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A review of the effects of contamination and temperature in *Solea solea* larvae. Modeling perspectives in the context of climate change

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Abstract :

The flatfish species *Solea solea* has been the subject of research for supporting their management in fisheries, restocking natural populations, domestication in aquaculture, and ecotoxicology research. Soles undergo a metamorphosis with drastic morphological and physiological changes and settlement, processes that make them potentially more vulnerable to pollutants than other fish. Up to date, efforts made to develop its aquaculture production in Europe have been limited. In the context of climate change (CC), this review aims i) to gather research conducted in *S. solea* larvae that summarizes the effects of increased temperature and contaminant exposures during larval development; and ii) to provide a summarized and synergistic view about its larval development. The review consists of 4 sections. Section 1 justifies the selection of this species from ecological and economic perspectives. Section 2 focuses on larvae ontogenesis, metamorphosis, rearing challenges, and further aquaculture production. Section 3 reviews studies dealing with the effects of temperature change (due to CC) and pollutants on larval development. Finally, section 4 provides a “how to go forward on ecotoxicological research” guideline, in which we highlight the methods that we found promising as tools to study the combined effects of CC and pollution. The section includes a multidisciplinary framework that proposes how existing data coming from different scientific domains can be synthesized to be useful for risk assessors and ecotoxicologists. To benefit from such a framework, it is necessary to reach consensus and nurture team effort from players that operate in different research disciplines.

Highlights

► Flatfish are relevant within ecotoxicity and environmental risk assessment. ► We summarize the effects of temperature and chemical exposure on *S. solea* larvae. ► Effects on larval development are of interest for multiple disciplines. ► We present a multidisciplinary framework to improve collaboration between disciplines. ► Physiology based models are promising tools for predicting anthropogenic effects on this species.

Keywords : Flatfish, Climate change, Contamination, Larval stage, Multidisciplinary

37 **Introduction: Working with flatfishes in ecotoxicology, why?**

38

39 Fish are a prominent group within ecotoxicity evaluation guidelines and subsequently within environmental
40 risk assessment for the aquatic environment. Up to date, there exist nine guidelines from the Organization for
41 Economic Co-operation and Development (OECD) that acknowledge the importance of fish toxicology, which
42 includes: the acute toxicity test (OECD 203, 1992), the short term toxicity test on embryo and sac-fry stages

43 (OECD 212, 1998), the fish juvenile growth test (OECD 215, 2000), the fish early life-stage toxicity test
44 (OECD 210, 2004), the 21-day fish assay: a short-term screening for oestrogenic and androgenic activity, and
45 vitellogenin and secondary sex characteristics (OECD 230, 2009), the fish sexual development test (OECD
46 234, 2011), the fish short term reproduction assay (OECD 229, 2012), bioaccumulation in fish: aqueous and
47 dietary exposure (OECD 305, 2012), and the fish embryo acute toxicity (FET) test (OECD 236, 2013).

48

49 Current integrated toxicity testing strategies consider that fish are an indispensable component, and in general
50 terms, this is mainly because:

- 51 - fish are an essential food resource for humans;
- 52 - the aquatic environment is the final reservoir for many chemicals;
- 53 - fish are critical in aquatic food webs by top-down and bottom-up regulation of nutrient and energy flow;
- 54 - fish have been and continue to be used as sentinels of water quality used for human consumption;
- 55 - fish kills following accidents are visible to the public and encourage them on the need to protect the aquatic
56 environment from pollutants; and
- 57 - fishing has recreational value in many cultures (Lammer *et al.*, 2009).

58

59 Among fish, flatfishes have been the subject of research supporting their management in fisheries, restocking
60 natural populations, and domestication in aquaculture. Also, they present unique ontogeny, metamorphosing
61 from bilateral to asymmetric, which makes them a compelling target for evolution and developmental studies
62 (Geffen *et al.*, 2015). Furthermore, flatfishes present similar gene regulation and consequently (except for few
63 exceptions) similar physiological responses to pollutants (Lammer *et al.*, 2009).

64

65 Specific characteristics, like metamorphosis and settlement, make flatfishes respond in a potentially different
66 manner when exposed to pollutants. To begin with, they undergo metamorphosis, with morphological and
67 physiological changes associated with eye migration, a 90° rotation in posture, and asymmetrical pigmentation
68 (Geffen *et al.*, 2007). Metamorphic larvae undergo drastic remodeling of tissues and physiological changes.
69 The transition from larval to juvenile stage involves developing most organs and tissues, the maturation of
70 different physiological functions, and the establishment of the immune system (Ferraresso *et al.*, 2013).
71 Flatfish larvae also undergo settlement, a behavioral and ecological change associated with a transition from a
72 pelagic to a benthic existence. As benthic animals feed on invertebrates, flatfishes are affected by sediment-
73 associated pollutants; so, their life history and habitat also make flatfishes valuable models for ecotoxicology
74 research (Geffen *et al.*, 2015). The benthic lifestyle potentially exposes flatfishes to a wide range of
75 contaminants, including -but not limited- to endocrine disruptors, heavy metals, polycyclic aromatic
76 hydrocarbons (PAH), and dioxins (Munsch *et al.*, 2010, 2011; Wessel *et al.*, 2010; Gilliers *et al.*, 2012; Geffen
77 *et al.*, 2015). This fact makes them useful sentinels for monitoring the biological effects of contaminants in
78 inshore and estuarine waters.

79

80 Besides physical changes, survival and growth after recruitment are often assumed to depend on the behavioral
81 and ecological changes associated with metamorphosis (Geffen *et al.*, 2007). Optimal cues, such as sensory
82 perception and interpretation, guarantee the survival of early juveniles, yet it is known that swimming activity,
83 exploration ability, and place preferences can be affected by exposure to PCBs and PBDE (Péan *et al.*, 2012).
84 Moreover, flatfish species are widely distributed, which facilitates conducting spatio-temporal comparisons.

85
86 The characteristics mentioned above (metamorphosis, benthic lifestyle, and behavioral adaptations) make
87 flatfishes unique and potentially more vulnerable to anthropogenic activities and estuarine contamination. It is
88 worth noting that the aquatic environment is the ultimate sink for many man-produced products, which are
89 continuously being released at estuaries, either due to direct discharges or to hydrologic and atmospheric
90 processes. The chemicals that end up in these habitats range from many thousands of organic trace pollutants
91 (polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons
92 (PAHs), polychlorinated dibenzofurans (PCDFs), and dibenzo- p-dioxins (PCDDs)) to compounds of
93 emerging interest, which include perfluoroalkyl substances (PFAS), nanoparticles, personal care products,
94 detergents, estrogen-like products, and flame retardants. All these substances are potentially interacting and
95 impacting the health of flatfish and represent a risk for the health of benthonic fish populations (van der Oost
96 *et al.*, 2003).

97
98 Finally, Geffen *et al.* (2015) argued that one of the most rapidly developing areas that would benefit from the
99 synergy of flatfish research in aquacultured and wild populations is the ecotoxicology domain. Unfortunately,
100 research on flatfishes is distributed across journals in several (sub)disciplines, and because the reference lists
101 of individual papers rarely extend across these disciplines, in order to gain a full benefit of knowledge
102 exchange, it is necessary to read widely.

103

104 **Objectives**

105

106 This review aims to gather works primarily focused on ecotoxicological research conducted in the flatfish
107 species *Solea solea* along with their larval development and early juvenile phase. We draw attention to this
108 life period because it is critical for fisheries and aquaculture development and for understanding anthropogenic
109 impacts on *S. solea* populations and ecosystem degradation. Additionally, a secondary objective is to provide
110 a summarized and synergistic view on research conducted in *S. solea* larvae that serves different disciplines
111 such as aquaculture, fisheries, marine ecology, and ecotoxicology.

112

113 We divided the review into four sections. The first section justifies the selection of this species and reviews *S.*
114 *solea* ecologic and economic relevance.

115

116 Section two focuses on the main particularities of this species. Soles, like all flatfishes, undergo an extreme
117 metamorphosis which translates into a susceptible larval stage. The section summarizes *S. solea* regular larvae
118 development by citing the most recent literature describing the optimal conditions for *S. solea* laboratory

119 rearing and growth. The main questions addressed in section 2 include: What are the main bottlenecks on the
120 aquaculture production of *S. solea*? Furthermore, what have been the scientific advances that have been made
121 to overcome these problems?

122
123 In the third section, we will focus on reviewing works studying the effects of climate change (CC, we only
124 focus on temperature change) and contamination on larval development. This section comprises the main focus
125 of the review and will include works from the molecular level until the population level, exploring and
126 comparing these different “tools” employed to understand, estimate, and predict the effects of human activities
127 on this species. The questions we intend to answer in section 3 include: What are the individual and combined
128 effects of CC and environmental pollution on larval development of *S. solea*? Furthermore, can we predict or
129 anticipate the effects of combined stresses?

130
131 Finally, in the last section, we will review the methods and efforts made to integrate different types of data to
132 1) have a holistic view on the subject and 2) to be able to extrapolate (if possible) to different levels of
133 biological organization or other species. This section is meant as a “how to go forward on ecotoxicological
134 research” guideline, in which we primarily highlight the methods that we found promising as tools to study
135 the combined effects of CC and pollution. The main questions addressed in section 4 include: What methods
136 allow us to understand the combined effects of CC and contamination? How can we reduce animal testing for
137 new chemicals and improve risk assessment? And finally, how much can we rely on numerical models?

138

139 **Section 1 - Why *Solea solea*?**

140

141 **1.1 Common sole life-cycle main events**

142 The flatfish species, *Solea solea*, also known as the common or Dover sole, and from now on simply denoted
143 as sole, is an important commercial species found in shallow waters (between 10 to 60 m depth but also down
144 to 200 m) of the eastern Atlantic Ocean. Its distribution range extends from the north-western coast of Africa
145 in the Mediterranean Sea to the southern coast of Norway (Zambonino-Infante *et al.*, 2013).

146

147 The common sole is a batch-spawner, and increases in temperature trigger spawning. Depending on the
148 geographical location, spawning events might occur from late winter to spring. In the Mediterranean Sea,
149 spawning occurs between January and April, with two peaks in February, while in the Bay of Biscay, spawning
150 occurs between December and May (FAO, 2019), depending on the latitude. In the North Sea, spawning
151 happens between April and June, and different sole stocks present differences in the average date of peak
152 spawning, with spawns occurring from late March in the Bristol Channel to mid-May in the Irish Sea and in
153 late May in the eastern-central North Sea (Fincham *et al.*, 2013). The optimal temperature for spawning ranges
154 from 7 to 12 °C (Lacroix *et al.*, 2013).

155

156 Spawning behavior in *S. solea* involves three stages, courtship, spawning rise, and a post-spawning return to
157 the seafloor. During courtship, the male follows a female on the bottom and then swims below the female. If

158 courtship is successful, the pair swims vertically towards the water surface with closely synchronized body
159 movements, and gametes are released at the apex of the rise, followed by a return to the seafloor (Baynes *et*
160 *al.*, 1994).

161
162 Fertilized eggs and larvae are both pelagic (Fig. 1), which allows for wide dispersion and exposes them to
163 environmental variability, notably temperature and food abundance. Fertilized eggs hatch after few days (5
164 days at 16 °C, Imsland *et al.*, 2003), and the larval stage -from hatching until complete metamorphosis- can
165 last between 26 and 15 days, at temperatures of 16 °C and 19 °C, respectively (De Swaef *et al.*, 2017; Richard *et*
166 *al.*, 1991).

167
168 The larval stage comprises a metamorphic period, where significant ontogenic, morphological, physiological,
169 and behavioral changes occur (Geffen *et al.*, 2007). The length of metamorphosis depends on temperature and
170 food availability, and by the end of it, juvenile soles are ready to change from pelagic to benthic lifestyle to
171 colonize shallow coastal waters in estuaries and bays (Zambonino-Infante *et al.*, 2013).

172
173 Newly metamorphosed juveniles settle in coastal areas for around two years until they reach maturity (Post *et*
174 *al.*, 2017). These areas serve as nursery grounds, where abundant food (epi and endobenthic prey) favors
175 growth. Further, the pelagic larval and the juvenile stage on nursery grounds are critical for common sole
176 recruitment, *i.e.*, the number of fish surviving to enter a fishery and contribute to the next generation
177 (Zambonino-Infante *et al.*, 2013). After settlement on estuarine nursery areas, *S. solea* individuals undergo
178 further ontogenic habitat shift (in some populations, juvenile soles progressively migrate tens of kilometers
179 from shallow estuarine waters to deeper coastal water).

180
181 At the adult stage, they migrate offshore (few or up to hundreds of kilometers depending on location) in
182 particular for reaching spawning grounds, and they eventually come back closer to the shore out of
183 reproduction season to reach feeding grounds (Vasconcelos *et al.*, 2014). Regardless of this migration, Diopere
184 and collaborators (2017) argue that the wide geographical area at which this species is found and the fact that
185 they do not conduct long migrations makes it reasonable to assume that sole in European waters belongs to
186 more than one population. However, the precise habitat range of this species remains unclear (Post *et al.*,
187 2017). Moreover, and because spawning grounds are distant from the nursery areas, the planktonic larvae have
188 a drifting period which ideally should end at an area that is suitable as a nursery for the juvenile stage. The
189 question is whether they do anything to maximize their probability of arriving there or whether it is a random
190 process (Duffy-Anderson *et al.*, 2015). Factors such as release time, location, stage duration, climate, and
191 hydrodynamic circulation play a crucial role in larval mortality rates and determine the transport of eggs, larvae
192 and the population renewal (Vinagre, 2007; Duffy-Anderson *et al.*, 2015).

193
194 Such a complex life cycle (Fig. 1) with progressive habitat shifts along *S. solea* ontogenesis makes it
195 challenging to determine how population dynamics are affected by habitat quality. In this regard, Vasconcelos

196 and collaborators (2014) showed in their review how little is known about habitat-specific demographic rates
197 on species of commercial interest, including the common sole. Knowledge about the relationship between
198 habitat characteristics and population dynamics is particularly valuable from a conservation and management
199 perspective since the vulnerability of coastal habitats to anthropogenic stressors is increasing (Rochette *et al.*,
200 2013). Specific recommendations include emphasizing the spatiotemporal scales in studies addressing the
201 reproduction, mortality, immigration, and emigration dynamics of *S. solea*. By this means, it will be possible
202 to define settlement habitats, the degree of connectivity between primary and secondary settlement habitats,
203 and the level of dependence of juvenile and adult populations on these essential coastal habitats (Vasconcelos
204 *et al.*, 2014).

205

206 **1.2 Ecological importance and habitat selection**

207 *Solea solea* is an r strategist species, characterized by a strong fecundity -with hundreds of thousands of eggs
208 spawned (per kg of female)- and low survival rates. The environmental fluctuations strongly influence survival
209 rates, egg and larval life history along the larval dispersion process, and the transition from a pelagic to a
210 benthic lifestyle (Savina *et al.*, 2016). At first, eggs derive towards nursery areas thanks to the action of winds,
211 tides, and currents (Savina *et al.*, 2010). After hatching, larvae start showing active vertical migration in the
212 water column triggered when feeding starts (Lagardère *et al.*, 1999). Larvae use vertical migration to enter,
213 leave, or remain in estuaries by either selecting the seaward outflow at the surface or the inflow near the bottom
214 (Duffy-Anderson *et al.*, 2015). Therefore, the successful arrival in nursery areas is dependent on hydroclimatic
215 conditions (Lagardère *et al.*, 1999).

216

217 Recently metamorphosed individuals settle in nursery areas and switch to a benthic lifestyle starting their
218 critical juvenile stage when their movements are limited, which will last for up to 2 years (Eichinger *et al.*,
219 2010). According to the “nursery size hypothesis” (Van der Veer *et al.*, 2000), juvenile survival depends on
220 the surface area of nurseries; as each nursery can only sustain a population in proportion to the size available
221 (Duffy-Anderson *et al.*, 2015), and it is during this period that soles become top predators (Amara *et al.*, 2001).
222 Indeed, early juvenile diet is mainly based on copepods, progressively changing to include short-lived, highly
223 productive benthic organisms, such as polychaete worms, mollusks, and small crustaceans (Amara *et al.*, 2001;
224 Rijnsdorp *et al.*, 2004).

225

226 Juvenile fish distribution is influenced, among other factors, by sediment type, food availability, and movement
227 ability, and the trade-off outcome of these factors determines their habitat selection. For *S. solea*, estuarine and
228 shallow coastal systems act as nurseries where the trade-off between growth and survival is optimized due to
229 abundant food resources, few predators, and favorable environmental conditions (Freitas *et al.*, 2012). This
230 fish-habitat interaction is of utmost importance at early-life stages when successful recruitment and
231 development of fish from larval to juvenile stages depends on the quality and the characteristics of nursery
232 areas (Post *et al.*, 2017).

233

234 For flatfishes, the type of sediment is also a decisive factor, as it can inhibit burial behavior or affect prey
235 availability (Post *et al.*, 2017). That is why habitat degradation, such as beach sand nourishment, directly
236 impacts recruitment success and species fitness. Coarsening the sediment in the coastal zone would increase
237 the energy requirement for juvenile sole through prolonged digging behavior, consequently impacting
238 individual growth and increasing their vulnerability to predation due to their smaller size (Post *et al.*, 2017).
239 Post and collaborators (2017) measured the sediment preference of soles by determining their relative
240 distribution over the sediment classes. Preference -defined as selecting a specific sediment type through their
241 presence at the end of the experimental period (4 days)- was tested in a circular preference chamber. Their
242 results demonstrated that juvenile soles prefer fine grains, and they suggest that such marked preference could
243 be a consequence of higher food availability and lower energy to bury (Howell and Canario, 1987; Post *et al.*,
244 2017).

245

246 The juvenile stage, where soles have a benthic lifestyle and inhabit coastal nursery areas, is the period at which
247 individuals are exposed to a broader range of contaminants, which includes not only hydrophilic compounds
248 dissolved in the water column but also hydrophobic contaminants that can be either directly absorbed from the
249 sediment or ingested from contaminated food (Rochette, 2011).

250

251 Given its characteristics and ecological importance, *S. solea* has been included within the EU Marine Strategy
252 Framework Directive (MSFD) as a model and indicator species for evaluating the good environmental status
253 in the marine environment in 2020.

254

255 Finally, readers seeking for more detailed information on niche ecology and habitat selection of *S. solea* are
256 encouraged to revise the works from Vinagre *et al.* (2006) that evaluates the species distribution and determines
257 the habitat quality using habitat suitability index models or the study by Vinagre and collaborators (2005),
258 which evaluates the niche overlap between European flounder and *S. solea*. Furthermore, there is an important
259 body of research that focuses on comparing *Solea senegalensis* and *S. solea* and their differential use of
260 estuaries (Cabral and Costa 1999), species distribution (Vinagre *et al.*, 2009), prey consumption (Cabral 2000;
261 Vinagre and Cabral 2008) and habitat-specific growth rates (Vinagre *et al.*, 2008) from populations inhabiting
262 the Tagus estuary, Portugal. The list of publications on related topics goes on, including Martinho *et al.* (2008),
263 where the authors compared the timing of estuarine colonization, the population structure, growth, and diet
264 composition of flatfish juveniles from the species *Dicentrarchus labrax*, *Platichthys flesus*, *S. solea*.

265

266 **1.3 Economic importance of soles**

267 The sole species, *Solea solea*, and *Solea senegalensis*, are characterized by high flesh quality and high market
268 value making them very promising candidates for European aquaculture (Howel *et al.*, 1997; Bonaldo *et al.*,
269 2011). Also, fisheries landings for *Solea spp.* have shown a reduction of over 43%, and the average size of
270 wild sole has also declined (Morais *et al.*, 2014). This reduction in the offer has promoted average prices of
271 different sole products (fresh, frozen, and farmed) to increase and consistently attain high prices in European

272 markets (Morais *et al.*, 2014). For example, in 2013, farmed products attained 12.25 € kg⁻¹ in MercaMadrid
273 (Morais *et al.*, 2014), and in May 2019, *S. solea* reached an average price of 23.8 € kg⁻¹ in France
274 (FranceAgriMer, <https://rnm.franceagrimer.fr/prix?SOLE&12MOIS>). Their high commercial value, the
275 growing market demand for adults and juveniles, and the increasing fishing pressure -not restricted to the coast
276 but also present in the estuary- make sole fisheries an attractive business from both the socio and economic
277 perspectives. These reasons have reinforced the interest and promise of developing commercial aquaculture
278 with these species. In fact, *S. solea* and *S. senegalensis* have been repeatedly identified as the most promising
279 species for aquaculture diversification in Europe for at least four decades now (Morais *et al.*, 2014).

280

281 As a result, during the last 30 years, sole species have attracted considerable research attention, and significant
282 investments have been made to develop rearing methodologies (Howell 1997; Dinis *et al.*, 1999). Nevertheless,
283 the production of *Solea spp.* has failed to reach successful commercial development until recently, and this
284 success is limited to *S. senegalensis*. The three major bottlenecks for *Solea spp.* production are 1) high larval
285 mortality rates related to nutrition and growth dispersion, 2) sub-optimal larval weaning strategies that result
286 in weaning onto formulated diets and lead to low growth and high juvenile mortalities; and 3) inadequate
287 control measures for common diseases. Additionally, the techniques for intensive rearing of the sole are yet to
288 be optimized, which limits the final volume of fish production (FAO, 2019; Morais *et al.*, 2014). Other
289 problems include a decreased growth rate at high stocking densities, pigmentation abnormalities,
290 malformations related to eye migration (Dinis *et al.*, 1999). Finally, the reproduction of cultured specimens is
291 complicated in F1 and later generations (especially in *S. senegalensis*, Ibarra-Zatarain *et al.*, 2020). In the next
292 section, we will summarize some of the research advances made to solve the above-mentioned problems.

293

294 **Section 2 - Importance of studying *Solea solea* larvae: development, the cost, and challenge of** 295 **metamorphosis**

296

297 External biotic and abiotic factors affecting larval development have consequences at later life stages (Howel
298 and Baynes, 2004). Fish larval stages represent a transitional period at which both differentiation and growth
299 lead to substantial changes in structure, physiology, size, and morphology. Compared to any other life stages
300 of a marine fish species, individuals at their larval stage will have the highest potential for growth, weight-
301 specific metabolic rates, natural mortality rates, and the highest sensitivity to environmental stressors
302 (Hutchinson *et al.*, 1998; Belanger *et al.*, 2010).

303

304 These factors highlight the importance of studying fish larvae. Early stages are usually characterized by strict
305 environmental requirements, where factors such as nutrition, microbial environment, and physical/chemical
306 conditions determine the healthy development and the survival rate. Moreover, all organs develop during the
307 embryonic and larval stages, and often, causes of permanent abnormal development occur during this early
308 stage, ultimately influencing how fish will perform later in life (The Research Council of Norway, 2009).

309

310 Up to date, only a few works have focused on reviewing research conducted on this species. Such works have
311 mainly focused on the potential of *Solea solea* and the closely related species *S. senegalensis* for commercial

312 aquaculture. These include the work by Baynes *et al.* (1993), which reviews the egg production by sole in
313 captivity, and Howel (1997) summarizes the challenges and benefits of cultivation of sole. However, the
314 information provided is limited to assessing the technical rather than the economic feasibility of farming sole.
315 More recently, the works by Imsland *et al.* (2003), Morais *et al.* (2014), and Munoz-Cueto *et al.* (2019) focused
316 on presenting the biology and discussing the potential use of *Solea spp.* -*S. solea* and *S. senegalensis* for
317 aquaculture. In this last reference, a chapter by Solé (2019) walks the reader through the use of these species
318 within ecotoxicological studies, particularly by measuring biomarkers in sole tissues.

319

320 To our knowledge, this is the first review that exclusively focuses on *S. solea* larval biology and ecotoxicology.
321 In our opinion, this could be partly explained given that only until recently, advances in culturing marine fish
322 species and in developmental biology -notably on model species such as zebrafish- have allowed for the entire
323 understanding of many biological processes and have made the field of larval ontogenesis more accessible for
324 experimental exploration (The Research Council of Norway, 2009). Added to this, the threats imposed by
325 climate change and pollution justify the need for summarizing those research works devoted to understanding
326 the individual or combined effects of CC and anthropogenic activities on larvae.

327

328 For this review, we searched various academic search services (Thomson Reuters, Web of Knowledge, and
329 Web of Science) for works published in the period between 2003 and 2020 using combinations of the following
330 topics: *Solea solea*, flatfish, larvae, larviculture, ontogenesis, chemical contamination, and climate change. We
331 choose to start our searching from 2003 onwards to avoid duplicating information already available in Imsland
332 *et al.* (2003).

333

334 **2.1 Why does fundamental research on larval biology matter?**

335 Fundamental research in larval biology is essential for assuring the ecosystem functioning of nursery areas.
336 Larvae biology is also crucial for marine fish farming. Because all organs and biological systems develop
337 during the embryonic and larval stages, early development strongly influences how the fish performs later in
338 life (Howel and Baynes, 2004). Therefore, knowledge about optimal larval development can substantially
339 impact further stages and make a big difference in economic and sustainable terms. An appropriate example
340 was reducing halibut discards due to incomplete eye migration, which accounted for up to 40-60% of halibut
341 fry. Such a problem was solved by only applying photoperiods during larval rearing (Solbakken and Pittman,
342 2004).

343

344 There is potential for improvements in aquaculture cost efficiency by increasing growth and survival rates and
345 reducing the prevalence of deformities, and the key for such improvements might be related to larval
346 development. Moreover, for wild stocks, knowledge about early life stages provides insight into how the fish
347 survive under various conditions by adapting to their environment. Several reasons pinpoint fish early-life-
348 stages (ELS) as more sensitive to pollutants than fully developed individuals from the same species
349 (Hutchinson *et al.*, 1998). These reasons are related to a combination of critical developments occurring during

350 ELS, the limited possibilities to compensate for the impact of physiological defects, and significant changes
351 in the composition of the developing tissue (Foekema *et al.*, 2012).

352

353 **2.2 Advances made on larvae survival during rearing**

354 Before standardizing a massive farming production of the sole, several problems are to be overcome, including
355 larval mortality, feeding behavior, susceptibility to disease, stocking density, and reproduction. Besides, there
356 are high mortality rates during juvenile production around metamorphosis and weaning (Bonaldo *et al.*, 2011).

357

358 For successful larvae rearing, the first factor to consider is related to the quality of the eggs, which can be
359 defined as the egg's ability to be fertilized, survive, and develop into a normal embryo (Lund *et al.*, 2008;
360 Parma *et al.*, 2015). As the egg must satisfy the nutritional needs of embryonic and larvae stages, and because
361 healthy larvae depend largely but not exclusively (hormones and other molecules transferred in eggs are also
362 crucial for embryonic and larval development), on the amount of fatty acids (FAs), the quality of eggs is usually
363 assessed by studying its lipid and FAs composition (Lund *et al.*, 2008; Parma *et al.*, 2015). Besides,
364 broodstocks' nutrition and life history has been shown to affect egg nutrients qualitatively and quantitatively
365 (Foekema *et al.*, 2012; Parma *et al.*, 2015).

366

367 In order to study the relationship between hatching rate, survival, and growth of larvae and egg quality, Parma
368 *et al.* (2015) analyzed and characterized the FAs composition of *S. solea* eggs obtained from domesticated
369 broodstock during an entire spawning season. According to their results, the authors conclude that the feed
370 supplied to broodstock before and during vitellogenesis has a significant role in determining the eggs' FA
371 profile in sole. Another objective of their work included defining the relationship between FAs and egg
372 viability parameters and larval survival as a means to provide information for improving -if necessary-
373 broodstock feed in common sole. Regarding this last objective, the authors observed that, on the one hand,
374 saturated and monounsaturated (MUFA) fatty acids were positively correlated with the hatching rate. On the
375 other hand, TL, 22:6n-3 (DHA), 20:4n-6 (ARA), polyunsaturated FAs of the (n-3) series (n-3 PUFA), and
376 polyunsaturated FAs of the (n-6) series were negatively correlated with the hatching rate. MUFA, 20:5n-3
377 (EPA), n-6/n-3 were positively correlated with survival rate, while DHA, n-3 PUFA, DHA/EPA were
378 negatively correlated. The relationships found between total lipids and FAs with egg and larval viability
379 parameters showed a positive correlation with egg quality, while n-3 PUFA, DHA, DHA/EPA, and ARA
380 content were inversely correlated with egg larval survival. This is typically different from many other farmed
381 marine fish species, suggesting the need for a specific broodstock feed for this species.

382

383 In order to elucidate if offspring quality was affected by culture-related conditions, Lund *et al.* (2008)
384 compared the eggs' quality -via FAs composition- and its relationship to egg size, fertilization, and hatching
385 rate in eggs coming from cultured and wild broodstock. The FAs profile allowed discriminating between
386 culture and wild origin. Eggs from wild broodstock were bigger, had higher levels of C16:1(n-7), C20:4(n-6),
387 and C20:5(n-3), and larval survival was significantly higher than that from cultured broodstock. Cultured

388 broodstock eggs had high levels of C20:1(n-9), C18:2(n- 6), and C18:3(n-3), and their fertilization and
389 hatching rates were generally lower than eggs from wild broodstock but not related to FAs eggs composition
390 (Lund *et al.*, 2008).

391
392 In a more recent series of experiments, De Swaef *et al.* (2017, 2019) aimed to achieve a more sustainable sole
393 larviculture. They developed several research tools designed for tackling some key issues hampering
394 commercial sole aquaculture. These tools include the design of a 24-well microplate rearing system (De Swaef
395 *et al.*, 2017), the evaluation of several probiotic and prebiotic candidates to increase sole larval health (De
396 Swaef, 2019), and the evaluation of disinfection protocols to control the bacterial disease vibriosis (De Swaef
397 *et al.*, 2018).

398
399 For the development of a 24 well microplate rearing system, the authors argue that by rearing larvae
400 individually, problems related to group housing, such as variation between replicates and changes in the water
401 quality of the system after the death of one larva, can be avoided (De Swaef *et al.*, 2017; De Swaef, 2019) and
402 consequently larvae mortality during rearing decreased. Consequently, developing a system for rearing fish
403 larvae individually guarantees that a dead individual will not affect the health of an adjacent one and facilitates
404 the monitoring of their health status, growth, and behavior. It also improves the experiment's reproducibility
405 and finally (because each well is considered as a replicate), it reduces the number of experimental animals
406 needed (De Swaef *et al.*, 2017).

407
408 Many of the problems faced during larval sole rearing relate to the fact that the molecular mechanisms
409 underlying physiological and morphological changes during the larval-to-juvenile transition are still unknown.
410 Because of this, efforts to sequence and characterize the transcriptome of *S. solea* have been done (Ferraresso
411 *et al.*, 2013; Benzekri *et al.*, 2014). Ferraresso *et al.* (2013) revealed that pre-metamorphic larvae display a
412 distinctive transcriptomic landscape compared to previous and later stages. The highest number of
413 differentially expressed genes was found between 1 day-post-hatching (dph) and 4 dph, with a total of 1,539
414 significant genes (974 over and 565 under-expressed in 4 dph larvae), while only 120 genes (81 up- and 39
415 down-regulated) displayed a change in expression between 11 and 13 dph. In detail, genes related to the
416 development of the visual system and neuronal processes are up-regulated during the early stages of larval
417 development, while muscle development and anaerobic energy pathways increase in expression over time.
418 Moreover, the expression profile of genes within the thyroid hormones cascade, the growth hormone, and the
419 insulin-like growth factor genes revealed their crucial role in fish growth and initiation of metamorphosis
420 (Ferraresso *et al.*, 2013).

421
422 The development of visual perception is essential for larval feeding. At hatching, the eyes are slightly
423 pigmented or non-pigmented and most likely non-functional. Therefore, successful first-feeding depends on a
424 well-developed eye and visual system since fish larvae are essentially visual feeders. Light intensity is among
425 the variables that could affect the survival of larvae during rearing. Bonvini *et al.* (2016) studied the effect of

426 different light regimes on *S. solea* larvae reared in the laboratory. They prepared 4 different rearing treatments
427 with the following light intensities 1000 Lux, 500 Lux, 50 Lux, and 3 Lux. Larvae were exposed to the different
428 light intensities from fertilization until metamorphosis, and effects on development, metamorphosis success,
429 and growth were evaluated. The authors conclude that sole larvae require different light intensities along
430 ontogenesis. High light intensity (between 50-500 Lux) is recommended during the pelagic stage (4–12 dph),
431 in which first feeding larvae would benefit from light to initiate feeding. Also, histological examination
432 revealed the importance of vision and light in the first feeding of this species (Bonvini *et al.*, 2016). After
433 metamorphosis, the full development of other sensory organs indicates that feeding activity is also mediated
434 by chemosensory perception, and thus low light intensity (3-50 Lux) is recommended to ensure better growth
435 from the onset of metamorphosis to the benthic phase (Bonvini *et al.*, 2016).

436

437 **2.3 Advances made on larvae weaning, nutrition, and susceptibility to disease**

438 Nutrition plays a key role in sustainable aquaculture development, where fish health and wellness are of prime
439 importance. Problems such as reduced growth, low survival, malpigmentation, and malformations are often
440 encountered when fish are subjected to stress or deficient diet (Piccinetti *et al.*, 2012). Moreover, standard
441 feeding regimes during larval and early juvenile stages represent a challenge for fish farmers due to the required
442 administration of live feed, which is usually characterized by variable availability, high price fluctuations, poor
443 hygienic conditions, and high levels of pathogenic bacteria (Bonaldo *et al.*, 2011). Thus, the feasibility of early
444 weaning in common sole is a key factor in the industrialization of farming processes of this promising
445 aquaculture species. In this context, Bonaldo and collaborators (2011) experimented with different feeding
446 regimes and evaluated the effects that weaning *Solea solea* larvae as early as 13 days post-hatching (dph) has
447 over metamorphosis success, survival, and fish size. The experiment lasted until 29 dph and consisted of two
448 groups, a control one fed with live feed from 4 dph until 27 dph; and a second one weaned after 13 dph using
449 three different microdiets (MDs), AgloNorse, Ewos (FR-A), Gemma, Skretting (FR-G) and O.range Large,
450 INVE (FR-O) (Bonaldo *et al.*, 2011). The authors did not find significant differences in survival nor the
451 metamorphosis development between control and the early-weaned group. However, the larvae that were
452 weaned earlier showed lower weight and length than the control ones (Bonaldo *et al.*, 2011). Besides, they
453 obtained size variations regarding the type of MD administrated, with FR-G showing the lowest standard
454 growth rate. The authors conclude that it is feasible to conduct very early weaning on common sole and that
455 this practice is advantageous as it reduces the number of *Vibrio spp.* in the water, preventing disease outbreaks
456 (Bonaldo *et al.*, 2011). Finally, because larvae length and fatty acid body composition were significantly
457 influenced (reduced) by MDs, growth was negatively affected by the early introduction of MDs. Thus, the
458 authors hinted at the need for further research linked to nutrient utilization and the digestive capability of sole
459 larvae as a means to further improve larvae performances.

460

461 On this last note, Parma *et al.* (2013) studied the molecular ontogeny and expression of digestive enzyme
462 precursors on early-weaned larvae. They aimed to gain further knowledge of the effects of early weaning on
463 the performances and physiological mechanisms during larval development of common sole, optimize

464 weaning protocols further and reduce mortality around this period (Parma *et al.*, 2013). For that, the authors
465 cloned partial sequences of the enzymes trypsin (trypsinogen 1 (tryp1), trypsinogen 3 (tryp3), trypsinogen Y
466 (trypY), chymotrypsin (chymotrypsinogen (chymt)), amylase (amy), and hepatic lipase (HL), which are among
467 the most important pancreatic enzymes in teleost fish (Parma *et al.*, 2013). The expression of the former genes
468 was assessed from the onset of exogenous feeding until the end of metamorphosis. Four weaning protocols
469 (WPs) were evaluated, a control one with weaning occurring only at 27 dph, two intermediate treatments at
470 which live food was provided until 13 and 18 dph, and a final treatment with larvae weaned to micro diets at
471 4 dph. Based on their results, Parma and collaborators conclude that the different WPs did not greatly influence
472 the expression of selected genes. On the contrary, survival, growth rate, and metamorphosis success were
473 influenced by the WP, notably for larvae weaned at 4 dph, which showed the lowest survival rate (18.6% vs.
474 a mean survival rate of $52.8\% \pm 3.1$ for the 3 remaining protocols) and the lowest growth rate for the first 13
475 days (SGR of 4.4 vs. a mean specific growth rate of 17.3 ± 0.4 for the 3 remaining protocols). Finally,
476 regardless that most larvae started metamorphosis on day 19 (71% of larvae), none had reached complete
477 metamorphosis by the end of the experiment in the early-weaned group (Parma *et al.*, 2013).

478
479 Regarding nutrient utilization, Piccinetti *et al.* (2012) tested the effects of a total or partial replacement of live
480 feed (mainly consisting of rotifers and *Artemia nauplii*, group A/control group) with a copepod-based diet
481 (mixed diet of live feed and dry copepods, named group B) on common sole growth, survival, lipid
482 composition, lipid assimilation, defense to stress and malpigmentation (Piccinetti *et al.*, 2012). Their motivation
483 was based on the observation that traditionally used live preys like rotifers and *Artemia* have nutritional
484 deficiencies that result in a general decrease in fish health, development, growth, and pigmentation (Piccinetti
485 *et al.*, 2012 and references therein). To develop rearing schedules suitable for market production, the authors
486 conducted an experiment in which live preys were replaced with preserved copepods - the natural food of
487 larvae- (Piccinetti *et al.*, 2012). For that, starting from day 9 post-hatching, group B larvae were fed a mixed
488 diet (co-feeding), while group A and C were fed on live feed and copepod exclusive diets, respectively. Their
489 results showed that including a complement of dry copepods (group B) within the diet increased survival by
490 over 8% compared with group A (fed with rotifers and *Artemia nauplii*). In addition, larvae fed with a mixed
491 diet started and ended metamorphosis faster than the group fed with live prey (group A: 16 dph- 25 dph vs.
492 group B: 13 dph - 20 dph). Malpigmentation was significantly lower in group B than group A. Finally, larvae
493 from group B had a higher standard growth rate both in terms of body weight (BW) and total length (TL) than
494 individuals from group A. The authors explain that live prey motion is an essential attribute in stimulating
495 larval predatory activity, which explains why the best results, in terms of survival, growth, and pigmentation,
496 were achieved when the preserved copepods were administered together with live preys (rotifers and *Artemia*).
497 Diet with only preserved copepods (group C) resulted in 100% mortality after 7 dph (Piccinetti *et al.*, 2012).

498
499 The outbreak of infectious diseases can impact survival in marine fish larviculture (De Swaef *et al.*, 2018;
500 Dinis *et al.*, 1999; Morais *et al.*, 2014; FAO, 2019). Microbiota can help to prevent and control diseases.
501 However, knowledge about the establishment and action of microflora in fish's gut during larval stages is still
502 scarce (Vino *et al.*, 2006). The most common members of the microflora of healthy marine fish are *Vibrio*

503 *spp.*, *Pseudomonas spp.*, and *Acinetobacter spp.* (Vino *et al.*, 2006). Vibriosis, a bacterial infection caused by
504 abnormal/unbalanced colonization of *Vibrio spp.*, is among the diseases of primary concern for *S. solea*, being
505 particularly challenging to tackle during early life stages (Austin and Austin, 2016; Novriadi, 2016).

506
507 Thus, another research subject in fish nutrition that has been primarily studied as an alternative for improving
508 disease resistance and nutrient absorption is the effects that dietary manipulation can have over fish gut
509 microbiota, mainly by the administration of prebiotics and probiotics (Ringø *et al.*, 2014; De Swaef, 2019).

510
511 During the last decade, many works have studied the effects of prebiotics and probiotics, not only for their role
512 in improving disease resistance (as an immunostimulant) but also as a functional dietary supplement. Details
513 on this subject escape from the scope of this work, and we refer the interested reader to consult the many other
514 reviews available on the subject (Bricknell and Dalmo, 2005; Avella *et al.*, 2011; Ringø *et al.*, 2014; Torrecillas
515 *et al.*, 2014; Vine *et al.*, 2006). However, in general lines, manipulation of gut microbiota has proven beneficial
516 in adult fish, enhancing growth and feed efficiency and immunity and disease resistance (Ringø *et al.*, 2014).
517 Regardless of many studies describing such beneficial effects, literature on the effects of prebiotics and
518 probiotics administration to fish larvae is somewhat limited (De Swaef, 2019). In this regard, and to reduce
519 losses in early stages of larval development and maximize production of captive common sole, Avella *et al.*
520 (2011) studied the effects of administrating *Enterococcus faecium* IMC 511, isolated from sole broodstocks,
521 as a probiotic candidate during *S. solea* larval development. During the first 50 days post-hatching, the
522 probiotic was administrated daily through live feeds. The authors evaluated the intestinal presence of *E.*
523 *faecium* IMC 511 during the experiment and the effects on intestinal microbial load, fish survival, welfare, and
524 growth. For that, morphometric and molecular approaches were conducted. As markers of animal welfare, the
525 authors monitored gene expression of cortisol and the 70 kDa Heat Shock Protein (HSP70). Their results
526 showed reduced *Vibrio* populations in *S. solea* larvae intestines treated with the probiotic *E. faecium* IMC 511,
527 but survival was not affected. The expression of stress markers (cortisol and HSP70) was significantly lower
528 in probiotic-fed larvae than control larvae. By the end of the experiment, sole juveniles treated with probiotics
529 had significantly higher body weight (BW) and total length (TL). Based on their results, the authors conclude
530 that 50 days of probiotic treatment can improve common sole larval growth (Avella *et al.*, 2011).

531
532 Finally, De Swaef (2019) assessed the impact of dietary supplementation in *S. solea* larvae of two prebiotic
533 candidates derived from the aquatic environment, the mannanoligosaccharide and alginic acid. Contrary to the
534 results previously cited, the tested prebiotics did not increase larval growth or survival and did not shift the
535 proliferation and apoptosis rate of the intestinal epithelium (De Swaef, 2019). On the contrary, the
536 administration of alginic acid had a negative effect on standard body length and the larvae supplied with 0.2%
537 alginic acid followed by a challenge test (consisting of infection by *V. anguillarum* WT at a final concentration
538 of 1×10^7 colony-forming units/mL at 10 DAH) displayed a lower survival in comparison with the challenged
539 larvae without any supplementation. These results reveal a possible negative impact of alginic acid
540 supplementation on common sole larvae (De Swaef, 2019) and highlight the need for further research on the

541 benefits of prebiotics, probiotics, or synbiotics (nutritional supplements combining a mixture of probiotics and
542 prebiotics in the form of synergism, Ringø *et al.*, 2014) in *S. solea* larvae.

543

544 Because fish are free-living organisms since their initial stages, they are exposed to numerous pathogens even
545 before their lymphoid organs, and consequently, their adaptive immunity has fully developed (Ferraresso *et*
546 *al.*, 2016). Therefore, to defend the host from infection, newly hatched fish rely on their innate immune
547 repertoire that acts in a non-specific manner. Ferraresso *et al.* (2016) argued that for developing effective
548 approaches for disease management at early life stages of *S. solea* is necessary to understand the mechanism
549 related to innate immune responses. For that, in their study, the authors characterized and assessed the
550 transcriptional onset of unexplored relevant genes of both innate and adaptive immune systems during the *S.*
551 *solea* ontogenesis, from hatching to the accomplishment of the juvenile form (Ferraresso *et al.*, 2016). Their
552 results showed that several genes involved in the innate immune repertoire are up-regulated during the first
553 stages (such as lysozyme, AMPs (hepcidin, b-defensin), PPRs, and complement components), supporting the
554 hypothesis of protection from environmental pathogens during early development. Moreover, the onset of the
555 expression of adaptive immune genes (i.e., Class I and class II MHC, TCRs) coincides with metamorphosis
556 and larvae-to-juvenile transition. Finally, data collected suggest that full maturation of the cell-mediated
557 immune system of *S. solea* is not completely mature by the end of metamorphosis (Ferraresso *et al.*, 2016).

558

559 **2.4 Pigmentation and eye migration**

560 Other developmental problems related to the successful commercialization of soles are problems related to the
561 appearance of the fish product, which can be insufficient for the market standards, such as malpigmentation
562 and incomplete eye migration.

563

564 Pigmentation problems are known to be related to vitamin A, fatty acid composition, and thyroid hormones.
565 However, in most cases, it is an unbalanced fatty acid composition of the feed the main reason for
566 malpigmentation of farmed flatfish, and the thresholds for concentrations of these essential fatty acids are
567 species-specific (The Research Council of Norway, 2009). In this regard, Lund and collaborators (2008)
568 studied the effects that diets supplemented with arachidonic acid (ARA) and eicosapentaenoic acid (EPA) have
569 on sole survival, metamorphic success, and notably pigmentation. They hypothesized that the induction of
570 malpigmentation (*i.e.*, hypomelanosis) was linked to a specific larval period or “a pigmentation window” at
571 which larvae sensitivity to ARA increases. Their results confirmed their hypothesis of the presence of an early
572 pigmentation window at pre-metamorphosis. Also, they revealed that in common sole larvae, ARA induced
573 malpigmentation, but no effect was associated with diets enriched with EPA (Lund *et al.*, 2008). Regarding
574 metamorphosis success, the initiation or advance of eye migration was not related to dietary ARA, EPA, but
575 the size of the individuals (Lund *et al.*, 2008).

576

577 **Section 3 - Effects of temperature and contamination on *Solea solea* larvae**

578

579 Temperature is the main factor regulating flatfishes' species range. Human-induced climate change has played
580 a central role in larval dispersal variability, leading to species losses and invasions, biogeographical and regime
581 shifts, and changes in the adaptation capabilities of organisms (Lacroix *et al.*, 2016). Also, temperature
582 modulates the duration of the egg and larval stages and, therefore, determines the distance between spawning
583 and nursery grounds (Gibson, 2015). Further, the species range and the average level and the annual variability
584 in recruitment regulate sole populations. The variability in recruitment is governed by two distinct processes,
585 the effect of food availability on the adult condition at spawning time and the density-dependent mortality of
586 juveniles on the nursery grounds (Gibson, 2015). Finally, temperature also affects fish growth rate and ultimate
587 size.

588
589 Chemical exposure from anthropogenic activities negatively impacts marine habitats, notably on coastal
590 habitats, where most of the human population is established (60% at 100 km from the water; Brown, 2006).
591 Moreover, several species of economic interest expend part of their life cycle on coastal habitats and estuaries,
592 where specific characteristics in temperature and physicochemical conditions guarantee their healthy
593 development (Seitz *et al.*, 2014). However, estuaries can accumulate and stock several pollutants in the
594 sediment compartment (Ridgway and Shimmield, 2002). Previous works that conducted inter-estuarine
595 comparisons showed that the chemical contamination status notably affects individuals' growth and survival
596 and thus the quality of the nursery area (Gilliers *et al.*, 2006; Amara *et al.*, 2007). In the case of *Solea solea*,
597 unwanted changes in nursery ecosystems could affect (i) juvenile settlement, (ii) survival of juvenile
598 specimens, and (iii) healthy development and growth, which in the long term could impact the renewal capacity
599 of the population –recruitment-, and impact the species fitness.

600

601 **3.1 Changes in spawning, larval development and survival, and recruitment related to** 602 **temperature**

603

604 In recent years, fish spawning patterns and growth rates have been changing. Increased temperatures are
605 believed to have various effects on fish populations, including effects on their phenology by altering growth,
606 the survival of juveniles and adults, and reproductive traits, such as the timing of spawning (Alix *et al.*, 2020).
607 Shifts in the timing of spawning will have both direct and indirect consequences for fishery management and
608 could have significant implications on species recruitment and population size. These effects include –among
609 other things– mismatches between the timing of larval development and the availability of planktonic food
610 sources, and a reduction in the effectiveness of fishery closures during spawning seasons (Fincham *et al.*, 2013;
611 Alix *et al.*, 2020).

612

613 In their work, Fincham *et al.* (2013) evaluated temperature data from coastal monitoring sites situated close to
614 spawning grounds, together with data on *Solea solea* timing of spawning. The last data came from market
615 sampling programs in ports in England and the Netherlands, where random samples of fish landed by
616 commercial fishing vessels are taken, and biological data, such as the proportion of spawning females amongst
617 the total number of sampled adult females, was calculated for each week of the year for a time series of 40

618 years (Fincham *et al.*, 2013). Their results showed that four out of the seven stocks they studied had a
619 significant shift in the time of spawning in response to increased sea surface temperature (SST). In detail, the
620 stocks in the Irish Sea, east-central North Sea, southern North Sea, and the eastern English Channel spawned
621 in earlier weeks of the year at a rate of 1.5 weeks per decade (Fincham *et al.*, 2013). They observed both spatial
622 and temporal differences, which were strongly correlated to temperature. They also found that winter SST is
623 inversely correlated with the timing of spawning, which is caused by the faster maturing of gonads due to
624 warmer temperatures during the winter. The authors conclude that in a warmer world, *S. solea* populations
625 from the English Channel will spawn earlier (Fincham *et al.*, 2013). However, as recruitment depends on food
626 availability, changes in the phenology can have important implications for the population dynamics (Gibson,
627 2015). Evidence suggests that the timing of peaks in larval food abundance may not necessarily advance with
628 warming temperatures (Wiltshire *et al.*, 2008). A mismatch between the timing of spawning and hatching
629 periods with the peak of the phytoplankton or copepods will reduce the recruitment success and abundance of
630 the species.

631
632 Although temperature seems to be the main driver to alter sole spawning phenology from 1970 until 2010, the
633 authors do not dismiss the effect of other factors, including population density, growth, and maturity at age.
634 However, and because the temperature has a positive influence on the growth of age-0 sole (Teal *et al.*, 2008),
635 if phenology changes were due to changes in growth rate, then the rising temperature should delay the timing
636 of spawning, resulting in bigger but younger fish at first spawning (Fincham *et al.*, 2013). Nevertheless, there
637 is evidence that sole size and age at first spawning have decreased (Mollet *et al.*, 2007), while the timing of
638 maturation may be delayed in smaller, relatively more abundant fish in their first year of maturation (Ramsay
639 and Witthames, 1996). Thus, if growth affects phenology, the rising temperature would lead to the reverse
640 effect of that observed by the authors, *i.e.*, delayed timing of spawning. This reasoning strongly suggests that
641 the observed phenological shifts are less likely to be the indirect result of changes in the size-age composition
642 of spawning fish but a more direct effect of winter temperatures on gonad maturation (Fincham *et al.*, 2013).

643
644 After the spawning, the transport of eggs and larvae from spawning grounds to nursery areas is primarily driven
645 by hydrodynamic processes. However, behavioral and environmental factors might also influence the final
646 dispersal pattern as well as the larval survival and abundance (Lacroix *et al.*, 2013). The survival of the early
647 life stages is dependent on environmental constraints -notably temperature- the availability of food, and the
648 presence of predators (Lacroix *et al.*, 2013). After that, fish recruitment is dependent on larval abundance and
649 success of metamorphosis at nursery areas. Therefore, understanding larval dispersal is of utmost importance
650 as it might explain recruitment patterns and has direct consequences for the conservation of fish, stock
651 management, the design of marine reserves, the management of non-indigenous invasive species, and the
652 prediction of climate change effects (Lacroix *et al.*, 2016).

653
654 On the question about larval dispersal, Lacroix and collaborators have notably contributed with a series of
655 works (Savina *et al.*, 2010; Lacroix *et al.*, 2013, 2016; Barbut *et al.*, 2019) for which the main focus has been
656 to understand the impact of interannual variability of hydrodynamics and temperature on larval dispersal and

657 supply to the nurseries. The works also aim to predict the potential changes in *S. solea* dispersal patterns under
658 warmer scenarios due to climate change in the English Channel and the North Sea. Other relevant works related
659 to sole larval dispersal in other regions include the works by Vinagre *et al.* (2007) in the Tagus Estuary in
660 Portugal and the study by Tanner *et al.* (2017) in the Iberian coast.

661

662 For evaluating larval dispersal, a larval transport model for sole coupled with a 3D hydrodynamic model of
663 the North Sea was implemented, and interannual variability of the transport of fish larvae during different
664 periods was investigated (Savina *et al.*, 2010; Lacroix *et al.*, 2013, 2016). Shortly, the study of 2013 showed
665 important interannual variability (coefficient of variation of 29.2%) of the total larval abundance at the
666 nurseries. The larvae dispersal patterns were broader than those reported in the first generation model by Savina
667 *et al.* (2010). In their work from 2016, the inclusion of wind speed and directions as factors in the model
668 together with simulations based on predicted scenarios from the “Warm+” IPCC showed an increase in the
669 dispersal distance (+70%) and an increase in the pelagic larval duration (+22%) which was in response to
670 reduced temperature (-9%) endured by larvae that hatched earlier (Lacroix *et al.*, 2016).

671

672 In recent work, Barbut *et al.* (2019) evaluated the relationship between dispersal capacity and larval traits, such
673 as behavior, pelagic larval duration, seasonality of reproduction, and structure of the water column and
674 currents, from populations of six commercially exploited flatfish species in the North Sea. Their results
675 demonstrate considerable variation in both connectivity and spatial distribution of all studied species, with two
676 groups emerging with similar life-history traits and dispersal patterns features (Barbut *et al.*, 2019).

677

678 The set of papers from this group tell a coherent story in which knowledge about larval dispersal increases
679 progressively. However, this group of researchers' methods and the model are common, and since they are
680 based on a single model, the level of uncertainty was not included. On this last point, we direct the reader to
681 Hufnagl *et al.* (2017 and references therein) work in which the variability that can be expected concerning
682 connectivity, uncertainty, and the order of magnitude of variability resulting from choosing different physical
683 models, was addressed by comparing a suite of 11 North Sea models (Hufnagl *et al.*, 2017).

684

685 On a final note, warmer scenarios may improve the survival of juveniles. Earlier spawning will prolong the
686 growing season of 0-group fish and may result in an increase in the body size during the 1st winter (Teal *et al.*
687 *et al.*, 2008), and since winter survival is positively related to body size, 0-group cohorts with enhanced energy
688 stores are more likely to survive (Post and Parkinson, 2001). However, evidence suggests that fish size may
689 shrink in response to climate-induced changes in temperature and oxygen (Baudron *et al.*, 2014). The
690 physiology behind the pattern is well established for aquatic ectotherms, higher temperatures decrease the
691 aerobic capacity, and individuals with smaller body sizes have a reduced risk of oxygen deprivation (Baudron
692 *et al.*, 2014). For *S. solea*, there is evidence showing that exposure to elevated temperatures during early
693 developmental stages alters the environmental adaptation performance of fish at later stages, making juveniles
694 more resistant to later in life hypoxia events (Zambonino *et al.*, 2013). However, and in contradiction to

695 previous results, Zambonino *et al.*'s (2013) results showed that higher temperatures during early stages resulted
696 in larger-bodied individuals.

697

698 **3.2 Changes in spawning, larval development, and survival, related to chemical exposure**

699

700 The presence of significant levels of pollutants in sole tissues can affect their physiological performance and
701 harm the health of consumers. Given this, several studies assessing the bioaccumulation capacity and toxicity
702 of different chemical contaminants (organic pollutants and metals) have been conducted using *Solea solea* as
703 target species (Dierking *et al.*, 2009; Wessel *et al.*, 2010; Trisciani *et al.*, 2011; Cannas *et al.*, 2013; Sanchez-
704 Nogué *et al.*, 2013; Sister *et al.*, 2013; Solé *et al.*, 2013; Cuevas and Zorita, 2018; Mounier *et al.*, 2020a,
705 2020b), some other study focused on differential mortality in age-0 juveniles (Guinand *et al.* 2011). However,
706 all these studies have focused on the juvenile stage and the ecotoxicity of contaminants at the early life stages
707 of *S. solea* it is still, up to date, understudied.

708

709 Although working with juvenile fish in laboratory conditions requires specific facilities and is logistically
710 challenging, most ecotoxicity tests conducted with soles are designed at this developmental stage because it
711 guarantees enough tissue for running tests in target organs (*i.e.*, biomarker and bioaccumulation
712 analyses). Moreover, working with juveniles reduces sex-related endpoints variability and does not consider
713 the influence of reproduction on bioaccumulation. Larval stages (*i.e.*, egg, yolk-sac larvae, and free-feeding
714 pre-metamorphic larvae) are considered to be the most sensitive stages of soles life cycle (Ferraresso *et al.*,
715 2013). As discussed in the previous sections, mortality under control conditions still reaches up to 60%
716 (Foekema *et al.*, 2008, 2014), and protocols for rearing larvae in the lab still need to be refined (De Swaef,
717 2019). At the same time, the fields of biology and toxicology have seen some important developments.
718 Advances in measurement technologies, computational capabilities, fundamental toxicological understanding
719 at the molecular level, and *in vitro* technologies have increased the amount of information produced following
720 ecotoxicity tests (Ankley *et al.*, 2010). This fact, together with the refinement of analytical protocols (Olsson
721 *et al.*, 1978; Thomson and Budzinski, 2000) and the proposal of easier to manipulate rearing methods (such as
722 the microplate early life stages adapted protocol presented in De Swaef *et al.*, 2017) opens the door for
723 conducting ecotoxicity tests with sole larvae.

724

725 There are three sources for contaminant exposure during the larval stages. The first is the result of parental
726 exposure. During fish vitellogenesis, most of the lipid resources are stored in the yolk, and during this process,
727 lipophilic pollutants are also transferred to the eggs (Foekema *et al.*, 2012). The second exposure route occurs
728 after hatching when pelagic larvae start the uptake and elimination (through detoxification mechanisms) of
729 pollutants from the water. Once the larvae deplete the yolk reserves, it will start feeding, and therefore, the
730 third exposure route is activated through ingestion of large amounts of lipid-rich -and potentially contaminated-
731 zooplankton.

732

733 As the first route of exposure involves maternally transferred contamination, there is the question of the effects
734 of lipophilic persistent organic pollutants (POPs) on early life stages (ELS).. For predicting sole's
735 bioaccumulation of POPs, such as polychlorinated biphenyls (PCBs), Foekema *et al.* (2012) adapted the
736 bioaccumulation model OMEGA (Hendriks *et al.*, 2001) to the ELS of sole turning it to ELS-OMEGA. The
737 model was validated with experimental data and proved effective in predicting the development of PCBs'
738 concentrations in the tissue of developing soles. The authors measured and modeled the concentrations of
739 PCBs (52, 110, 101, 118, 138, and 153) in water and tissue during the development of an embryo into a juvenile
740 fish. The model showed good fits to the measured data, and according to the simulations, tissue concentrations
741 of pollutants with log octanol-water partition ratio (K_{ow}) > 5 peaks when the lipid reserves are depleted at the
742 moment that larvae become free-feeding, revealing that maternally transferred highly lipophilic compounds
743 are barely excreted (Foekema *et al.*, 2012). Moreover, these peaks may exceed two to four times the
744 concentration in the spawning parent, concentrations that could result in extreme body burdens during critical
745 stages of larval development, with potentially severe consequences for larval survival. Once the fish starts
746 feeding externally, fish increase rapidly in weight, and dilution by growth plays an essential role in tissue
747 concentration decrease (Foekema *et al.*, 2012).

748
749 The authors highlight the risk of underestimating the effects of maternally transferred PCBs (especially those
750 with K_{ow} > 5) at the early-life-stages, principally because of the exposure to waterborne substances of a fish
751 embryo inside the egg is limited compared with the post-hatch situation (Foekema *et al.*, 2012). Unfortunately,
752 the ELS toxicity test focus on exposing eggs via the water, and very often, the experiment will end just before
753 the end of the yolk-sac stage, the moment at which the internal concentration will peak to its maximum
754 (Foekema *et al.*, 2012). The authors argue that terminating ELS tests before the yolk-sac stage ends is valid
755 for testing substances with log K_{ow} < 5. They, however underline that when testing more hydrophobic
756 substances, mixtures of substances, or unknown mixtures (such as those present in effluents or contaminated
757 sediments), the toxic effects of the lipophilic compounds will be seriously underestimated (Foekema *et al.*,
758 2012).

759
760 The previous statement is supported by data from two more works by Foekema *et al.* (2008, 2014), in which
761 delayed effects after exposure during the egg stage were observed on *S. solea* larvae. In their earlier work, the
762 authors exposed *S. solea* eggs to a concentration series of PCB 126 in seawater until 4, 8, 10, and 15 days post
763 fertilization (dpf), and the development of the larvae was followed up under unexposed conditions until larvae
764 were fully metamorphosed (up to 50 dpf) (Foekema *et al.*, 2008). Their second work aimed to recreate the
765 effects of maternally transferred POPs in early larvae development (Foekema *et al.*, 2014). The results from
766 both studies indicate that ELS fish tests underestimate the effect of highly lipophilic compounds, which
767 continue to affect survival and metamorphosis success long after the exposure, showing that, at least for sole,
768 the moment of observation has a significant impact on the determination of the effect concentration
769 (approximated by the LC_{50} in Foekema's works), even without further exposure (Foekema *et al.*, 2008; 2014).

770

771 **Section 4 - Methods that integrate the data -bridging the gap-**

772

773 Studying the effects of temperature and contamination along *Solea solea* larvae ontogenesis is relevant for
774 several disciplines (Fig. 2). Researchers in fisheries and ecology want to know if the stocks will be secured in
775 future years and, what will be the species biogeographical distribution, the impact of invasive species (e.g.,
776 Marras *et al.*, 2015), and how spawning and larval dispersal might be affected by increased temperatures
777 (Pankhurst & Munday, 2011). Aquaculture research is interested in understanding larvae's physiology and
778 developing protocols for rearing them while minimizing the cost and maximizing survival. Environmental
779 chemists develop analytical protocols to detect smaller and smaller concentrations of environmental pollutants
780 that might be present in several matrixes (water, pore water, sediments, and biota). Their efforts orientate
781 towards understanding and predicting bioaccumulation and transfer of pollutants in sole into the trophic web.
782 Moreover, their results are input for fate and transport models. Finally, ecotoxicologists aim to predict and
783 understand the effects of combined stress from exposure to a mixture of chemicals under future climate change
784 scenarios to provide relevant data for chemical regulation.

785

786 Generating relevant data for risk assessment and chemical regulation is the major challenge that
787 ecotoxicologists have and keep facing up to today. In short, the ecotoxicology domain aims to answer two
788 impossible questions i) which of the millions of species are at risk from pollution? And ii) which of the tens
789 of thousands of chemicals contribute most to the risk? As Ashauer and Jager (2018) stated, the great pitfall in
790 ecotoxicology is that there is no theory to link the effects of toxicants at the molecular or cellular level to
791 changes in life-history traits. Moreover, at the cellular level, there are plenty of examples that demonstrate that
792 responses are cell-type and species-specific and conditioned by age, size, gender, season, and temperature,
793 among many other biotic and abiotic variables (van der Oost *et al.*, 2003; Au, 2004; Martínez-Álvarez *et al.*,
794 2005; Hardman, 2006). Also, multiple types of effects depend on the pollutant or the mixture of pollutants,
795 and that can vary depending on the exposure route (Hardman, 2006). Even with a fast pace of research
796 production, there is no way that we can catch up with the speed at which the industry produces new chemicals.
797 So, how can we catch up with xenobiotics production? Furthermore, which are the tools that we need to bring
798 together to bear on climate change and pollution effects?

799

800 Our poor knowledge of physiological modes of action (pMoA) and how they vary across species and toxicants
801 has been recently identified as a major knowledge gap (Ashauer and Jager, 2018). Physiological MoA refers
802 to how chemical stress (single compound or mixture) affects an organism's energy allocation and,
803 consequently, life-history traits. It can be determined using Dynamic Energy Budgets (DEB) models, which
804 have strong theoretical foundations (Kooijman, 2010). In their paper, Ashauer and Jager (2018) proposed
805 pMoA as a key element to extrapolate from small to larger biological levels. We believe that the authors'
806 proposition is correct and that researching pMoA is a promising venture.

807

808 According to the authors, knowing pMoAs is an excellent way to boost and complete the information that the
809 two main approaches in ecotoxicology -data from chemical stress ecology and high-throughput bioassays of
810 cellular or molecular markers ELS- offer to ecotoxicologists (Ashauer and Jager, 2018). Shortly, chemical
811 stress ecology treats pollution as one of the many factors that influence individuals, populations, communities,
812 and ecosystems. On the one hand, this approach aims to provide ecological relevance and realism to
813 environmental risk assessments, but it fails to discriminate if the observed effects are due to a single pollutant
814 or a mixture of them, giving little relevant information from the regulatory point of view. On the other hand,
815 high-throughput bioassays orbits on the promises to upscale results from bioassay to organisms and beyond,
816 rallying around the idea of quantitative adverse outcome pathways (AOPs) first proposed by Ankley *et al.* in
817 2010. The AOP framework aims to provide a useful structure within which existing knowledge can be
818 organized, from which key uncertainties and research priorities can be identified, and through which we can
819 improve predictive approaches needed to advance regulatory ecotoxicology. It integrates evidence from *in*
820 *vitro* assays linking a molecular initiating event (e.g., a molecular interaction between a xenobiotic and a
821 specific biomolecule) and an adverse outcome in the context of a pathway that leads to an adverse outcome at
822 a biological level of organization relevant to risk assessment (Ankley *et al.*, 2010). However, to answer which
823 of the millions of species are at risk from pollution, it is necessary to extrapolate quantitative AOPs across
824 species. For that, we need to assume that molecular pathways are qualitatively and quantitatively conserved,
825 and data suggest that a significant fraction of receptors and target sites are not conserved between species
826 (Gunnarsson *et al.*, 2008; Rand-Weaver *et al.*, 2013).

827
828 For covering the present needs in ecotoxicology, focusing on determining the pMoA would be a strong tool as
829 it would represent a middle point between AOPs and stress ecology. As organisms require resources to grow,
830 develop, and reproduce, it is these traits that we ultimately require to link AOPs to ecological theory and
831 upscale to the population level and higher (Ashauer and Jager, 2018). Changes due to toxicant exposure or
832 environmental stress in energy-demanding traits -such as growth and reproduction- logically imply changes in
833 the energy budget and model parameters. By using energy-budget models and linking the related parameters
834 to AOPs, we will be able to 1) identify where energy allocation has changed due to a stressor -the physiological
835 mode of action- and by how much, and 2) justify the observed changes -if any- with data from AOPs, which
836 will therefore allow us to interpolate from the individual to the subcellular level. Further, pMoA acts at the
837 individual level, and there are theories available in ecology that allow extrapolating from individual to
838 population and community level (e.g., Physiologically Structured Population model (PSP), Caswell *et al.*,
839 1997; Ghwila and Willms 2019). Thus, pMoAs could be the missing link between subcellular levels and higher
840 levels of biological organization. Nevertheless, it is important to highlight that, on the possible types of pMoAs
841 within a CC and contaminated scenario, one important dichotomy involves toxicant-induced changes that alter
842 the ability of an organism to respond to CC stressors (toxicant-induced climate susceptibility [TICS]), in
843 contrast to climate-induced toxicant sensitivities (CITS), which is the scenario where CC affects the toxicity
844 of chemicals (Hooper *et al.*, 2013). We invite the reader to seek further details regarding pMoAs and AOPs in
845 Ashauer and Jager (2018) and Perkins *et al.* (2019), respectively.

846 Finally, the reason why studying combined effects of temperature and chemical exposure is challenging is that,
847 as previously mentioned, the main approaches followed by ecotoxicologists are incapable to either discriminate
848 the separate effect of each stressor (as in chemical stress ecology) or measuring the mixed effect and translate
849 it to a relevant level of biological organization (when using high-throughput bioassays). The implementation
850 of DEB models provides the means to overcome this issue while taking advantage (or making use) of the
851 information provided by these two ecotoxicological approaches. The ecological theory is indispensable to
852 extrapolate from the individual to the population level, and subcellular data complements the information from
853 model parameters, allowing us to understand the molecular mechanisms behind the pMoA. Further,
854 concentrating efforts from different disciplines into a common modeling goal directly translate in reducing
855 animal testing (by reusing data from tests already conducted), and since DEB models offer a real opportunity
856 for species extrapolation (see Kooijman, 2010 for examples of model parameters variation related to body size;
857 Baas *et al.*, 2015; Sardi *et al.*, 2019), it also translates in reducing costs for research associated to risk
858 assessment.

859

860 We hope that within this section, we have managed to convey the following message: existing data can be
861 synthesized in a useful way to risk assessors and ecotoxicologists, but it demands consensus and team effort
862 from players that operate in different ways disciplines of research.

863

864 **Further research recommendations and conclusions**

865

866 Working with DEB and derived models (GUTS see Jager and Ashauer, 2018a and b, and DEBtox, Kooijman
867 and Bedeaux, 1996 or Jager and Zimmer, 2012) offers an excellent opportunity to answer (individually or by
868 coupling them to other models) to several -if not all- the research questions asked in many different disciplines,
869 and shortly stated at the beginning of the previous section (Fig. 2).

870

871 In DEB models, temperature and food availability are forcing variables (Kooijman, 2010). The last means that
872 we could run simulations for higher temperatures and provide insights about the effects of sea warming on
873 energy budgets allocation, growth, maturation, and reproduction success. Besides, DEB models can be coupled
874 with larval dispersal and hydrodynamic models (Huret *et al.*, 2012), which will provide information about the
875 time and size of fish at spawning for a given temperature and the dispersal of hatched larvae. Also, by
876 combining DEB models with ecosystem models, it is possible to build habitat quality maps (*e.g.*, Teal *et al.*,
877 2012) at which the effect of climate change on the spatio-temporal fish dynamics can be explored. Moreover,
878 this type of approach can be conducted in similar species with different temperature optima (such as it is the
879 case between *S. solea* and *S. senegalensis*) and provide insights about changes in species biogeographical range
880 or native species displacement by invasive ones (see Solé, 2019).

881

882 In ecotoxicology, coupling DEB with TKTD models (DEBtox model, Kooijman and Bedeaux, 1996; Billoir
883 *et al.*, 2007; Jager *et al.*, 2004; Augustine *et al.*, 2012; Sadoul *et al.*, 2019) allows for depicting the pMoAs
884 which are potentially the missing link between subcellular processes and effects on higher levels of biological

885 organization. DEB and DEBtox models are so far the most powerful available tool that could allow for species
886 and chemical extrapolation, and their standard implementation could be the necessary information for
887 improving environmental risk assessments and chemical regulation.

888

889 In aquaculture research, larvae rearing methods could benefit from the information coming from simulations
890 done with DEB models, in which growth is fitted for different temperature and food conditions. Also, DEBtox
891 or GUTS models can be applied to evaluate the effect of antibiotics, probiotics, and disinfection methods often
892 applied for reducing larvae mortality. This practice will potentially reduce the experimental cost and render
893 the business profitable. This last point is not only crucial from a commercial perspective. Improving rearing
894 systems and developing the aquaculture on this species also impact the food security and human health (by
895 preventing humans from consuming fish that have bioaccumulated pollutants), and decrease the fishing
896 pressure over natural populations, which might face difficulties in their renewal owing to the changes in
897 temperature and pollution projected for future years (Fig. 2).

898

899 Finally, DEB models coupled to bioaccumulation models would provide the means to predict contaminant
900 dilution by growth effect and to estimate the concentration of chemicals ingested by individuals along
901 ontogenesis, both common questions in environmental chemistry research (Arnot and Gobas, 2006).

902

903 In a nutshell, even though the development of DEB models requires significant effort and investment -as it
904 requires the user to understand DEB theory, its mathematical foundation, notation, and interpretation of model
905 parameters- its application is potentially beneficial for many disciplines, opening the door for interdisciplinary
906 research. In this line of thought, we would like to propose an interdisciplinary framework that aims to bring
907 together and take full advantage of already existing data (often coming from different research fields) and tools
908 (often operating at different levels of biological organization) to bear on climate change and pollution effects
909 on *S. solea* larvae ontogenesis. Figure 3 illustrates such a framework. At least five different disciplines have
910 an interest in studying the combined effects of temperature and chemical contamination in sole larvae, though
911 their research questions (outer blue circle) differ. Answering those questions requires collecting/generating
912 different types of data (purple circle), which are often used as input for calibrating models (dark pink circle)
913 that operate at different levels of biological organization. Finally, all these models can be used either
914 individually or in combination, but we can only achieve full understanding regarding the effects of
915 contamination or temperature stress at the individual level after determining the pMoAs (light pink circle) and
916 linking these to processes at the sub-cellular level with AoPs (yellow circle, Fig. 3).

917

918 To conclude, models are simply tools to deduce quantitative conclusions from a set of assumptions and data.
919 In this case, the DEB family of models offers an incredible opportunity to ecotoxicologists searching for the
920 missing link between effects at subcellular level and effects at the population and community level and,
921 consequently, producing and presenting data in a way that is useful to environmental risk assessors.

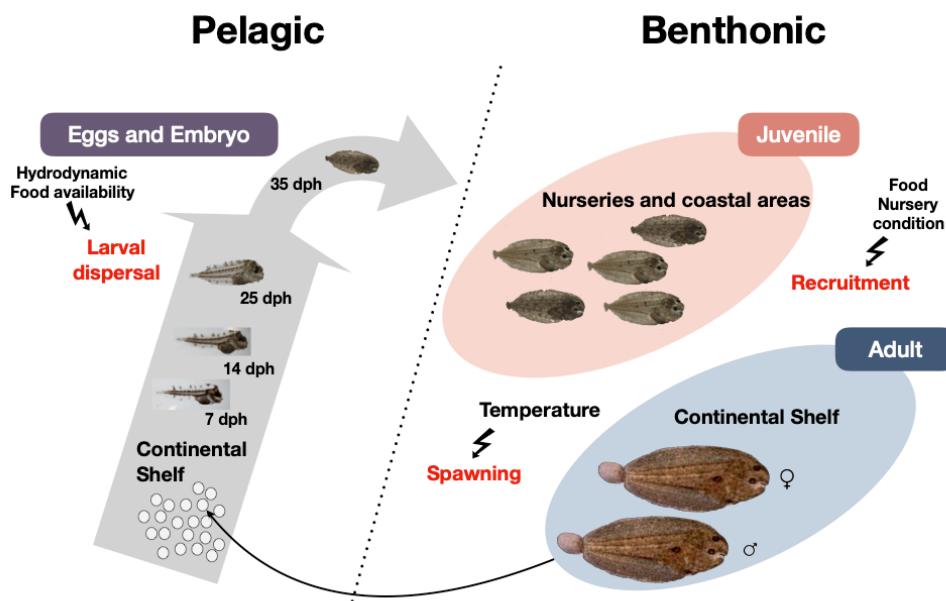
922

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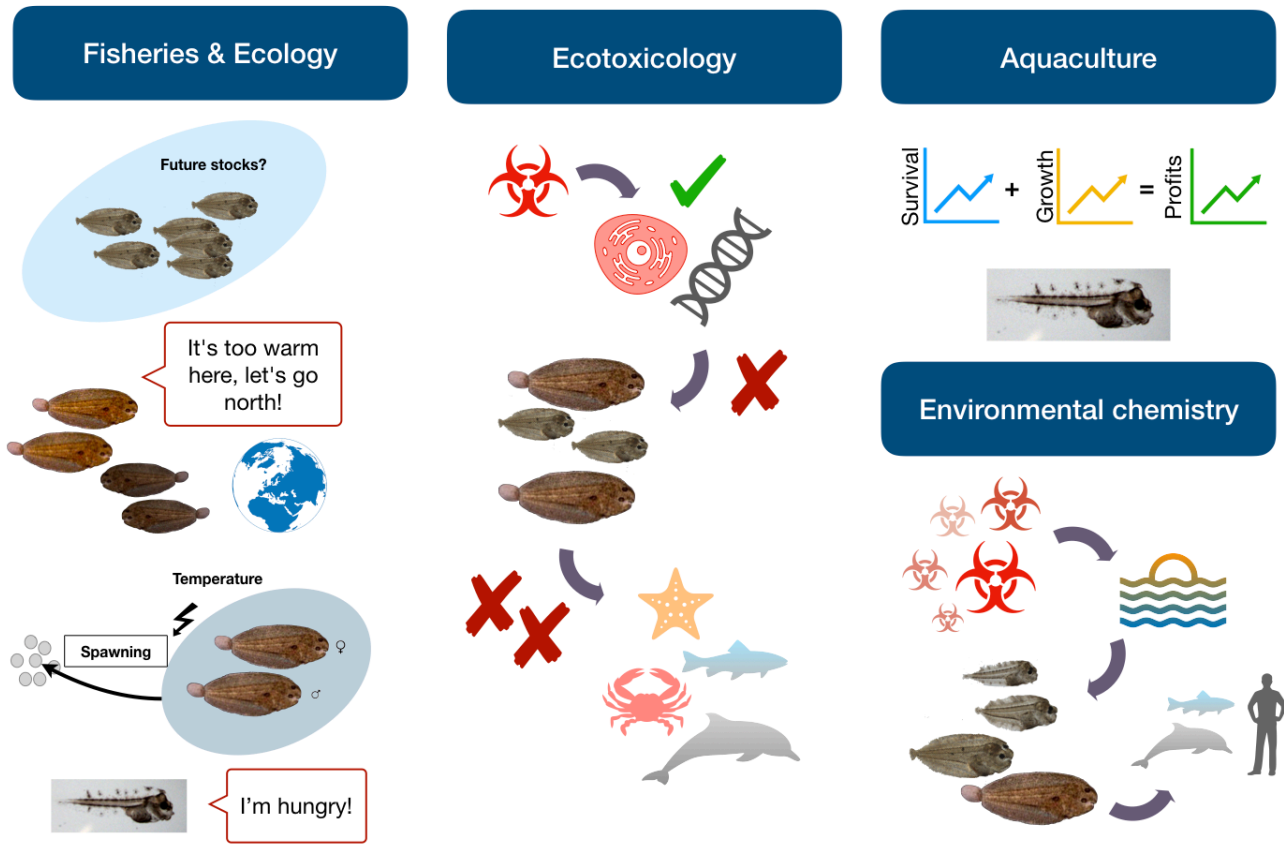
925
926 Figure 1. *Solea solea* life cycle. Soles have a complex life cycle that depends on biotic and abiotic variables.
927 In the continental shelf, temperature triggers spawning from benthonic adults. After hatching, the pelagic
928 larvae undergo extreme metamorphosis and migrate towards the nursery and coastal areas, all while
929 undergoing metamorphosis. Migration and metamorphosis success depends on hydrodynamics and food
930 availability. The length of metamorphosis varies depending on temperature and food availability, but it can
931 last up to 15 days at 16 °C. The abbreviation dph stands for days-post-hatching.

932
933



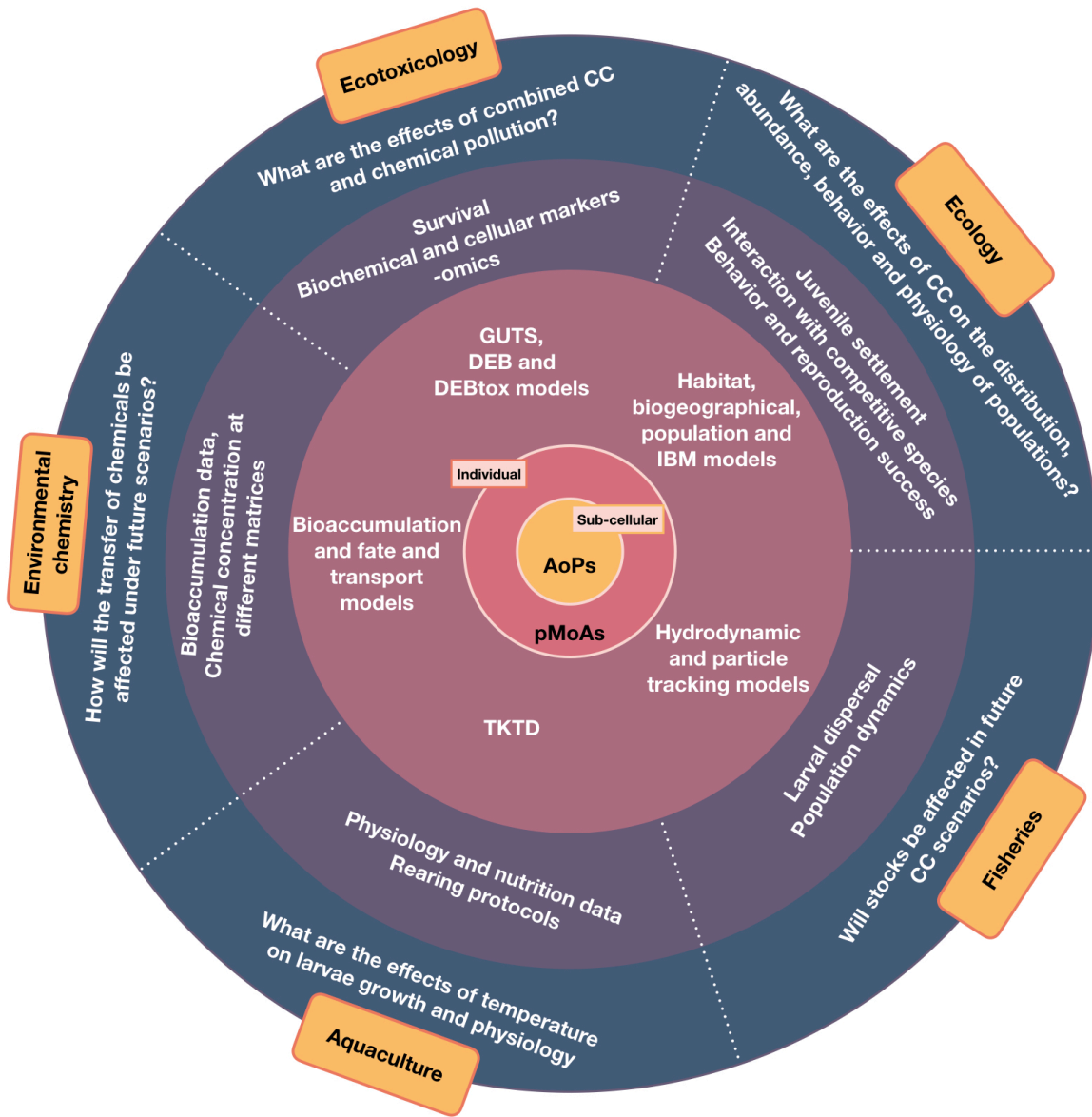
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939 Figure 2. Scheme illustrating some of the research questions related to sole and larval ontogenesis for
 940 different research disciplines. See the text for proposed tools and solutions.



941
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 945

946 Figure 3. Interdisciplinary framework to enhance ecotoxicology and climate change-related research in
 947 fish larvae. Abbreviations stand for Climate Change (CC), GUTS (General Unified Threshold model
 948 of Survival), DEB (Dynamic Energy Budgets), TKTD (Toxico-kinetic toxico-dynamics), IBM
 949 (Individual-Based Model), AoPs (Adverse outcome pathways), and pMoAs (physiological modes of
 950 actions).
 951
 952



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