling@ebc.uu.se

380 **Journal of Fish Biology**

381 **Supporting Information:**

382 Images of sampled testes in stage 3 (ICES 2010) for flounder *Platichthys flesus* sampled in ICES  
383 subdivision (SD) 21 (A), SD 25 (B) and SD 28 (C and D). KA refer to fish sampled in Kattegat (SD  
384 21) spawning at a salinity of 30-35, BSDS to fish sampled in the Baltic deep basins (SD 25 and SD  
385 28) spawning at a salinity of 10-18, and BSCS to fish sampled in coastal areas (SD 28) spawning at a  
386 salinity of  $\approx 7$  (Table I; Fig. 1). Shown are examples of testes that were omitted in analysis based on  
387 color and shape as not being in early stage 3 (see ICES 2010), and testes for a 26 cm (total length) fish  
388 from the respective area/population (KA, BSDS and BSCS); the size of frames is the same for all  
389 images. N.B. the photos have been adjusted to show images at the same magnification.

390

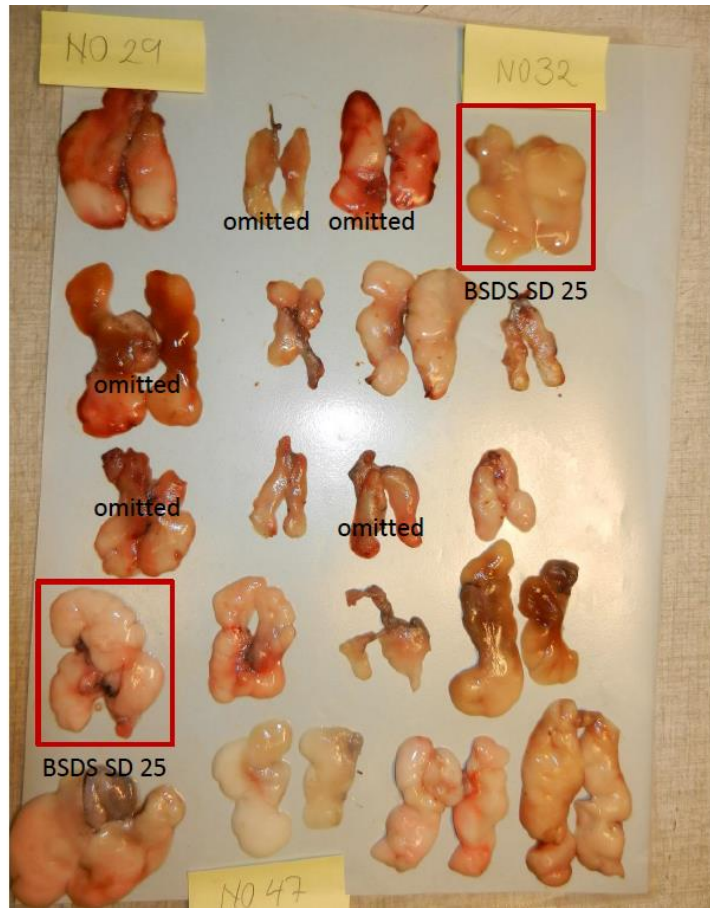
A)



391

392

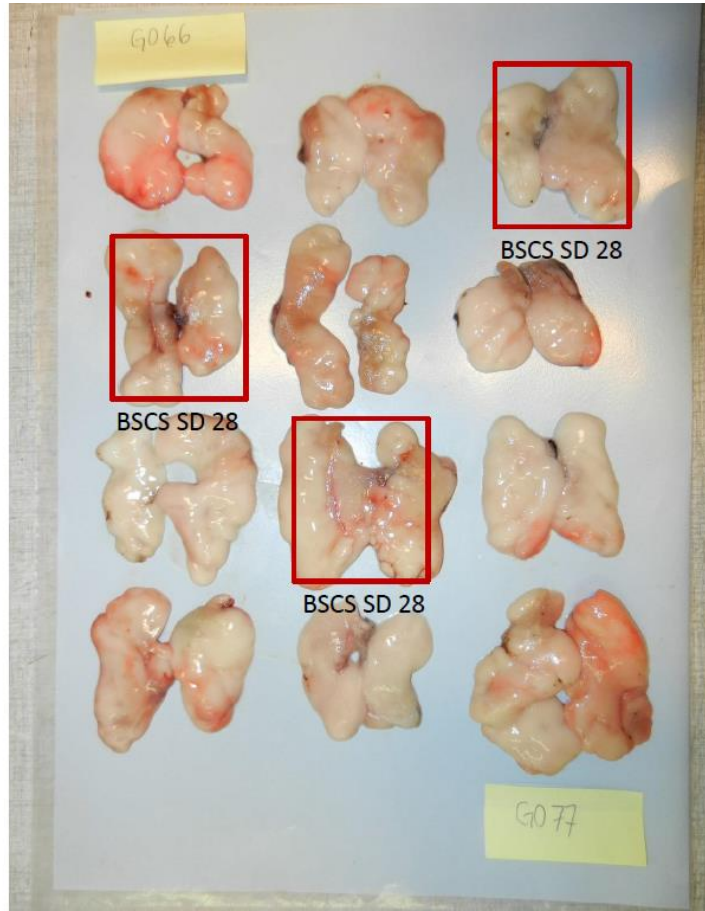
B)



393

394

C)

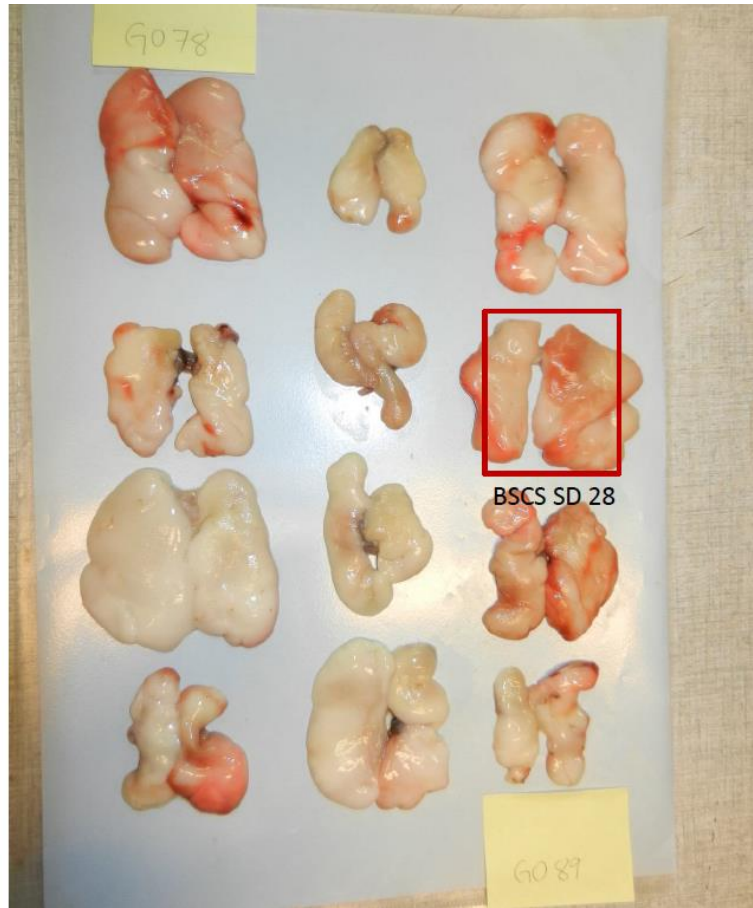


395

396



D)



397