Corrosion inhibition of copper in 0.5 M NaCl solutions by aqueous and hydrolysis acid extracts of olive leaf

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1 **Timing of oocyte recruitment within the ovulatory cycle of Macedonian**

2 **shad, *Alosa macedonica*, a batch spawning fish with indeterminate**

3 **fecundity**

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13

Abstract

14

15 The recruitment of primary growth (PG) oocytes to the secondary growth (SG) phase within

16 the ovulatory cycle, i.e. time interval between two sequential ovulation/spawning events, has

17 rarely been examined in detail in a quantitative manner for batch spawning fishes with

18 indeterminate fecundity. In the present study we analyzed the ovarian dynamics of

19 Macedonian shad, *Alosa macedonica*, an iteroparous batch spawning clupeid with

20 indeterminate fecundity, with the main goal to define the timing of PG recruitment and relate

21 it to the ovulatory cycle. The latter was classified into four different phases (pre-ovulatory,

22 running, post-ovulatory, intermediate) through postovulatory follicles and presence/absence

23 of hydrated oocytes. Various indices of ovarian dynamics, including the formation and mean

24 diameter of the advanced oocyte batch, the ovarian developmental stage, the oocyte size

25 modality and the gonadosomatic index, varied among the ovulatory phases, evidencing

26 cyclicity. Relative fecundity of newly recruited SG oocytes was used as an index of PG

27 recruitment intensity and was shown to follow a specific pattern within the ovulatory cycle;

28 PG recruitment occurred in a stepwise manner and in parallel with ovulation of the advanced

29 oocyte batch, and synchronization of these two processes kept the ovary in a state of dynamic
30 equilibrium.

31

35

1. Introduction

37 Many fish species are classified as batch (or multiple, partial, serial, heterochronal; [1,2])
38 spawners, since they release their oocytes in multiple events within a spawning season [3-5].
39 Thus, during the ovulatory cycle, i.e. time interval between two sequential
40 ovulation/spawning events, of a batch spawner, ovarian developmental processes take place
41 so the first event will be followed by the second, the second by the third, and so on. In case
42 of batch spawners with determinate fecundity type, these ovarian processes pertain mainly
43 to the secondary growth phase (SG) and the development of the advanced batch to be
44 spawned next [6]. On the contrary, in batch spawners exhibiting indeterminate fecundity type,
45 the ovarian processes that occur within the ovulatory cycle include both the formation of the
46 advanced oocyte batch and the recruitment of oocytes from the primary (PG) to the SG phase
47 (i.e. PG recruitment) [6].

48 The development of the advanced oocyte batch can follow different patterns among fishes
49 [7] and has been studied extensively in both determinate (e.g. [6,8]) and indeterminate (e.g.
50 [9-12]) spawners. The process of PG recruitment has also been examined both qualitatively
51 (e.g. [13-15]) and quantitatively (e.g. [16-21]). During the last decade, significant progress has
52 been made in terms of small-sized oocyte characterization and quantification, mainly by the
53 development of the oocyte packing density theory (OPD) [22], which led to advanced and very
54 detailed distinction of small-sized PG and SG oocytes into developmental stages and
55 estimation of their numbers in each of these stages [17-20]. In specific, Korta et al. (2010)
56 combined advanced OPD, stereological techniques and the auto-diametric method [23] to
57 examine the early cycle oocyte recruitment dynamics in European hake, *Merluccius merluccius*
58 [17]. Kjesbu et al. (2011) established the "grid method", which is a simpler version of OPD, to
59 quantify PG and SG oocyte production in Atlantic cod, *Gadus morhua*, [18]. OPD was also

implemented to study the ovarian dynamics of the European anchovy, *Engraulis encrasicolus* [19]. Moreover, Serrat et al. (2019) analyzed the seasonal ovarian dynamics in European hake via advanced OPD [20]. Most of these studies were focused on broad temporal scales, such as the reproductive season or the spawning season. To our knowledge, only the paper of Schismenou et al. (2012) provided suggestions regarding the underlying mechanisms of PG recruitment within the narrow temporal scale of the ovulatory cycle [19]. In specific, the latter study suggested recruitment "in rapid pulses of less than 24-h (possibly few hours) activated by the hydration of the spawning batch" in European anchovy.

To fill this gap, we analyzed the ovarian dynamics of Macedonian shad, *Alosa macedonica*, an iteroparous batch spawning clupeid with indeterminate fecundity [24, authors unpublished data], within the ovulatory cycle, with the main goal to define the timing of PG recruitment and relate it to ovulation cyclicity. To do so, the ovulatory cycle was classified into four different phases. Ovarian developmental indices were compared among these phases to assess ovulation cyclicity in the best detail possible and to define the timing of PG recruitment. This study analyzed both the formation of the advanced oocyte batch and PG recruitment within the ovulatory cycle and intended to find a temporal association between these two ovarian processes.

2. Materials and methods

Fish were collected with vertical gill nets during the night on five sampling dates in June 2016 (Table 1), i.e. during the spawning season [24]. All fish were immediately stored in 10% neutral buffered formalin. The biometric measurements included total length (in mm), total weight (in g), eviscerated weight (W_{ev} in g) and gonad weight (W_g in g). Gonadosomatic index was also estimated (GSI = 100 x W_g/W_{ev}).

Ovarian subsamples from 91 mature females were processed histologically (~2mm thick hand-cut cross sections, paraffin embedding, 4-μm sections, hematoxylin/eosin staining) and through whole-mount procedure (Table 1). Each histological section was digitized into a high-resolution photomicrograph using a BASLER acA1920-40uc camera fitted on a Zeiss Axio Lab.A1 microscope and the Microvisioneer software, and each whole-mount subsample was digitally imaged using a Jenoptik Progress C3 camera mounted on a Euromex NZ 80 stereo microscope. Prior to digitalization, whole-mount subsamples were stained with hematoxylin to increase oocyte opacity and prevent possible overlook due to their original transparency.

92 The photomicrographs enabled, for each female: (i) identification of the distinct oocyte

93 developmental stages at both the PG and the SG phase, (ii) detection of postovulatory follicles

94 (POF), measurement of the cross-sectional area of the biggest POF in the section, POF_{XSA} (see

95 [25]), and classification of each POF_{XSA} as large or small based on its histomorphological

96 characteristics [26,27] and its value, and (iii) definition of the ovarian developmental stage as

97 the stage of the most advanced oocytes in the histological section. We identified: cortical

98 alveolar (CA) oocyte stages, three vitellogenic (VIT-1 – VIT-3) stages, the germinal vesicle

99 migration (GVM) stage and the hydration (HYD) stage [28].

100 Whole-mount photos were analyzed through the auto-diametric method and particle analysis

101 [23] to estimate oocyte sizes and to create the oocyte size frequency distributions (OSFDs).

102 Each OSFD was subsequently analyzed through the Bhattacharya method [29] to identify any

103 clearly distinguished modes and to estimate the size (i.e. number of oocytes) and the mean

104 oocyte diameter (OD) of each mode, as has been previously done for Allis shad, *A. alosa* [21]

105 and Alewife *A. pseudoharengus* [30]. The advanced mode (AM) was separated in all OSFDs

106 (see Results section) and consequently, its relative fecundity (i.e. number of oocytes per g of

107 W_{ev}) was estimated through the gravimetric method [2] using the formula:

$$ RF_{AM} = \frac{n_{AM} \times \dfrac{W_g}{W_{ss}}}{W_{ev}} $$

108

109 where n_{AM} was the number of oocytes of the AM and W_{ss} the weight of the whole-mount

110 subsample. AM corresponded to the spawning batch, i.e. to the oocytes that are released

111 during a single spawning episode, and thus RF_{AM} was equivalent to relative batch fecundity

112 (number of oocytes released per spawning event per g of W_{ev}; RF_b). To avoid possible bias, RF_b

113 was estimated only from hydrated females prior to an ovulation event, i.e. those classified as

114 in pre-ovulatory phase (see Results section).

115 To estimate relative total fecundity (i.e. number of all SG oocytes per g of W_{ev}; RF_t), we set the

116 size threshold between PG and SG oocytes at 200 μm, based on previous values reported for

117 Allis shad [21] and American shad *A. sapidissima* [31]. RF_t was estimated gravimetrically for

118 each female by using the formula:

$$ RF_t = \frac{n_t \times \dfrac{W_g}{W_{ss}}}{W_{ev}} $$

119

120 where n_t was the number of all SG oocytes of the whole-mount subsample.

4

121 Recruitment intensity was assessed through the relative fecundity of the small-sized SG

122 oocytes, i.e. those with diameter between 200 and 320 μm ($RF_{200-320}$), based on previously

123 implemented methodology [16]. The ratio of RF_t to mean RF_b of females at the pre-ovulatory

124 phase (fecundity ratio) was used as a proxy of the number of co-occurring SG oocyte batches,

125 as has been done in previous study [12]. In females caught while ovulating, (running; see

126 Results section), the fecundity ratio was estimated by subtracting the remaining hydrated

127 oocytes from RF_t and adding unity to the resulting RF_t/RF_b quotient to counterbalance the

128 missing ovulating batch.

129 Cyclicity was examined by comparing the numerical formation of the AM and the mean

130 diameter of the AM (OD_{AM}), the ovarian developmental stage, the modality of OSFDs and GSI

131 among the different ovulatory phases (see Results section).

132 We used R 3.5.2 [32] to perform the statistical analyses and to create the plots. The specific R

133 packages used in this study were: ggplot2, ggridges, ggpubr [33-35]. Normality was tested by

134 the Shapiro test.

135

136 ## 3. Results

137 *3.1 Ovulatory phases classification*

138 Multiple SG oocyte developmental stages were present in each ovary. All females were

139 spawning capable (i.e. with oocytes at an advanced stage, capable of spawning during the

140 current reproductive period; [28]). High proportion of females (87%) had POF in their ovaries.

141 Most of these ovaries (82.5%) had POF from two different cohorts (new and old), which

142 originated from two sequential daily spawning events (Fig. 1). Thus, POF degeneration period

143 was constantly longer than the ovulatory cycle. POF_{XSA} of the newest cohort in each ovary,

144 classified as large, had values > 0.05 mm^2, while small POF_{XSA} had values < 0.02 mm^2 (Fig. 2).

145 Four phases were identified within the ovulatory cycle based on the presence/absence of HYD

146 oocytes and the presence of large or small POF_{XSA} (Table 2): (1) pre-ovulatory (PRE), (2) running

147 (RUN), (3) post-ovulatory (POST), and (4) intermediate (INT). The criterion of POF_{XSA} of the

148 newest cohort was clear-cut, especially for distinguishing PRE from RUN and POST from INT

149 females (Fig. 3). The few females without POF in their ovaries had HYD oocytes and were

150 classified as PRE.

151

152   *3.2 Ovarian dynamics cyclicity*

153   The profiles of AM formation, as overviewed in Figure 4, revealed that AM was discernible in

154   all four ovulatory phases. However, there were clear differences; the AM was gradually

155   becoming completely separated from the smaller oocytes as ovulatory cycle progressed from

156   POST to RUN females (Fig. 4). Cyclicity was evinced also through the ovarian developmental

157   stage; both PRE and RUN females were at the HYD stage, POST females were at the VIT-2 or

158   VIT-3 stage, and all INT females had progressed to VIT-3 or GVM ovarian stage (Fig. 4).

159   Detailed analysis of OSFDs revealed co-occurrence of various distinct modes within the

160   smaller oocytes (SM1, SM2, etc.) (Fig. 5). In general, as females were approaching an ovulation

161   event (PRE females), the different modes were becoming more distinct (Fig. 5).

162   GSI and mean OD_{AM} also exhibited a clear pattern, evidencing the continuity of ovarian

163   dynamics within the ovulatory cycle (Fig. 6). Prior to an ovulation event (PRE females), GSI and

164   OD_{AM} were at their maximum values (mean GSI ± SD = 21.6 ± 3.3; mean OD_{AM} ± SD = 864 ± 36

165   µm). Subsequently, both GSI and OD_{AM} followed a decreasing trend and dropped to their

166   minimum values (mean GSI ± SD = 8.6 ± 2.3; mean OD_{AM} ± SD = 500 ± 33 µm) in POST females.

167   As females approached readiness to ovulate again (INT females), both GSI and OD_{AM} increased

168   (mean GSI ± SD = 9.5 ± 1.5; mean OD_{AM} ± SD = 604 ± 50 µm). Mean GSI and OD_{AM} values were

169   significantly different from one ovulatory phase to the next (least significant difference, 95%

170   confidence level, $P < 0.05$). The only two exceptions were the lack of statistically significant

171   difference in GSI between POST and INT females and in OD_{AM} between PRE and RUN females

172   (least significant difference, 95% confidence level, $P > 0.05$).

173

174   *3.3 Timing of PG Recruitment*

175   PG recruitment occurred stepwise and in parallel with the ovulation of the advanced oocyte

176   batch. RUN females had higher mean (± SD) $RF_{200-320}$ values (411 ± 162 oocytes per g)

177   compared to PRE (323 ± 118 oocytes per g) and INT (270 ± 99 oocytes per g) females (least

178   significant difference, 95% confidence level, $P < 0.05$; Fig. 7a). In addition, RUN females had

179   higher mean (± SD) fecundity ratio (3.5 ± 0.9) than PRE (2.9 ± 0.6), POST (2.3 ± 0.8) and INT (2

180   ± 0.4) females (least significant difference, 95% confidence level, $P < 0.05$; Fig. 7b). The mean

181   RF_b of PRE females used to estimate the fecundity ratio values was 415 oocytes per g.

182

4. Discussion

Batch spawning fishes with either determinate or indeterminate fecundity display ovulation cyclicity, i.e. processes occur within the ovulatory cycle to accomplish egg release in multiple sequential events during their spawning period. The ovarian processes that take place within the ovulatory cycle in indeterminate batch spawners include mainly the formation of the advanced oocyte batch to be spawned next and the recruitment of oocytes from the primary (PG) to the secondary (SG) growth phase (PG recruitment). The formation of the advanced batch has been frequently analyzed, whilst the PG recruitment has often been overlooked and rarely examined in detail and in a quantitative manner at the fine temporal scale of the ovulatory cycle. In this study, we evinced cyclicity and we analyzed both the formation of the advanced batch and PG recruitment within the ovulatory cycle in an indeterminate batch spawning fish, Macedonian shad *A. macedonica*. Our results enabled the detection of the timing of PG recruitment within the ovulatory cycle and revealed a temporal association between ovulation of the advanced oocyte batch and PG recruitment.

Ovulation cyclicity in batch spawning fishes has been demonstrated in different ways, such as behavioral observations [36,37] and analyses of hormone profiles [38-40]. Especially, cyclicity in ovarian processes has been evinced through different indices and in many species [9-12,38,41]. In case of *A. macedonica*, we showed that ovarian processes were occurring within the ovulatory cycle by comparing several indices among four different ovulatory phases: the AM numerical formation and the OD_{AM}, the ovarian developmental stage, the modality of OSFDs and GSI. All these indices displayed specific patterns within the ovulatory cycle, and thus evinced the continuity of ovarian dynamics between sequential ovulation events. GSI and OD_{AM} followed an increasing trend from the completion of an ovulation event and prior to the next. The modality of OSFDs was getting more obvious and the AM was becoming more separated from the smaller oocytes as females were approaching an ovulation event. In addition, the developmental stage of the AM was more advanced in females that were prior to an ovulation event than in those that had just ovulated.

The two main ovarian processes that take place within the ovulatory cycle of indeterminate batch spawners have been analyzed disproportionately. The formation of the advanced oocyte batch has been analyzed extensively (e.g. [9-12,38,41,42]), whilst detailed analyses of PG recruitment have been conducted at a far less extent. The studies focused on PG recruitment were either qualitative, resulting in detailed description of the sequential developmental oocyte stages prior and after the recruitment process [15,43,44], or

216 quantitative [16, 18-20]. Most of these latter studies used advanced quantification techniques
217 to analyze PG recruitment process mostly during broad temporal scales, such as the
218 reproductive season and the spawning season [18-20], or rarely during the narrower temporal
219 scale of the ovulatory cycle [19]. In this study, we implemented the auto-diametric method
220 [23] with a few modifications (hematoxylin staining to increase PG and small SG oocyte
221 opacity), which may be less informative regarding the stages of oocytes but has been proven
222 effective in quantifying small-sized oocytes (e.g. [21,45]).

223 Comparisons in recruitment intensity and fecundity ratio among females at different
224 ovulatory phases revealed a clear pattern of PG recruitment. Females caught during ovulation
225 had higher recruitment intensity and fecundity ratio compared to females at earlier or later
226 ovulatory phases, revealing that a new batch of SG oocytes was recruited from the pool of PG
227 reserves at a specific time-frame, during the ovulation of the advanced oocyte batch. Our
228 conclusion is corroborated by previous reports for another indeterminate batch spawning
229 clupeid, European anchovy [19]. More specifically, the latter study suggested that oocyte
230 recruitment occurred in swift pulses triggered by the hydration of the spawning batch.

231 Figure 8 depicts a conceptual model of the ovarian dynamics within the ovulatory cycle in an
232 indeterminate batch spawner. In summary, PG recruitment and ovulation of the advanced
233 batch are synchronized, keeping the ovary in a state of a dynamic equilibrium regarding RF_t
234 and number of SG batches in the ovary, while GSI and OD_{AM} fluctuate between relatively
235 constant minimum and maximum values. The concept of a dynamic equilibrium in the ovaries
236 of actively spawning indeterminate batch spawners has been previously suggested. Mature
237 ovaries of Northern anchovy *E. mordax* were shown to contain a stable number of oocyte
238 batches; result attributed to the theoretically continuous recruitment of small oocytes in
239 parallel with spawning activity [46]. Additionally, model simulations suggested that PG
240 recruitment kept pace with spawning of the advanced oocyte batch, and thus sustained a
241 balanced number of oocyte batches and total fecundity in indeterminate batch spawners [47].
242 Finally, detailed analysis, through the OPD [22], suggested that oocyte numbers in the ovaries
243 were in a steady state, displaying an equilibrium, also in European anchovy [19]. Our study
244 evinced the concept of dynamic equilibrium within the ovulatory cycle and reported a
245 temporal association between ovulation of the advanced oocyte batch and PG recruitment.
246 The described association did not provide any justification of a cause-effect relationship
247 between the two ovarian processes and we suggest this matter to be analyzed in future
248 studies.

256

257 **Declarations of interest**

258 Declarations of interest: none

259

260 **Table titles**

261 **Table 1.** Number (N) of females analyzed histologically and through whole-mount analysis,

262 and total length (in mm) range, mean and standard deviation (S.D.), per sampling date

263 **Table 2.** Phases identified within the ovulatory cycle, based on the presence/absence (+/-) of

264 hydrated (HYD) oocytes, presence/absence (+/-) of postovulatory follicles (POF) and cross-

265 sectional area of the biggest POF (POF_{XSA}).

266

267 **Figure captions**

268 **Figure 1.** Photomicrograph of an ovarian histological section (hematoxylin/eosin staining)

269 showing postovulatory follicles (POF) from two different ovulation events (old and new POF

270 cohorts).

271 **Figure 2.** Frequency distribution of the cross-sectional area (in mm^2) of the biggest

272 postovulatory follicle (POF_{XSA}) in each of the 79 ovaries analyzed. Two POF_{XSA} size classes were

273 distinguished: small (area < 0.02 mm^2) and large (area > 0.05 mm^2).

274 **Figure 3.** Violin plot of the cross-sectional area (in mm^2) of the biggest postovulatory follicle

275 (POF_{XSA}) in each ovarian histological section for females at different phases within the

276 ovulatory cycle: prior to an ovulation event (pre-ovulatory phase), during ovulation (running

phase), immediately or closely after an ovulation (post-ovulatory phase) and in-between two ovulation events (intermediate phase). Colors and shapes represent the ovarian developmental stages, i.e. the stage of the most advanced oocytes in each ovary. VIT-2 = secondary vitellogenic stage, VIT-3 = tertiary vitellogenic stage, GVM = germinal vesicle migration stage, HYD = hydration stage.

Figure 4. Size frequency distributions of oocytes at the secondary growth phase for females at different phases within the ovulatory cycle: prior to an ovulation event (pre-ovulatory phase), during ovulation (running phase), immediately or closely after an ovulation (post-ovulatory phase) and in-between two ovulation events (intermediate phase). Color of each distribution represents the ovarian developmental stage, i.e. the stage of the most advanced oocytes in the ovary. Within each panel, distributions are displayed in order of increasing (bottom to top) mean oocyte diameter of the secondary growth oocytes. VIT-2 = secondary vitellogenic stage, VIT-3 = tertiary vitellogenic stage, GVM = germinal vesicle migration stage, HYD = hydration stage.

Figure 5. Size frequency distributions of oocytes at the secondary growth phase and the corresponding ovarian histological sections for females at different phases within the ovulatory cycle: (a–b) immediately or closely after an ovulation (post-ovulatory phase; POST), (c–d) in-between two ovulation events (intermediate phase; INT), and (e–f) prior to an ovulation event (pre-ovulatory phase; PRE). AM = advanced mode, SM1 = first subsequent mode, SM2 = second subsequent mode, SM3 = third subsequent mode, PG = primary growth stage, CA = cortical alveolar stages, VIT-1 = primary vitellogenic stage, VIT-2 = secondary vitellogenic stage, VIT-3 = tertiary vitellogenic stage, HYD = hydration stage, POF = postovulatory follicle.

Figure 6. Line plot of mean values and confidence intervals of: (a) gonadosomatic index (GSI) and (b) diameter (in µm) of the advanced oocyte batch (OD_{AM}), for females at different phases within the ovulatory cycle: prior to an ovulation event (pre-ovulatory phase), during ovulation (running phase), immediately or closely after an ovulation (post-ovulatory phase) and in-between two ovulation events (intermediate phase). Statistically significant pairwise comparisons of mean values are shown with asterisks ("**" for $P < 0.01$, "***" for $P < 0.001$).

Figure 7. Line plot of mean values and confidence intervals of: (a) relative fecundity of small oocytes at the secondary growth phase, i.e. with diameter between 200 and 320 µm (Relative fecundity$_{200\text{-}320}$), and (b) fecundity ratio (total fecundity/batch fecundity), for females at different phases within the ovulatory cycle: prior to an ovulation event (pre-ovulatory phase),

during ovulation (running phase), immediately or closely after an ovulation (post-ovulatory phase) and in-between two ovulation events (intermediate phase). Statistically significant pairwise comparisons of mean values are shown with asterisks ("*" for P < 0.05, "***" for P < 0.001).

Figure 8. Conceptual model of the ovarian dynamics within the ovulatory cycle in an indeterminate batch spawning fish. Prior to an ovulation event (pre-ovulatory phase), gonadosomatic index (GSI) and diameter of the most advanced oocytes (OD_{AM}) are at their maximum values and any postovulatory follicles (POF) present originated from a previous ovulation (old POF cohort), are already partially absorbed, and thus have small area. In parallel with ovulation of the advanced batch (running phase), a new oocyte batch is recruited from the primary growth to the secondary growth phase (PG recruitment), resulting in an oocyte size frequency distribution (OSFD) with an additional mode. Concurrently, GSI begins to decrease and old POF continue to shrink, while a new POF cohort is forming. Immediately or closely after the ovulation (post-ovulatory phase), the OSFD is comprised by several overlapping modes, GSI drops to its lowest values, old POF continue to decrease in size and new POF start to degenerate. As females approach readiness to ovulate again (intermediate phase), the advanced mode becomes gradually separated in the OSFD, GSI and OD_{AM} start to elevate again and old POF absorption is likely complete, while new POF degeneration continues. The OSFDs in the lower panel are "snapshots" of the secondary growth phase of the ovarian development for the different phases within the ovulatory cycle.

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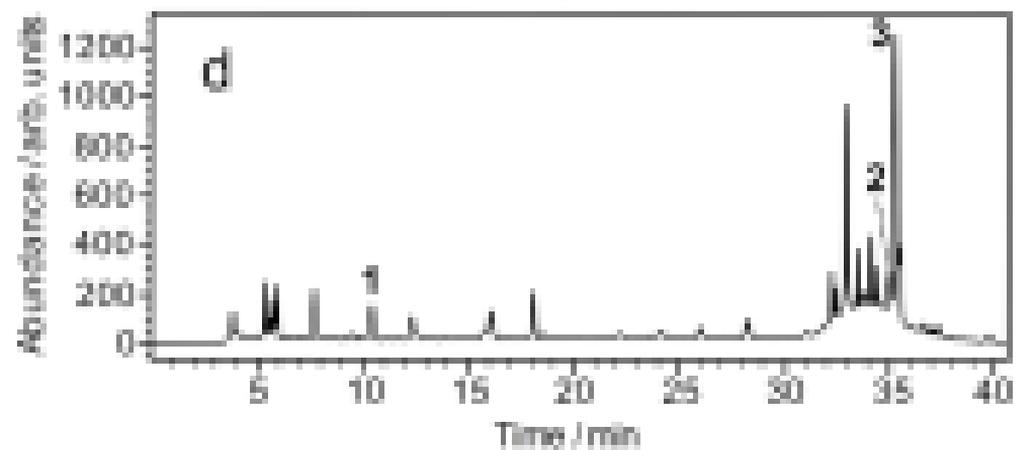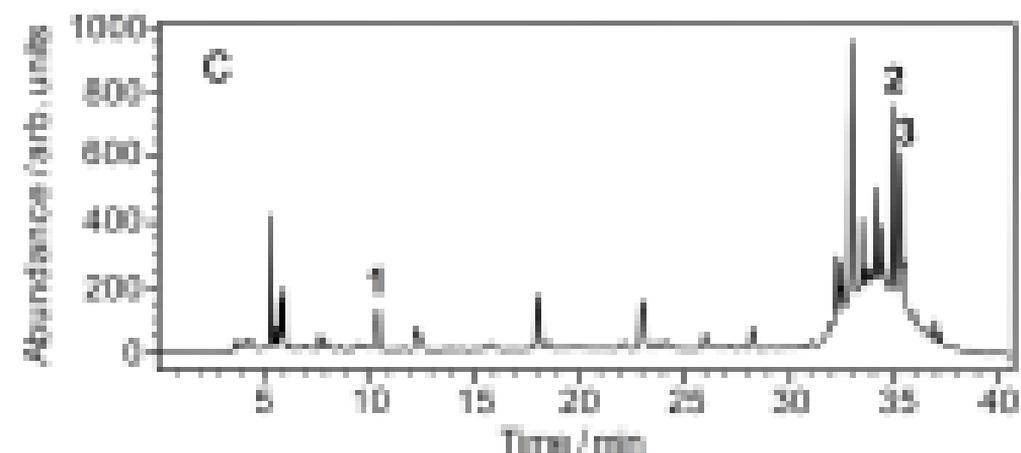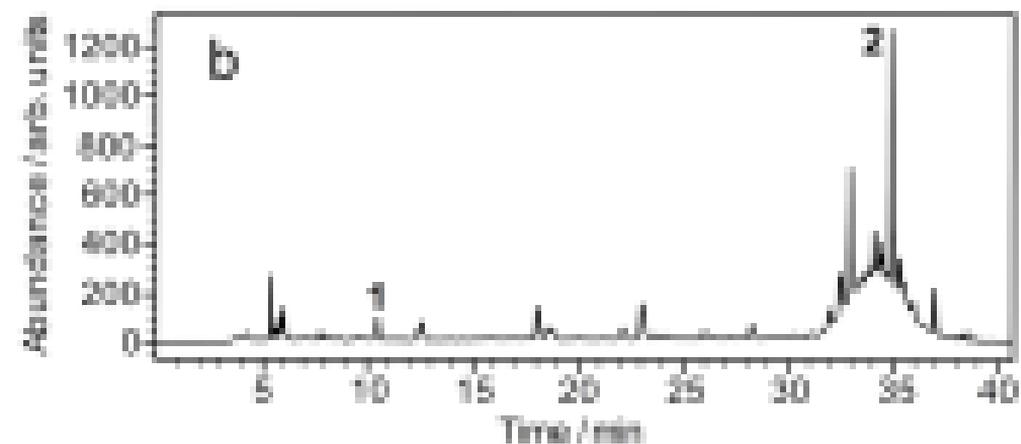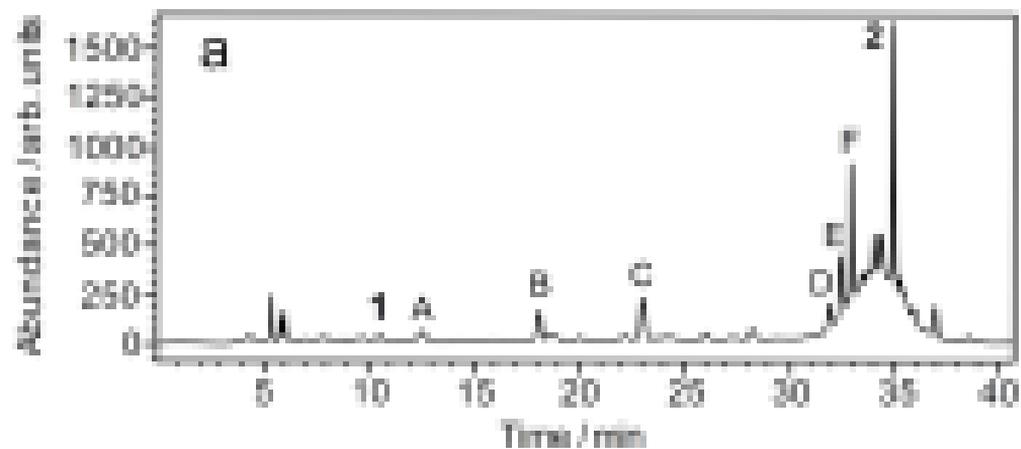
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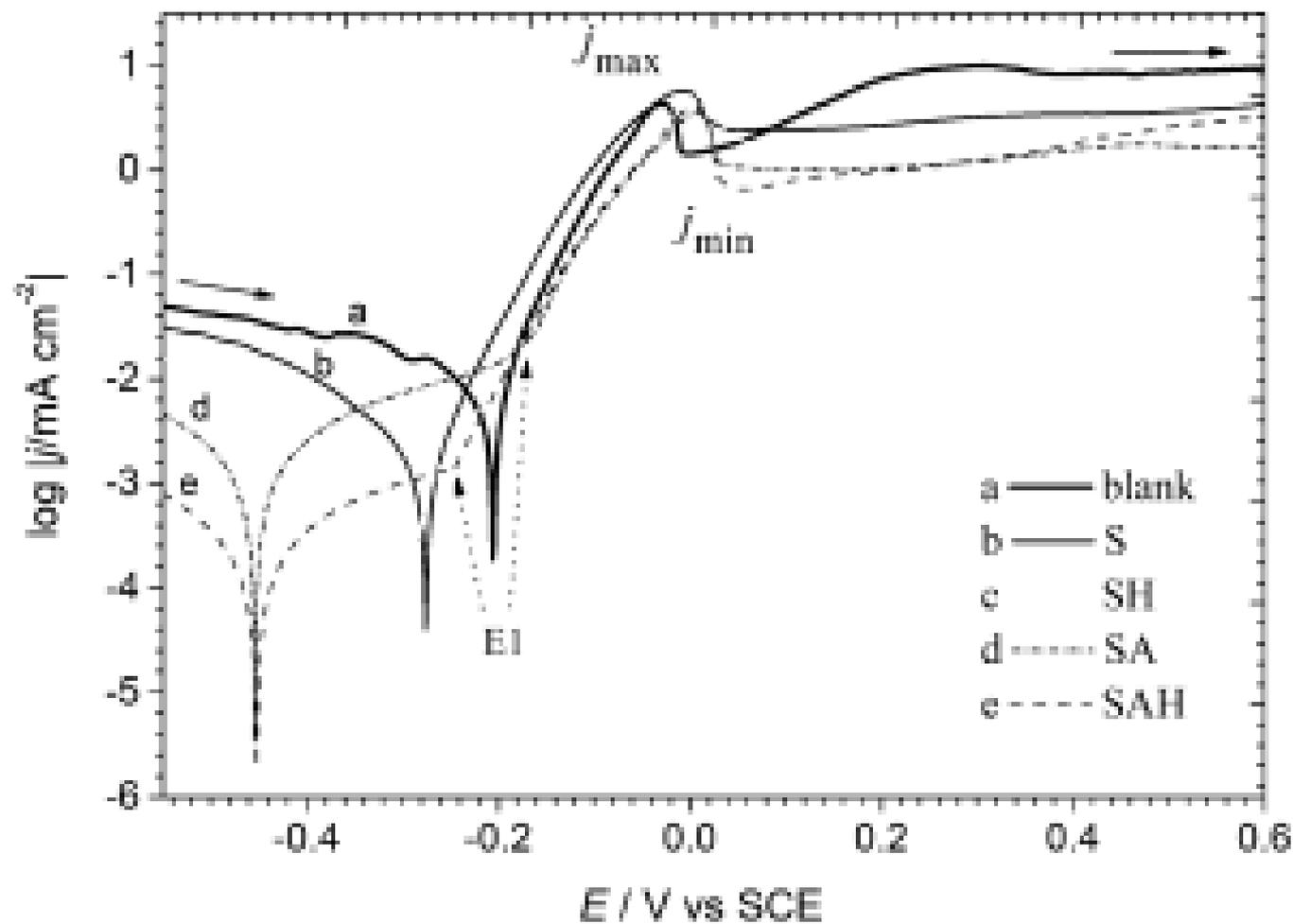
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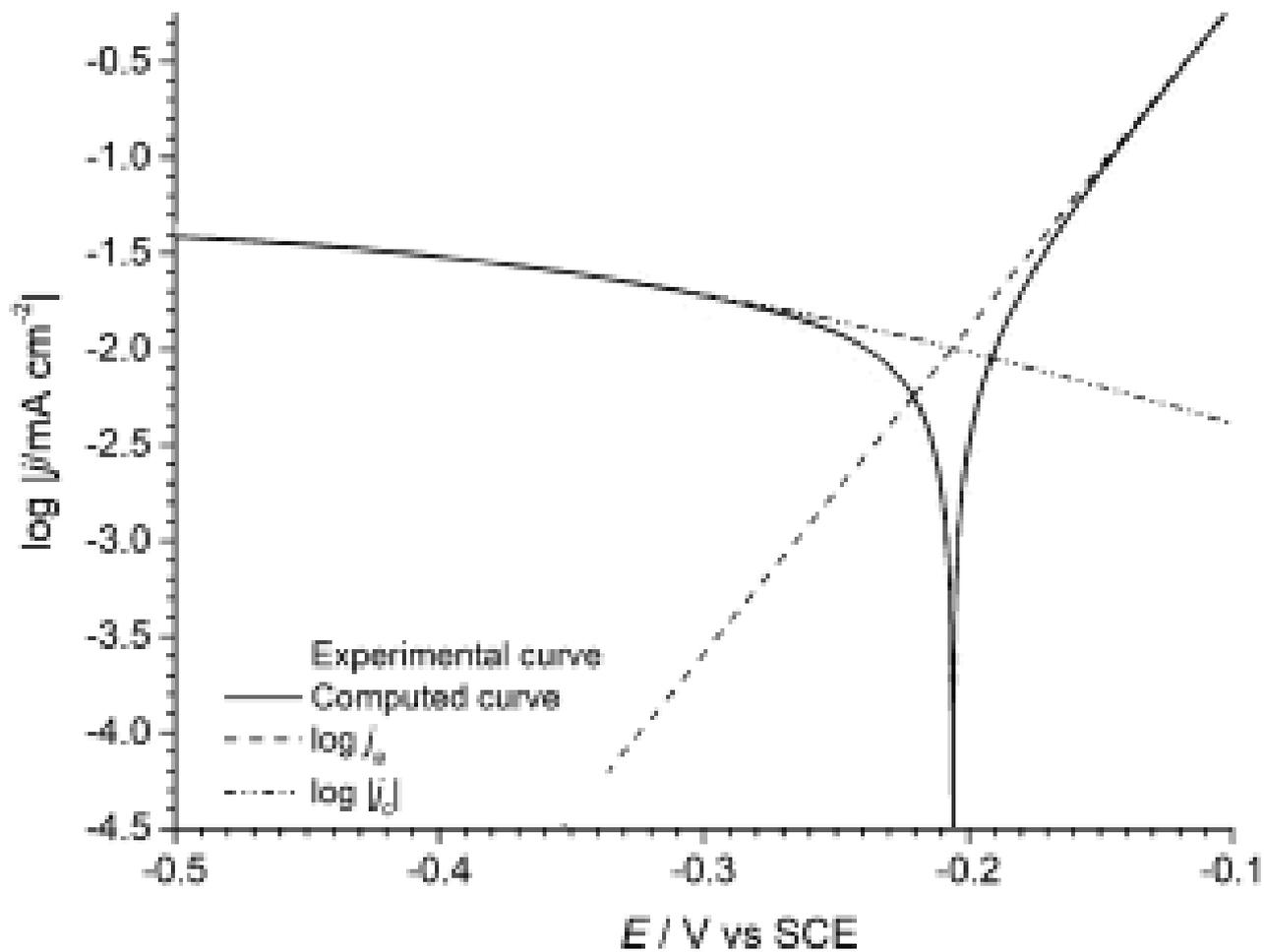381   2011;3:92–105. doi: 10.1080/19425120.2011.555714

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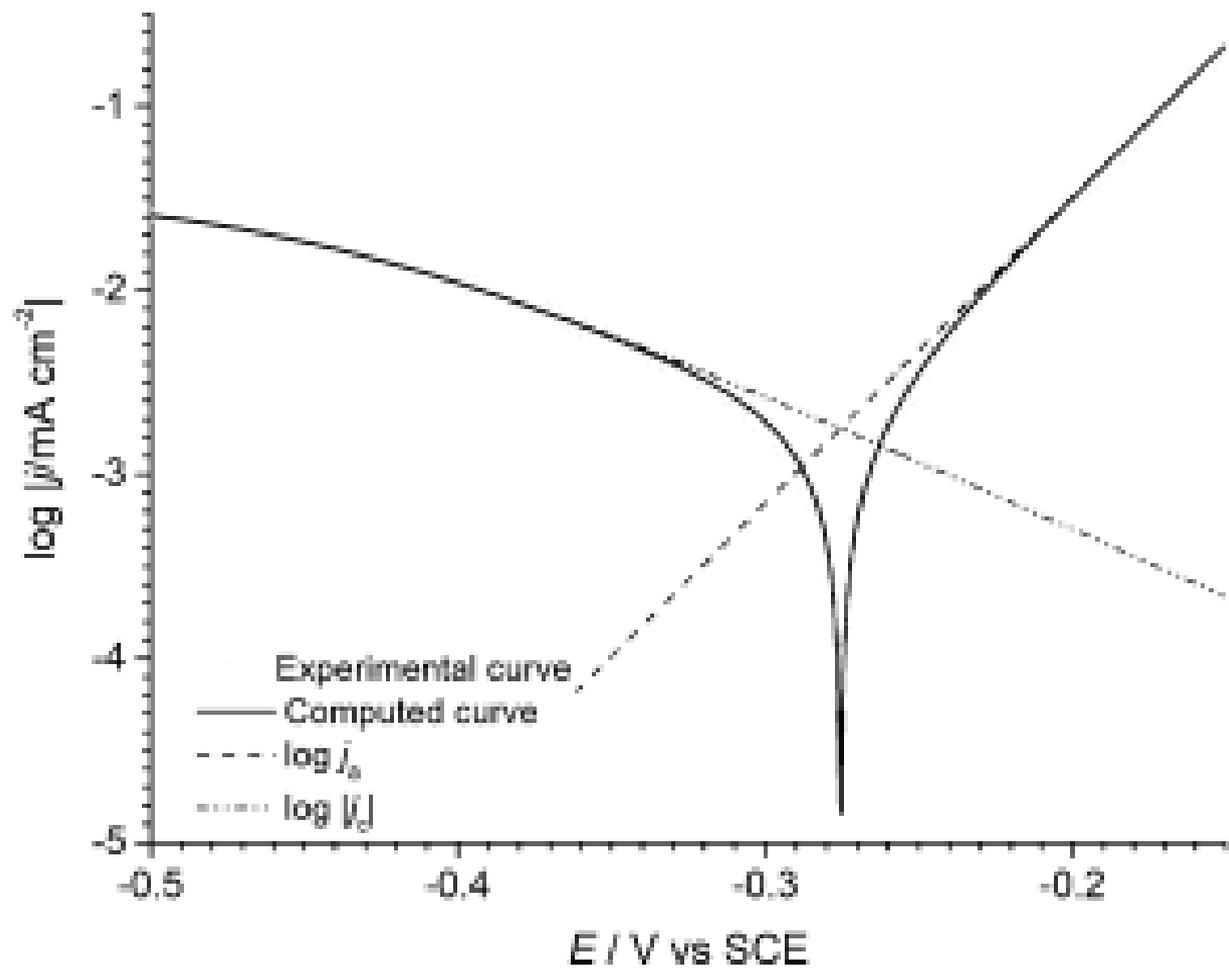420   10.1080/00028487.2013.862178

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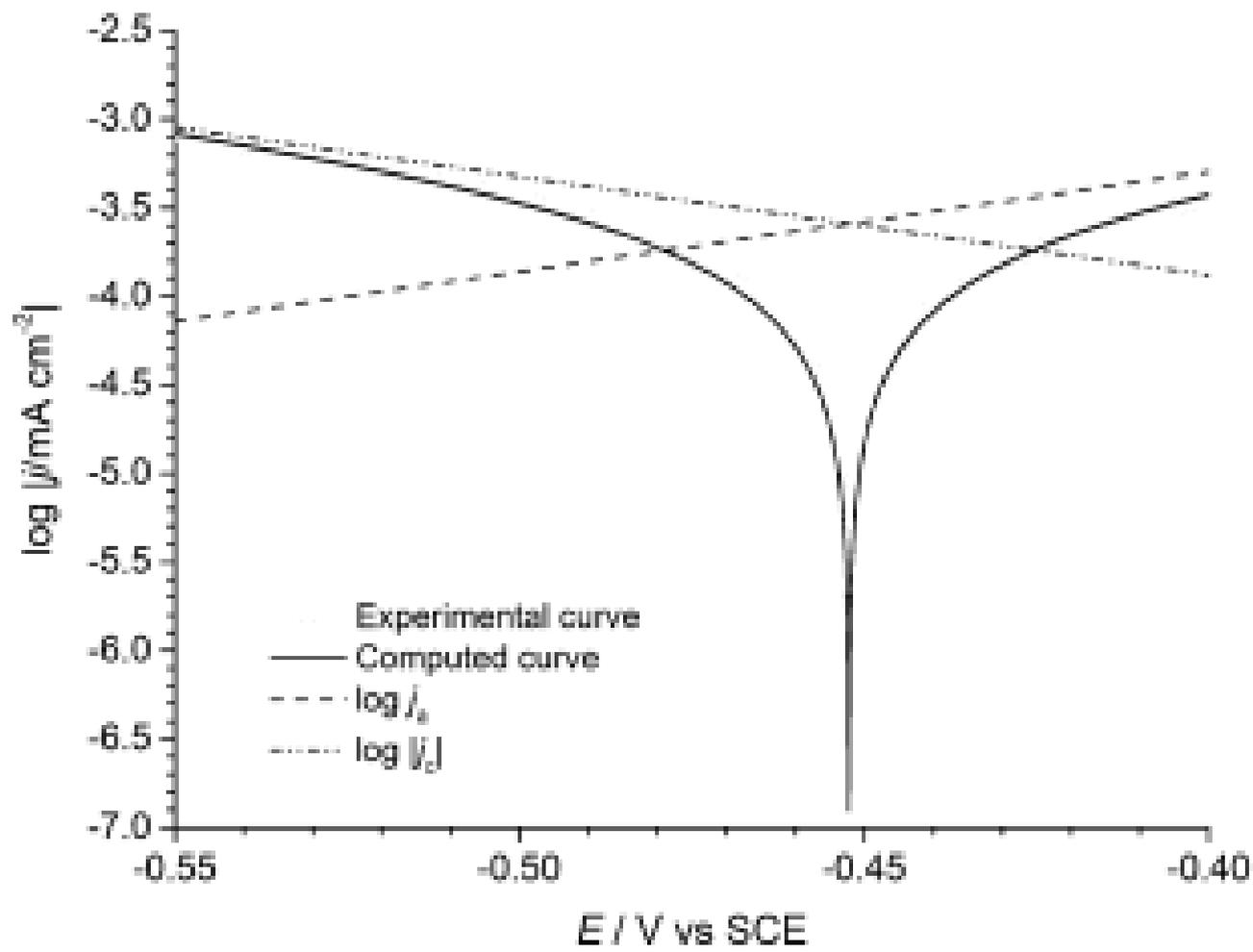457   mordax. Figh Bull 1981;79:215–30.

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Figure: Plot of $\log (j/\text{mA cm}^{-2})$ versus $E / \text{V vs SCE}$ showing curves labelled j_{max}, j_{min}, a, b, and $E1$.

a	blank	
b	SH	
c	SH	
d	SA	
e	SAH	

Experimental curve
—— Computed curve
- - - $\log j_a$
-·-·- $\log |j_c|$

Axis labels: E / V vs SCE (horizontal), $\log |j|/\mathrm{mA\,cm^{-2}}$ (vertical)

Experimental curve
Computed curve
$\log j_a$
$\log |j_c|$

(a) A circuit diagram with R_s in series, branching into Q_f and a parallel path containing R_f, followed by Q_{dl} in parallel with R_{ct}.

(b) A circuit diagram with R_s in series, branching into Q_f and a parallel path containing R_f, followed by Q_{dl} in parallel with R_{ct} and W.

Experimental curve
Computed curve