CHAPTER 22

European eel – an integrated approach to establish eel hatchery technology in Denmark

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Introduction

As catadromous fishes, anguillid eels have a stage-structured life cycle involving continental juvenile stages followed by diverse oceanic reproductive and larval stages. Within the latter, silver eels and larvae (leptocephali) travel thousands of kilometres to complete their life cycle. For the European eel (*Anguilla anguilla*), knowledge about their natural reproduction and spawning habitats in the Sargasso Sea is negligible and insights into the ecophysiology of their offspring is scarce. Therefore, development of hatchery technology involves dedicated experimental work filling gaps in knowledge related to their complex reproductive biology, early life history ecology and physiological requirements throughout ontogeny. Such targeted research, steadily has advanced development of broodstock feeds and assisted reproduction methods for enhanced production of viable offspring as well as egg incubation and larval culture technology leading to first-feeding trials. The next challenge is to identify effective diets and to establish suitable culture conditions for growth of leptocephalus larvae. In Denmark, research on European eel reproductive biology, offspring developmental competence and larval ecophysiology has progressed during the past 10 years, using an integrated, multidisciplinary approach to generate new insights. Here, we summarize and discuss the implications of these results in relation to the establishment of innovative hatchery technology. Moreover, such new insights in eel reproduction and early life history may contribute to understanding the natural life cycle of European eel, thereby assisting management and conservation plans for this critically endangered species.

Background

Aquaculture plays a key role in sustainable market development by virtue of its potential to contribute to increased food production while helping to reduce pressure on natural fish populations as fishery resources (OECD, 2019). Based on its dynamic performance over the last 30 years, and with relatively stable catches from commercial fisheries, it is anticipated that the future growth of the fish and shellfish sector will mainly originate from aquaculture (FAO, 2016). This development relies on closing the life cycle for fish species in captivity, thereby replacing capture-based fish farming (Teletchea, 2015). Here, closed life cycle propagation promotes the success of aquaculture by ensuring high-quality, year-round hatchery production of offspring to sustain commercial farming and facilitate breeding programmes.

The future growth and sustainability of European eel aquaculture extensively depends on closing the life cycle in captivity. Here, the natural stock has declined and glass eel recruitment is at a historically low level, which has rendered the species critically endangered and led to trade restrictions (CITES, 2007; Jacoby and Gollock, 2014). Therefore, as eel farming continues to be capture based, hatchery production of glass eels is required in order to remove constraints on aquaculture production and markets for European eel. In addition, self-sustaining aquaculture production may support management and conservation measures by reducing fishery pressure on continental life history stages, including glass eels, yellow eels and silver eels. However, a background effort of applied research and technology development is imperative for overcoming obstacles related to the complex reproductive control mechanisms and inscrutable early life history stages of eel, which challenge development of suitable breeding and larval culture technologies.

Life cycle challenges for controlled propagation

As catadromous fishes, eels have a stage-structured life history, involving continental juvenile stages, followed by diverse oceanic reproductive, embryonic and larval stages (Figure 22.1a). Within the oceanic stages, silver eels and leptocephalus larvae travel thousands of kilometres to complete their life cycle. While the European eel spawning area is assumed to be the Sargasso Sea, due to presence of early larval stages, knowledge about their natural reproduction and spawning habitat is negligible and insights into the ecophysiology of their egg and early larval stages remain scarce (Tesch, 2003). First insights into the oceanic early life stages originate from leptocephalus larvae captured in the Sargasso Sea and along their migration route (Munk et al., 2010; Schmidt, 1923), followed by the well-known continental stages from glass eels via elvers to yellow eels and silver eels, that is, the spawning migration stage. The dotted line in Figure 22.1a indicates these undisclosed, enigmatic phases of the European eel natural life cycle.

This limitation of information challenges the development of hatchery innovation. As natural conditions cannot be consulted, the development of reliable culture technologies for different life history stages relies on targeted experimental research and analytical techniques, generating the knowledge base needed (Figure 22.1). This involves broodstock management, including dietary requirements, rearing conditions and protocols for gamete production, and fertilization. For the

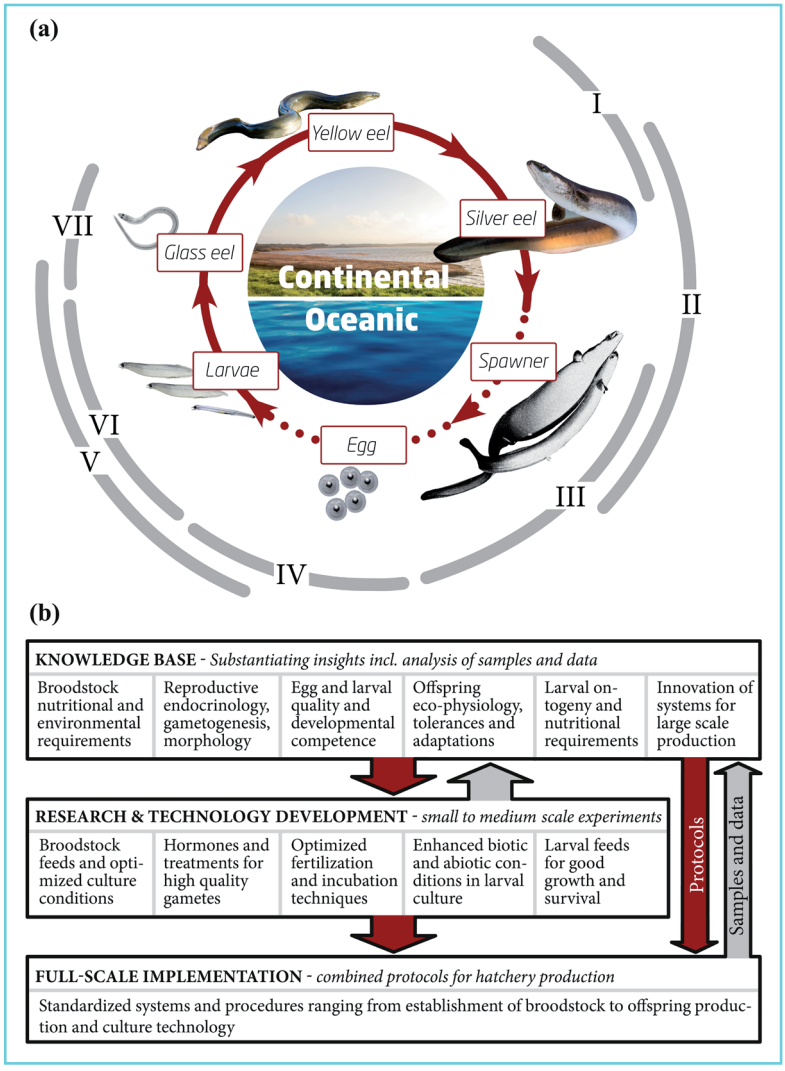

Figure 22.1 (a) Life cycle of European eel including life history stages related to the oceanic and continental phases. The solid line of the inner circle represents the known part in nature, while the dotted line shows the still unknown part. The outer ring illustrates phases of hatchery research and technology development: I Broodstock establishment: dietary requirements and conditioning; II Induction of gametogenesis and broodstock culture technology; III Follicular maturation, ovulation and sperm production; IV Fertilization techniques, egg incubation and embryonic development; V Bio-physical requirements and culture technology; VI Larval ontogeny and dietary requirements; and VII Metamorphosis and glass eel production. (b) Research concept using a systematic incremental approach linking basic science to application and technology development for implementation in full-scale production. Here, a knowledge base covering the reproductive phase and early life history stages is gradually substantiated through controlled experimental tests and dedicated analysis of samples and results. The insight gained is used to develop products, enhance protocols for application development, establish standardized full-scale production, and engineer culture systems. Photographs: Sune Riis Sørensen, DTU Aqua, and for Stage III Spawner, Inge Boëtius and Paul Juhlin, Danish Institute for Fisheries and Marine Research, now DTU Aqua.

offspring, it includes egg incubation and larval culture techniques and technology, insights into larval ontogeny and dietary requirements, and ultimately metamorphosis to obtain glass eels. In this context, a primary challenge for closing the life cycle of anguillid eels lies in an intricate hormonal control at the brain–pituitary level related to silvering and spawning migration, which inhibits sexual maturation and gamete production when inhabiting continental habitats (Dufour et al., 2003, 2005). Here, dopamine exerts an inhibitory control on gonadotropic function, that is, the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH), thereby preventing gametogenesis in both sexes (Jolly et al., 2016; Vidal et al., 2004). In female eels, the follicular maturation and ovulation does not proceed, because signals that hold oocytes in meiotic arrest are not released (Yamauchi, 1990). Consequently, the development of breeding protocols involves assisted reproduction using hormonal treatments to induce gamete development and for females, additional administration of maturation inducing steroid (MIS) (Kagawa et al., 1995; Ohta et al., 1997; Yamauchi, 1990). Another major obstacle is lack of information about the ambient environment of spawners, gametes, and early life history stages, which challenges the development of larval culture technology. Additional challenges refer to the biology and ecology of the leaf-like leptocephalus larvae, unique to the Elopomorpha superorder, where limited knowledge about their natural diet and nutritional requirements hampers the establishment of efficient culture technology throughout the larval stage until their transformation into glass eels (Ayala et al., 2018; Miller et al., 2012; Okamura et al., 2018; Riemann et al., 2010; Tomoda et al., 2018).

In recent decades, intensified research addressing these challenges has substantiated insights throughout ontogeny and led to significant progress in terms of closing the life cycle of anguillid eels. Most advanced is the technology development related to the Japanese eel (*A. japonica*), where glass eel production has been accomplished (Kagawa et al., 2005; Tanaka et al., 2003) and now efficient upscaling for aquaculture is targeted (Masuda et al., 2012; Okamura et al., 2014; Tanaka, 2015). In the beginning of this century, breeding protocols described for the Japanese eel by Ohta et al. (1997) were implemented with modified methodologies for European eels resulting in embryonic development and larval hatch in a few cases (Palstra et al., 2005; Pedersen, 2003, 2004). Further refinement of methodologies and techniques has led to a steady production of viable offspring (Mordenti et al., 2014; Tomkiewicz, 2012) and progress in culture technology towards larval first feeding for this species (Butts et al., 2016; Di Biase et al., 2017; Politis et al., 2018a; Sørensen et al., 2016). Now, a prominent challenge includes identifying effective diets and establishing suitable culture conditions for growth of the leptocephalus larvae of European eel.

To respond to the goal of enabling a closed production cycle in a hatchery context, our research strategy uses a systematic, incremental approach linking basic science to application and technology development, for implementation in full-scale production (Figure 22.1b). Here, multidisciplinary experimental work and advanced analytical methodologies complement existing and otherwise emerging knowledge thereby helping to fill gaps in knowledge throughout their life history. In order to close the life cycle, we have addressed (1) broodstock establishment considering dietary requirements for high gamete quality, (2) assisted reproduction methodologies for induction of gametogenesis, follicular maturation, and gamete production, (3) efficient, standardized fertilization techniques and incubation technology sustaining embryonic development and (4) larval culture techniques and technology with present focus on larval growth and dietary requirements.

Insights advancing viable gamete production

The availability of high-quality broodstock and gametes is a prerequisite for closing the life cycle in captivity of any aquaculture species. The advantages of a closed production cycle include fertilized eggs and viable juveniles being produced on a year-round basis and ability of implementing selective breeding programmes. However, raising broodstock of farmed origin often requires nutritionally enhanced diets differing from on-growing feeds in order to meet the species' requirements for specific fatty acids, amino acids, vitamins and minerals ensuring high-quality gametes and viable offspring production (Izquierdo et al., 2001). In some cases, further acclimatization using environmental cues and/or hormonal induction of the gametogenesis and/or follicular maturation is required (Mylonas and Zohar, 2009; Mylonas et al., 2010). This applies to anguillid eels, where hormonal control of the entire reproductive development is required in order to obtain viable gametes.

Female broodstock origin and nutrition

Researching eel broodstock dietary requirements, in order to advance reproductive performance and offspring quality, is intricate, as eels cease feeding during the silvering process prior to spawning migration and gamete development in nature (Tesch, 2003). Moreover, the nutrient composition of eggs, embryos and yolk-sac larvae from the native spawning area cannot serve as a basis for diet development. Consequently, analyses of gametes produced by wild-caught broodstock using assisted reproduction serve as a benchmark for developing enhanced broodstock feeds for European eel. Studies comparing the egg composition of farmed and wild-caught female broodstock revealed differences in lipid content and fatty acid profiles, in particular arachidonic acid (20:4 n-6, ARA), eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA). For instance, eggs from wild-caught silver eels were characterized by higher levels of ARA and lower levels of EPA in neutral lipids than levels in farmed eels (Heinsbroek et al., 2013; Støttrup et al., 2013). However, the content of essential fatty acids and their ratios in the eggs can be successfully adjusted via tailored feeds. For the European eel, increasing the dietary ARA level for farmed female broodstock to attain ~2–3% of the total fatty acids in the eggs led to higher fertilization rates as well as increased embryonic and larval survival (Kottmann et al., forthcoming; Støttrup et al., 2016). In contrast, studies on Japanese eels showed no marked relationship between egg quality and fatty acid levels of eggs, while fertilization success was negatively correlated with n-6 fatty acids in neutral lipids and with ARA in polar lipids (Furuita et al., 2003). A subsequent study by Furuita et al. (2007) furthermore suggested that a high ratio of n-6 to n-3 fatty acids negatively affected embryogenesis in Japanese eel. Moreover, optimal vitamin C and E levels in combination with highly unsaturated fatty acids (HUFA) improved egg and larval quality (Furuita et al., 2009a, 2009b) and high vitamin C level and vitamin E to HUFA ratio as well as optimized levels of vitamin A and E produced larvae with highest survival rates. These studies indicate that vitamin levels in Japanese eel play a major role defining high-quality eggs in combination with HUFA. In contrast, elevating vitamin C and E levels in broodstock diets for European eel did not affect the lipid peroxidation products in the eggs, indicating that applied vitamin levels were sufficient (Støttrup et

al., 2016). An important finding for broodstock nutrition in European eel is that the feeding period needs to be long in order to change the female broodstock lipid profile prior to onset of maturation (Kottmann et al., forthcoming; Støttrup et al., 2016). At this time, gonads are still undeveloped, and as feeding ceases in readiness for silvering and the spawning migration, the main sources of lipids and protein for gamete development consist of muscle tissue and visceral fat (Støttrup et al., 2013).

Male broodstock origin and nutrition

Compared to female broodstock, dietary influences on male broodstock performance in terms of sperm volume and quality is little investigated, however, a few studies on male eel sexual maturation and viable gamete production exist. For example, Mazzeo et al. (2010) suggested that polyunsaturated fatty acids (PUFA), owing to their increase in testes and high concentration in milt, play an important role in sexual maturation of European eel. We studied the impact of varying levels of the essential dietary fatty acids, ARA, EPA and DHA, on muscle and liver lipid composition prior to induced maturation and quantified the resulting sperm composition and performance (that is, total volume and motility) (Butts et al., 2015). Results showed that optimal feeds for male European eel should contain high levels of DHA for production of high milt volumes, high EPA levels (even >7%) for higher motility, medium levels of ARA, while the n–3/n–6 ratio should be close to or >2. Likewise, Baeza et al. (2015a) found that dietary fatty acid composition impacted characteristics of sperm and milt production, while Baeza et al. (2015b) highlighted the important role of lipid metabolism affecting spermatogenesis. Moreover, the impact of male eel diets varying in amino acid composition were investigated (Butts et al., forthcoming). Here, diets supplemented with amino acids did not influence morphometric indices (body and organ-somatic indices: liver, testes, gonadosomatic index GSI) and testes morphology, while certain amino acids positively affected spermiation and sperm motility. Taken together, this new information may prove useful for developing male broodstock diets to improve sperm quality and subsequently, offspring production for this species.

While analyses of the composition of milt from wild-caught male silver eels have not yet been performed, fertilization success and offspring developmental competence have been compared among male broodstock, that is, wild-caught silver eels and farmed male broodstock. The comparison showed that male origin did not significantly affect early life history traits, that is, fertilization success, hatching success and larval deformities (Benini et al., 2018), despite some variability. Although male impact was less pronounced than female, the study demonstrated that some parental combination were highly successful producing viable offspring, while other combinations yielded significantly higher proportions of larvae with deformities. This indicates that male–female compatibility could be an issue of potential interest when defining the pool of males to be crossed with the female broodstock in order to improve the quality of offspring. Here, available genomic resources targeted for eel (Henkel et al., 2012; Jansen et al., 2017) can provide future opportunities creating tools and detecting genetic markers for the development of tests to predict female-male compatibility reducing the risk of deformities.

Gonadotropic function and induced gamete development

The complex mechanisms controlling anguillid eel gametogenesis (Dufour et al., 2003, 2005) are caused by dopaminergic control inhibiting the gonadotropic function, that is, the production and release of FSH and LH from the pituitary gland, while inhabiting continental habitats (Jolly et al., 2016; Vidal et al., 2004). This inhibition must be released, when the eels approach or reach the spawning area, but the cues and signalling pathways remain uncertain in spite of dedicated endocrine studies of the European and Japanese eel. Consequently, the development of breeding protocols presently involves assisted reproduction using hormonal treatments to induce gamete development (Mylonas et al., 2010).

As in all vertebrates, the brain–pituitary axis controls gonadal maturation, thus regulating the onset of reproduction through external and internal signals initiated at the brain level by activating gonadotropin-releasing hormone (GnRH). In response to GnRH stimulation, gonadotropic cells produce and release the gonadotropins, FSH and LH, which stimulate gonadal functions, that is, steroidogenesis and gametogenesis (Mylonas and Zohar, 2009; Rousseau et al., 2009). Anguillid eels in continental waters, however, remain juvenile influenced by a strong dopaminergic inhibitory effect on GnRH-induced gonadotropin synthesis and release (Dufour et al., 1988, 2003, 2005; Jolly et al., 2016; Vidal et al., 2004). Since the beginning of this century, studies of these intricate endocrine regulation mechanisms at the molecular level have significantly substantiated insights into function of the brain–pituitary–gonadal axis in European eel (Ager-Wick et al., 2013; Campo et al., 2018; Lafont et al., 2016; Minegishi et al., 2012; Morini et al., 2015; Pasquier et al., 2012; Rojo-Bartolomé et al., 2017).

The assembly and annotation of the European and Japanese eel genomes has accelerated acquisition of new knowledge, thereby providing a source of data to discover and identify new genes, characterize gene expression and identify genetic markers (Coppe et al., 2010; Henkel et al., 2012; Jansen et al., 2017). While continued endocrine research has the potential to develop novel methodologies that can release the dopaminergic inhibition, the present techniques for induction of gametogenesis typically rely on provision of gonadotropins using pituitary extracts (PE) from salmon or carp for females, while treatment for males includes human chorionic gonadotropin (hCG) (Mylonas and Zohar, 2009) or eel gonadotropins produced using recombinant technology (Kobayashi et al., 2010; Peñaranda et al., 2018).

Follicular development, maturation and oocyte quality

For female eel, the first protocols for induction of vitellogenesis that produced viable eggs used biweekly injection of carp pituitary extract (CPE) for the European eel (Bezdenezhnykh et al., 1983; Prokhorchik, 1986) and salmon pituitary extract (SPE) for the Japanese eel (Kagawa et al., 1995, 2003; Ohta et al., 1997). Subsequent experimental research on European eel typically has applied weekly injection, while varying in dosage and pituitary extract, that is, SPE (Da Silva et al., 2016; Pedersen, 2003; Tomkiewicz, 2012) and CPE (Mordenti et al., 2013; Palstra et al., 2005; Pérez et al., 2011), while research on Japanese eel primarily applies a constant dose of SPE (Okamura et al., 2014; Tanaka, 2015). During a period of 12–20 weeks of PE treatment at around 20 mg kg^{-1}, the follicles develop and ovaries increase in size (Figure 22.2a). At lower or variable dose, the developmental period is longer, however, oocyte stages and ovarian

Figure 22.2 European eel broodstock reared in custom-designed recirculating aquaculture systems at the EEL-HATCH facility. Column (a) female broodstock handling, hormonal induction of ovarian development, and anaesthetized female in final maturation stage. Column (b) induced maturation in male broodstock, developed testis in abdomen, and milt production. Photographs: Sune Riis Sørensen, DTU Aqua.

development are similar (Da Silva et al., 2016; Tomkiewicz and Jarlbæk, 2008). However, responsiveness to treatment varies, which for European eel has led to tests of supplementary stimulation of sexual maturation and ovarian development including environmental conditions, for example, temperature, light and swimming (Mazzeo et al., 2014; Mordenti et al., 2012; Palstra et al., 2009; Pérez et al., 2011) as well as administration of androgens (Di Biase et al., 2017). For the Japanese eel and New Zealand freshwater eels, treatment using androgens has shown a

positive effect on ovarian synchronization (Lokman et al., 2015; Matsubara et al., 2003), while feminization using estradiol is well established in propagation of Japanese eel, promoting growth, responsiveness, and ovarian development (Okamura et al., 2014; Tanaka, 2015).

While pituitary extracts enable oocyte growth, follicular maturation and ovulation require administration of a MIS, often preceded by a priming dose of pituitary extract (Ohta et al., 1997; Yamauchi, 1990). While an increase in female body weight can be used as an initial indicator, oocyte appearance, staging development according to germinal vesicle migration, coalesces of oil droplets and germinal vesicle breakdown can be used as indicators for optimal timing of MIS provision (Kagawa et al., 1995; Palstra et al., 2005). Further optimization of indicators include estimation of oocyte size and oil droplet diameter in preparation to priming and provision of MIS (da Silva et al., 2018a, 2018b; Unuma et al., 2011). After oocyte maturation, ovulation takes place and meiosis is reactivated and completed upon fertilization.

The induction of spawning must be precisely synchronized with the acquisition of follicular maturational and ovulatory competence (Mylonas and Zohar, 2009). Fish that are treated too early or too late will either not ovulate/spawn or produce eggs of low quality or poor fertilization capacity. Among various factors interfering with the induction of follicular maturation and subsequent gamete quality, is the heterogeneity in the development of the oocytes in the ovary. This asynchronous or group-synchronous development indicates batch spawning, which agrees with the ability of female eels to spawn several times and produce fertilizable eggs (Bezdenezhnykh and Prokhorchik, 1984; Da Silva et al., 2018a, b; Lokman and Young, 2000; Pedersen, 2003; Tomkiewicz and Jarlbæk, 2008). Moreover, the batch fecundity is high ranging from 0.7 to 2.6 million eggs per female (Boëtius and Boëtius, 1980), averaging approximately 80,000 per 100 g initial body weight with approximately the same batch size in the first and second batches (Tomkiewicz et al., 2013), which is promising for hatchery production.

Milt production and sperm quality

For male eel, spermatogenesis can be induced by administration of one or two injections of hCG (Miura et al., 1991; Ohta et al., 1996). However, in order to sustain spermatogenesis, spermiogenesis, and spermiation, weekly hormonal treatment with hCG or gonadotropins is common practice in the reproduction of European eel. In males that are given weekly hCG injections, spermiation starts in the first males around the fifth week of treatment (Asturiano et al., 2005, 2006; Pérez et al., 2000). Temperature has been shown to profoundly affect the progression of spermiation in European eel, reaching spermatogenesis earlier at higher temperatures (Baeza et al., 2014; Peñaranda et al., 2016). Figure 22.2b shows testes development and spermiation in European eel. Treatment schemes vary in dosage, but generally yield high-quality sperm. Here, stripping 24 hours after a priming injection shows optimal sperm motility (Asturiano et al., 2005). Furthermore, recombinant DNA technology has been used to produce homologous reFSH and reLH for Japanese eel (Kobayashi et al., 2010), as well as for European eel (Herranz-Jusdado et al., 2019; Peñaranda et al., 2018). These recombinant gonadotropins were shown to stimulate gonadotropin receptors in vitro and in vivo and appeared generally efficient to induce spermiation in European eel. As for female eels, different environmental scenarios to enhance spermatogenesis have been tested to optimize treatment, for example, temperature in combination with recombinant hCG (Gallego et al., 2012). The duration of spermatogenesis and the

spermiation period depends on the treatment (that is, hormone dosage and injection frequency) (Asturiano et al., 2005; Peñaranda et al., 2010), with a continuous generation of spermatocytes and spermatozoa for several months as long as hCG is administered (Tomkiewicz et al., 2011). Here, the composition of different germ cell stages in the testis tissue remained constant with limited fluctuations for a period of 18 weeks until the end of the experiment. Such a reproductive strategy would match a batch spawning strategy of female eel.

Eel sperm quality is assessed widely using motility and velocity, by means of computer assisted analysis, CASA (Gallego et al., 2018; Mylonas et al., 2017; Vílchez et al., 2016, 2017), which serves as an objective measure of sperm swimming patterns. Alternatively, this can be evaluated by light microscopy, where motility is characterized using an arbitrary scale where 0: represents no motile sperm: I <25%; II 25–50%; III 50–75%; IV 75–90%; and V 90–100% of motile spermatozoa (Pérez et al., 2009). Using this method, sperm motility values of >IV are typically obtained and used for experimentation (Butts et al., 2014; Sørensen et al., 2013, 2015; Tomkiewicz, 2012).

Additionally, sperm density is quantified in order to standardize the sperm to egg ratio. Here, sperm cells are counted using a Neubauer hemocytometer, a method that is classified by the World Health Organization as the 'gold standard' for sperm density quantification (WHO, 1999). Faster counting methods (Fauvel et al., 2010), which correlate with Neubauer hemocytometer counts, have also been tested for European eel (Sørensen et al., 2013). Here, spermatocrit, CASA, and flow cytometry were positively correlated to haemocytometer counts. Additionally, it was shown that European eel sperm could be rapidly and accurately measured using a spectrophotometer (Butts et al., 2014).

Fertilization techniques, egg incubation and embryonic development

In common with most fishes, eels are external fertilizers, where gametes are released into the aquatic environment. As catadromous fishes, they naturally spawn in the marine environment, where the eggs are activated under hyper-osmotic conditions (Browne et al., 2015; Cosson et al., 2008). In hatcheries, common fertilization techniques include tank spawning with subsequent collection of fertilized eggs or manual stripping of gametes followed by in vitro fertilization (Mylonas and Zohar, 2009; Mylonas et al., 2010). Both procedures have been applied to European eel resulting in fertilized egg and larval production with increasing success over time (Butts et al., 2014; Di Biase et al., 2016; Mordenti et al., 2014; Palstra et al., 2005; Pedersen, 2003, 2004; Tomkiewicz, 2012). We utilize manual stripping followed by in vitro fertilization as these procedures can be controlled and standardized, thereby reducing noise in experimental results, allowing accurate determination of the floating egg layer and fertilization success (Butts et al., 2014; Sørensen et al., 2015; Tomkiewicz, 2012).

The in vitro fertilization protocol aims to optimize the sperm to egg ratio, considering sperm quality (motility, density) and post stripping ageing of eggs (Butts et al., 2014). An immobilizing medium mimicking seminal plasma (Asturiano et al., 2004; Peñaranda et al., 2010) is used for dilution and maintaining sperm cells in a quiescent state until in vitro fertilization (Butts et al., 2014). The pre-diluted milt enables a standardized sperm to egg ratio when mixing the gametes,

while addition of artificial seawater at a volume dependent ratio enables a reproducible osmotic environment during gamete activation. Moreover, timing of fertilization has proven crucial, as egg quality deteriorates rapidly after ovulation (Butts et al., 2014), which also has been shown for eggs of Japanese eel (Ohta et al., 1996). After fertilization, buoyant eggs are incubated in artificial or treated natural seawater (Sørensen et al., 2015). This in vitro fertilization technique reliably yields fertilization rates up to 80–100% (Butts et al., 2014).

During the first hours after fertilization, a perivitelline space (PVS) forms as the chorion separates from the plasma membrane and the egg swells thereby enhancing the egg buoyancy (Figure 22.3) (Sørensen et al., 2016). Embryonic developmental success positively relates to the degree of swelling with failure of PVS development leading to no hatch (Tomkiewicz and Jarlbæk, 2008). During embryonic development, as well as in the fertilization procedure, both salt type and salinity significantly influence egg swelling and subsequent hatch (Sørensen et al., 2015). For instance, the use of treated natural sea water consistently produces larger eggs with diameters ranging up to 1.6–1.8 mm, which is comparable to wild-caught Japanese eel eggs (Tsukamoto et al., 2011; Yoshinaga et al., 2011). Buoyant fertilized eggs typically contain several smaller oil droplets that slowly fuse to form one large oil droplet (Figure 22.3). At 20°C, the first cell cleavages are observed at ~1 hour post fertilization (hpf), while the 16-cell stage is reached within ~4 hpf (Sørensen et al., 2016). At this time, fertilization success can be accurately assessed (Butts et al., 2014; Sørensen et al., 2015). The first somites become visible at ~24 hpf. At ~32 hpf, the embryo evolves two noticeable eye capsules and several somites. Thereafter, the tail bud forms, the yolk-sac becomes ellipsoid and hatching occurs at ~48 hpf (Sørensen et al., 2016). Rearing temperature significantly affects the duration of embryonic development and survival with detrimental effects above 22°C (Politis et al., 2017) as described below.

Embryonic development is influenced by gamete quality involving nutrition and cytoplasmatic factors, such as maternal RNA and activation of the embryo's genes (Lubzens et al., 2017). Differences in gamete quality among wild-caught and farmed broodstock receive much attention in aquaculture breeding and are of consequence also for eel, for example, in terms of essential fatty acids (Kottmann et al., forthcoming; Støttrup et al., 2013, 2016). While male broodstock origin, either farmed or wild-caught silver eels, did not affect fertilization and hatching success in a study by Benini et al. (2018), Kottmann et al. (forthcoming) found that enhancing essential fatty acid contents in eggs from farmed broodstock significantly improved fertilization success and embryonic survival, although survival and hatching success was lower than for wild-caught female broodstock. Here, the critical period for embryonic survival appeared to be between 8 and 16 hpf. This concurs with the timing of the expression of specific mRNA transcripts during embryonic development, that is, the transition from reliance of maternal mRNA to expression of the embryo's own genome (Lubzens et al., 2017). A study by Rozenfeld et al. (2016) showed that egg batches of farmed female European eel with either high or low hatching success did not differ in mRNA abundance between groups at 0 and 5 hpf, while they differed significantly at 30 hpf. At this point, embryos of the high hatch group showed higher abundance of five genes analysed (CPT1a, CPT1b, β-Tubulin, PHB2, and PIGF5 transcripts) than the low hatch group, suggesting that a general up-regulation of embryonic genes after the mid-blastula transition is needed to sustain embryonic development and hatching success.

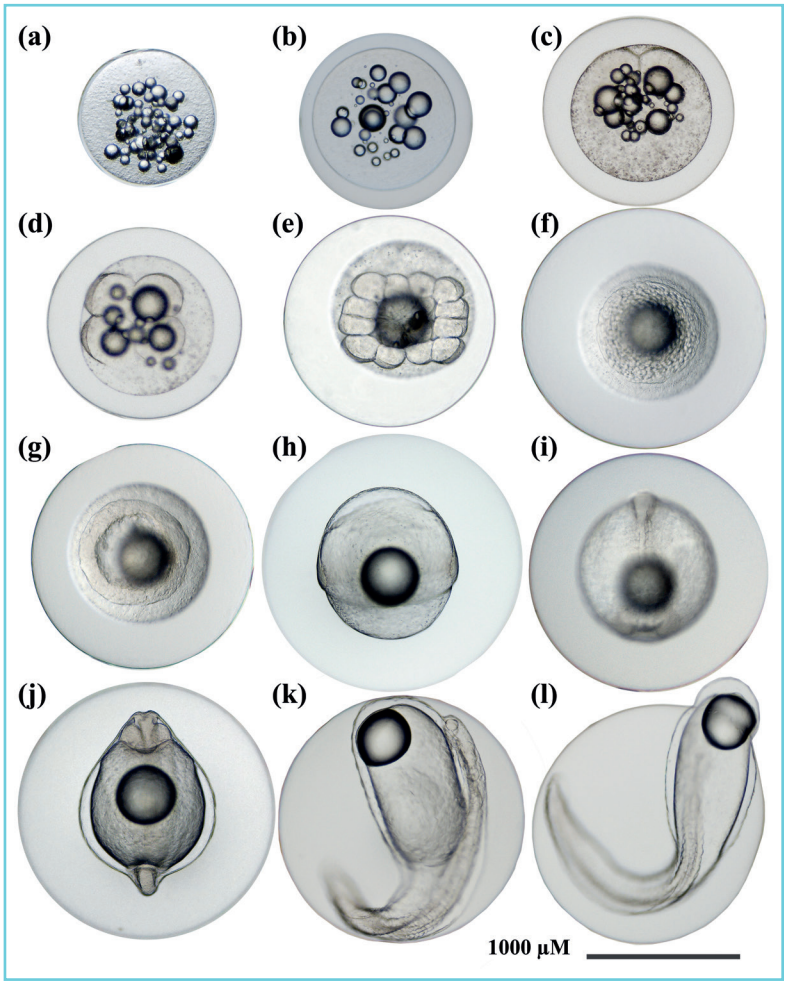

Figure 22.3 Embryonic development of European eel at 20°C. (a) Egg 5 min after extrusion by stripping, chorion not distended, multiple smaller oil droplets. (b) 0 h 25 min after fertilization, chorion has distended slightly. (c) 0 h 50 min, first cleavage and partial fusion of oil droplets. (d) 1 h 45 min, second cell cleavage and variable degree of oil droplet fusion (occasional full fusion of droplets at this stage). (e) 3 h, 16-cell stage and regular full fusion of oil droplet. (f) 8 h 30 min, early blastula stage, 100% fused oil droplets. (g) 13 h 0 min, germ ring formed. (h) 15 h, 1/2 epiboly. (i) 22 h 30 min, blastopore closure and first somites. (j) 32 h 15 min, embryo formed, eye vesicle visible and somites along most of the embryo, tail bud positioned downwards in figure. (k) 40 h, embryo has clearly distinguishable head, eyes and somites (> 20), occasional movements and onset of heart beat. (l) 45–48 h hatching starts by intense wriggling and repeated punch of head tip against chorion wall. Scale bar: 1 mm. Source: Sørensen et al. (2016).

Ontogeny of European eel pre-leptocephalus larvae

Russian scientists were the first to obtain and describe European eel embryos and larvae living up to 3.5 days (Bezdenezhnykh et al., 1983; Prokhorchik, 1986). Later, Pedersen (2004) reported on

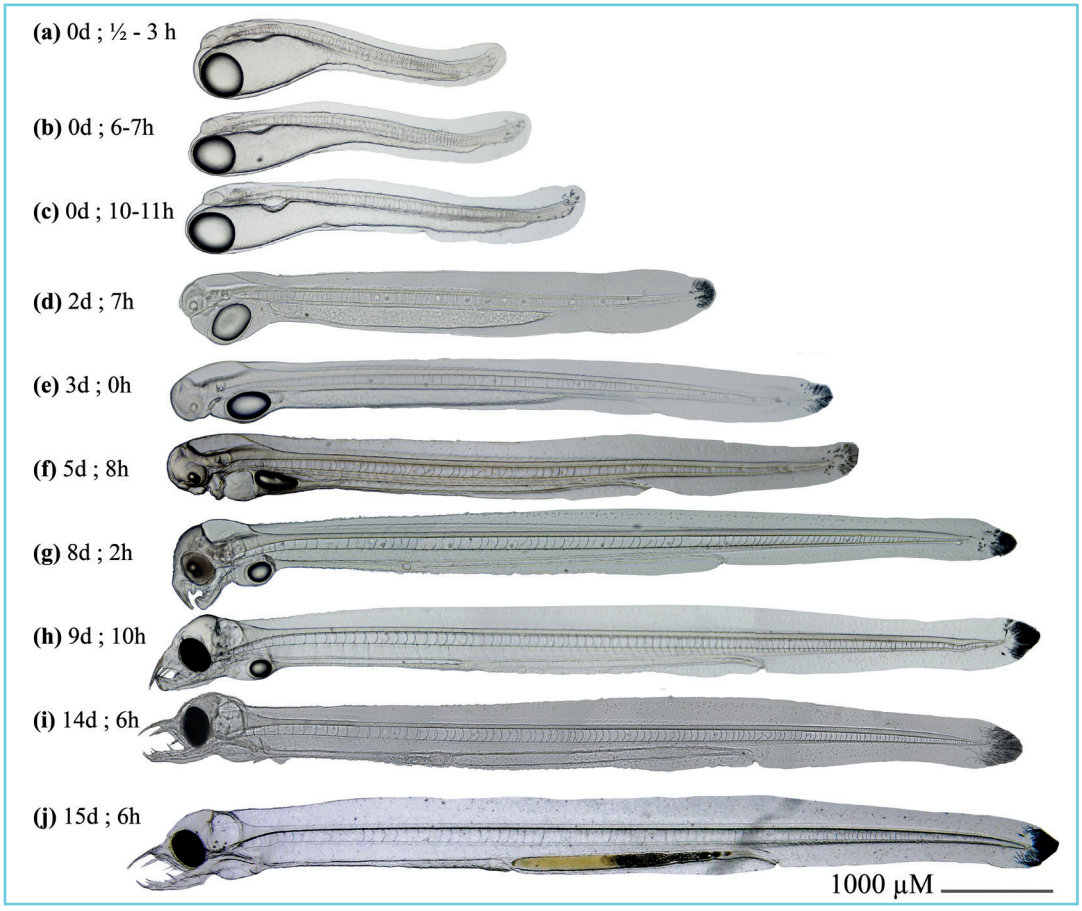

Figure 22.4 Development of European eel larvae from newly hatched to first-feeding stage [in hours post hatch (hph) and days post hatch (dph)] at 20°C. (a) 1/2–3 hph, larva still undeveloped, large yolk-sac with significant oil globule. (b) 6–7 hph, eye primordium apparent. (c) 10–11 hph prolonged tail, abdomen extends into yolk-sac, caudal pigments develop. (d) 2 dph, larval head protrudes anteriorly of yolk-sac, broad primordial fin apparent. (e) 3 dph, heart and brain clearly visible. (f) 5 dph, mouth starts to develop and eyes become pigmented. (g) 7 dph, upper and lower jaw distinguishable. (h) 9 dph, head angle changes, mouth points forward. (i) 14 dph, eyes clearly pigmented, gastro-intestinal tract enlarged, first-feeding stage. (j) 15 dph, first-feeding larva with food (rotifer paste) in the intestine. Scale bar: 1 mm. Adapted from Sørensen et al. (2016).

the chronology of embryological stages of European eel, while Sørensen et al. (2016) described early ontogeny and larval development throughout the yolk-sac stage, lasting 2 weeks when reared at 20°C. These results based on enhanced embryonic survival and mass hatching in the EU-FP7 project PRO-EEL outlined and illustrated for the first time the development throughout the pre-leptocephalus stage (Figure 22.4). At hatch, European eel larvae are little developed with a prominent yolk-sac encompassing a large oil droplet. During the first days, the larvae allocate resources to increase in length, develop optical capsules, a visible hindbrain and pericardium, as well as a wide primordial fin with a well-defined, rounded and pigmented tail. Towards

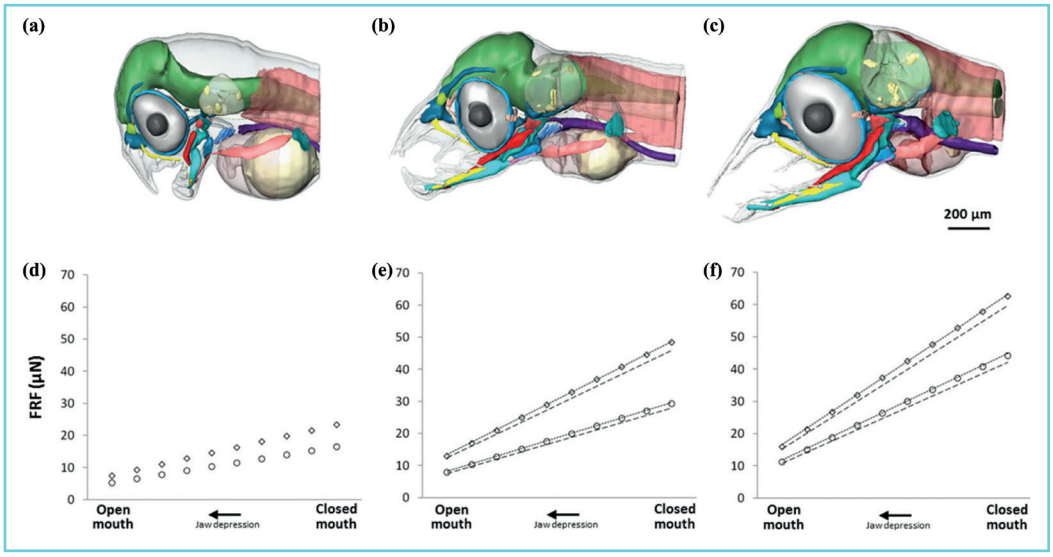

Figure 22.5 European eel larval head morphology: left lateral view on original 3D reconstructions. (a) 9 days post fertilization (dpf), (b) 12 dpf and (c) 15 dpf larvae of European eel. Cartilage is represented in blue, bone in yellow and muscles in red/pink. Graphical representation of food reaction forces (FRFs) for the (d) 9 dpf, (e) 12 dpf and (f) 15 dpf larvae. Circles and diamonds represent the force estimations at the level of first and third dentary teeth, respectively. All forces are calculated over a range of depression angles (15°, 30° and 40°) for the three investigated larval stages (9, 12 and 15 dpf) between a completely opened and closed mouth of the larvae. Modified from Bouilliart et al. (2015).

5 days post hatch (dph), the oral opening is observed as a small channel posterior to the eyes. At ~8 dph, the eyes start to pigment, upper and lower jaws emerge, and developing teeth appear. Towards 14 dph, yolk reserves appear utilized at 20°C (Sørensen et al., 2016) (Figure 22.4). During development, the angle between the head and trunk increases to enable the jaws pointing forward, finally resulting in the formation of the feeding apparatus with the characteristic, protruding teeth (Figure 22.5) (Bouilliart et al., 2015).

Early life history has been outlined for other anguillid eels including the Japanese eel (Ahn et al., 2012; Okamura et al., 2007; Yamamoto, 1981), American eel (*A. rostrata*) (Oliveira and Hable, 2010) as well as for hybrids of *A. dieffenbachii* ♀ × *A. australis* ♂ (Lokman and Young, 2000), *A. anguilla* and *A. japonica* (Okamura et al., 2004), and *A. australis* ♀ × *anguilla* ♂ (Burgerhout et al., 2011). Common to these species and their hybrids is that the larvae hatch at a relatively undifferentiated state, for example, little developed head morphology and digestive system compared to larvae of model species such as the zebrafish, *Danio rerio* (Kimmel et al., 1995). A characteristic that also applies to other members of Anguilliformes such as Japanese conger eel (*Conger myriaster*), daggertooth pike conger (*Muraenesox cinereus*) and the family of Sawtooth eels (*Serrivomeridae*) (Miller, 2009). Owing to species-specific temperature optima and rearing temperatures in embryonic and larval culture, the duration of the development phases differ. Thus, comparing morphological stages using degree day units (°D) as a measure of physiological time, starting at fertilization (Sørensen et al., 2016) shows that hatching time occurs

at 38.3–40 °D for *A. anguilla*, 26.7–35.8 °D for *A. rostrata*, 37.6–39.4 °D for *A. australis* ♀ × *A. anguilla* ♂; 32.6–40.7 °D for *A. dieffenbachii/australis*, and 39.4–42.2 °D for *A. japonica*. Likewise, eye pigmentation in yolk-sac larvae of *A. anguilla* and hybrids *A. australis* ♀ × *A. anguilla* ♂ occurs at 220 °D, while at 196 °D for *A. japonica*.

Bio-physical requirements and culture technology

In almost all species, mortality is highest during early life, where subtle differences in survivorship can cause large variability in offspring production (Houde, 2008). In fish, the bio-physical conditions in the ambient environment during early life history stages are vital for their survival. Similarly, in hatchery practice, the bio-physical requirements need to be determined and suitable rearing conditions established (Conceição and Tandler, 2018). The choice of species-specific rearing conditions and prey/diets used for larval culture is typically based on knowledge originating from ambient conditions and food availability in their natural environment, supplemented by insights obtained from experiments. However, in the case of European eel where the natural environmental regimes of embryos and yolk-sac larvae remain unclear, focus is on assessing tolerances and optima using experimental studies of captive-produced offspring (Figure 22.1). Our research has focused on the impact of microbial activity, temperature, light, and salinity. Here, the goal has been to identify biotic and abiotic factors influencing larval survival and welfare in order to improve rearing conditions and increase numbers of survivors for first-feeding larval production, exploring first-feeding regimes enabling a successful transition to exogenous feeding.

Microbial activity

A major factor influencing hatchery production of offspring is the species' microbial sensitivity (Attramadal et al., 2012; Vadstein et al., 2013, 2018) that drives mortality and suboptimal development during early life stages (Skjermo and Vadstein, 1999). During the embryonic stage, the chorion provides protection against the external microbial environment (Finn, 2007; Laale, 1980). However, microbial activity on the egg tends to impede embryonic development (Salvesen and Vadstein, 1995) possibly caused by insufficient gas exchange and waste product secretion across the chorion (Salvesen et al., 1997). Sørensen et al. (2014) studied the effects of microbial interference on embryonic survival and larval hatch in European eel using different levels of microbial control and egg disinfection treatments. Here, survival was related to the efficiency of antibiotics to impede microbial activity, with low microbial interference resulting in increased embryonic survival, hatching success, and larval survival, thereby extending larval longevity. These results emphasize the importance of a low-level, stable microbial community in eel offspring culture (Sørensen et al., 2014).

Temperature

Identifying temperature regimes is essential for optimization of rearing conditions in larval culture, as temperature controls fundamental biochemical processes in living organisms thereby influencing developmental rates and survival of fish larvae (O'Connor et al., 2007). In the closely

related Japanese eel, early ontogeny was found to be strongly influenced by temperature, with optimum thermal rearing conditions (~25°C) similar to those found in their recently identified natural spawning area (Ahn et al., 2012; Tsukamoto et al., 2011). In this regard, European eel larvae originate in the Sargasso Sea, which is a water mass characterized by a relatively constant salinity of 36.5 practical salinity units (PSU), a seasonal thermocline (~18°C) at a depth of ~300 m, and an upper warm water layer of 20–28°C (Castonguay and McCleave 1987; Worthington, 1959). In order to assess the optimal rearing temperature for European eel larvae, we selected a thermal regime ranging from 16 to 24°C (Politis et al., 2017), representing the thermal environment occurring between 600 m depth and close to the surface of the Sargasso Sea.

Temperature influenced all traits investigated such as time to hatch, hatching success, larval morphometrics and deformities, providing important insights on thermal phenotypic sensitivity. Moreover, this included the underlying gene expression of the molecular mechanisms relating to stress (heat shock proteins) as well as growth and development (growth hormone and insulin-like growth factors) (Politis et al., 2017). As no larvae hatched at 24°C, temperatures beyond this point were considered to be deleterious. Moreover, 22°C signalled the upper and 16°C the lower thermal tolerance limits, as ~90 and ~50% of larvae were deformed when reared at these temperatures, respectively. An optimal intermediate (18–20°C) thermal environment was identified with efficient growth and fewer deformities as well as high growth hormone expression (indicating better growth) and low heat shock protein expression (indicating lower stress).

Similarly, the expression of genes relating to the thyroid hormone (TH) signalling pathway, known to regulate growth, development, and metabolism in vertebrates, was followed and the sensitivity of this mechanism to temperature elucidated. Generally, THs are key regulators of growth, development, and metabolism in vertebrates (Power et al., 2001; Tata, 2006; Warner and Mittag, 2012), while evidence increases that they play important roles during early life development and metamorphosis in fish (Campinho et al., 2010; Infante et al., 2008; Marchand et al., 2004; Walpita et al., 2007). TH is produced in the thyroid gland (or thyroid follicles) mainly as T4 (thyroxine), which is then metabolized by deiodinase (DIO) enzymes in peripheral tissues, whereas their action is mostly exerted by binding to a specific nuclear thyroid hormone receptor (THR). The Japanese conger eel was previously chosen as a model species to investigate the TH role on development and metamorphosis of Anguilliformes (Kawakami et al., 2003), while a subsequent investigation regarding *thrs* showed differentially regulated expression during development and metamorphosis in Japanese eel (Kawakami et al., 2013). In European eel larvae, Politis et al. (2018b) identified three deiodinases (*dio1*, *dio2* and *dio3*) as well as two isoforms of *thra* (*thraA* and *thraB*) and two isoforms of *thrβ* (*thrβA* and *thrβB*), which showed high similarity to other mammalian, bird, amphibian or fish species, but highest similarity to the closely related Japanese eel.

Generally, regarding gene expression, the warmer the temperature the earlier the expression response was observed for a specific target gene. Moreover, in real time, the expression profiles appeared very similar and only shifted with temperature, while in developmental time, expression of all genes differed across selected developmental stages, such as hatching, during teeth formation or at first feeding. As such, understanding the biological responses, limits and adaptabilities or preferences to extrinsic environmental factors, such as temperature, provides enhanced knowledge for the optimization of rearing techniques.

Light

Light influences development and behaviour during early life history of fish (Downing and Litvak, 2002). Most animals possess well-developed photoreceptors and neuronal networks to organize, identify, and interpret sensory information through the perception of light in order to react to environmental changes and adapt accordingly (Meissl et al., 1986). Before hatch, the retinae of most teleost larvae consist of only cone photoreceptors and development is not completed until metamorphosis (Blaxter and Staines, 1970). Primarily under the control of rods, scotopic vision seems impaired in early life stages (Powers and Raymond, 1990), but early developed pineal photoreceptors are generally well-suited for detecting luminance changes (Kusmic et al., 1992; Meissl and Ekström, 1988). We performed a study on the reaction of European eel embryos and larvae to photoperiod, light intensity and wavelength (Politis et al., 2014). Here, embryonic survival was highest when embryos were reared under a 12:12 hour light/dark photoperiod and low-intensity light regime, while low-intensity light also led to the highest hatching success. However, timing and development of colour sensitivity is not clearly understood, as eel embryos were not affected by spectral composition, probably due to the perceptive mechanism and corresponding receptors not being fully developed at this stage. On the contrary, European eel larvae were sensitive to all physical parameters of light. Here, larvae reared in low-intensity light had higher survival than those reared in high-intensity light, larvae reared in a 12:12 hour photoperiod had higher survival than those reared in constant light, and larvae reared in red light had higher survival than those reared in green or white light.

Furthermore, a high-degree of offspring survived and developed when reared under continuous darkness, similar to the best intensity-photoperiod-spectral composition light regime. As such, rearing in darkness or under a red, low-intensity and 12 hour photoperiod light regime seems to be of benefit for European eel yolk-sac stage pre-leptocephali (Politis et al., 2014). However, the incidence of first feeding and percentage of gut fullness increased with light intensity (Butts et al., 2016), clearly indicating a shift in light sensitivity and preference at later developmental stages.

Salinity

Most fish species are hyper-osmotic in freshwater, where plasma osmolality is higher than the environment and hypo-osmotic in seawater, where plasma osmolality is lower than the environment (Marshall and Grosell, 2005). Thus, in freshwater they need to actively take up ions to counteract the diffusive ion loss and osmotic water gain, while in seawater they need to maintain osmotic balance through a desalting process to counteract osmotic water loss (Evans, 2008). Eels are euryhaline species that have adapted to cope with both hyper- and hypo-osmotic environments, likely due to regular salinity changes in their habitats and migrations between freshwater and marine environments at different developmental stages (Tesch, 2003). Eel offspring naturally occur in a hyper-osmotic environment in the ocean (Castonguay and McCleave, 1987), and interestingly, Okamura et al. (2009) showed that reducing salinity during early life history rearing under culture conditions, results in better growth and survival of Japanese eel larvae.

We tested this proposition also for European eel by rearing yolk-sac larvae under different salinity reduction scenarios with the goal to ease osmotic 'stress' by adjusting salinity towards

iso-osmotic levels (Politis et al., 2018c). The results showed that gradually reducing salinity towards iso-osmotic conditions, that is, from 36 to 18 PSU, improved growth and lead to a four-fold increase in survival. Thus, the larvae were able to keep energy metabolism at stable levels judged from the related gene expression (*atp6*, *cox1*). Moreover, the lower salinity allowed for an energy surplus by reducing metabolic demands associated with osmoregulation and stress revealed by lower expression of $Na^+K^+2Cl^-$ *cotransporters, aquaporins* and *heat shock proteins*. This likely facilitated efficient use of energy for conversion of endogenous resources into somatic development, reflecting improved survival, biometry, and growth efficiency. Additionally, salinity reduction decreased the amount of severe deformities such as spinal curvature and emaciation but tended to induce an oedematous state of the larval heart. Here, the most balanced survival/deformity ratio was obtained when salinity was decreased from 3 dph and onwards at 2 PSU per day.

Ontogeny of the immune system and feeding apparatus

Overall, the identification of bio-physical requirements and regulated underlying molecular processes during the embryonic and yolk-sac larval stage has advanced our understanding on larval physiology. Here, the new insights gained by morphologically and molecularly defined physiological tolerance limits and preferences have gradually improved protocols for incubation and pre-leptocephalus larval culture technology and resulted in high numbers of healthy larvae capable of reaching the first-feeding stages. This increase in larval survivorship, allowed us to initiate comprehensive experimental studies on the development of the larval immune system and feeding capabilities.

Larval immunity

Teleost fish were one of the first phylogenetic groups to develop an immune system that possesses both the innate and adaptive arm of the immune response, characteristic to higher vertebrates (Uribe et al., 2011). However, evidence has accumulated that newly hatched fish larvae are particularly sensitive to pathogens as their immune system is not fully developed throughout the first weeks of life. During this period, they solely rely on the innate arm of the immune system, while exposure to pathogens intensifies due to hatching, mouth opening and first feeding (Ferraresso et al., 2016; Magnadóttir et al., 2004; Vadstein et al., 2013). Moreover, temperature is a fundamental modulator of the immune system of fish (Bowden, 2008) and has been shown to affect immunity during fish early life history (Dios et al., 2010). Therefore, in the light of our previous results, showing notable microbial sensitivity and temperature influence (Politis et al., 2017; Sørensen et al., 2014), we studied the molecular ontogeny of the immune system considering temperature as an immunomodulatory factor (Miest et al., 2019).

The molecular ontogeny of both the innate and the adaptive arm of the immune system during yolk-sac larval development was tested experimentally at different temperatures to evaluate the sensitivity of the early larval stages to microbial interference (Miest et al., 2019). The expression patterns of 11 immune genes were analysed throughout development, from hatch until the first-feeding stage. Interactions with temperature and immune competency were

reduced at the lower end of the thermal spectrum (16°C), while close to the upper thermal limit (22°C) larvae showed signs of thermal stress. At the larvae's thermal optimum (18°C), the pattern of immune gene expression revealed an immunocompromised phase between hatch and teeth development (0–8 dph), caused by a lag period between initial protection and development of inherent immune competence. These results, together with the previous studies, emphasize the importance of a low-level, stable microbial community in eel offspring culture (Sørensen et al., 2014) in order to reduce detrimental fish–microbe interactions that commonly cause high and unpredictable mortality in the first weeks after hatching of marine fish larvae (Vadstein et al., 2013).

Insights leading to first feeding larval trials

Generally, when initiating first feeding, larvae detect prey through a range of chemical, visual and physical stimuli (Rønnestad et al., 2013). For instance, natural or synthetic chemo-tactic stimulants attract larvae, foster orientation, and promote the initiation of prey capture and ingestion (Barroso et al., 2013; Kamstra and Heinsbroek, 1991; Reig et al., 2003). However, nutritional requirements of fish larvae are species specific and differ across developmental stages within a species mainly due to morphological and physiological changes during ontogeny (Zambonino-Infante and Cahu, 2001). As such, scientists have aimed at revealing the natural feeding sources of larval eel, but despite increasing insights relying on advanced analyses of stomach content, their natural diet is not fully uncovered (Ayala et al., 2018; Miller et al., 2012; Riemann et al., 2010; Tomoda et al., 2018). At the same time, increased scientific inquiry has been directed towards developing suitable first feeding diets for eel larvae. In the case of Japanese eel, larvae are attracted to and successfully feed on a slurry diet based on shark egg yolk (Tanaka et al., 2003), hen egg yolk, exoskeleton-free Antarctic krill (Okamura et al., 2013), or a protein hydrolysate-based diet (Masuda et al., 2016). Additionally, a minute rotifer (*Proales similis*) was suggested as an alternative diet closer to natural larval trophic levels (Wullur et al., 2013). Similarly, for European eel, suitable feeds for larval culture covering the nutritional requirements need to be tailored and tested. First insights on potential feed items are revealed from investigating the morphology of the feeding apparatus.

In order to estimate the biting force and size of ingestible particles, the musculoskeletal anatomy of the larval head was investigated by histological 3D-reconstruction of the somatic structure of the feeding apparatus (Bouilliart et al., 2015). All cartilaginous and bony elements, as well as ligaments, tendons, and muscles were traced and identified in larvae close to the first-feeding stage, that is, 9, 12 and 15 days post fertilization. Here, Figure 22.5 shows the teeth morphology and orientation, the presence of branchial arches (no gills), a non-rigid operculum, and the ligament between the hyoid and lower jaw. Bigger anatomical structures were also reconstructed including eyes, brain, heart and otic vesicle. Using this reconstruction, both the biting force and kinematic efficiency of the present hyoid four-bar mouth opening mechanism showed minimal biting force. Still though, the distinct attachment sites of the first pair of teeth, in both the upper and lower jaw, indicate that these teeth are involved in biting. As a combined result, a rather weak bite force (± 50 μN) and a relatively small maximum gape angle of the lower jaw (± 25°) suggests a preference for very soft and/or small food organisms and/or particles (Bouilliart et al., 2015).

First feeding in European eel larvae

The stable production of larvae reaching the feeding stage and indication of potential diets led to first feeding experiments, studying, for example, feeding behaviour, ingestion rate and mechanisms regulating ingestion, digestion, and growth (Figure 22.6). Here, we aimed at determining whether tailored diets, chemo-attractants, and light conditions impact the incidence of first feeding, gut fullness, and behaviour in the larvae. First results by Butts et al. (2016) showed that up to 50% of cultured larvae in the experiment ingested a diet composed of enriched rotifers (*Brachionus plicatilis*), concentrated and emulsified into a paste, with or without potential stimulants of feeding. Moreover, it was documented that first-feeding eel larvae are able to execute a complex goal-oriented motor response, where highly distinctive modes of swimming were observed from short-term bouts, slow steady-state cruising to quick lunges for either prey attacks or spontaneous escape behaviours. Swimming activity increased over ontogeny, co-varied with the frequency of attacks and increased in the presence of live rotifers or chemo-attractants, probably by increasing the awareness of food availability (Figure 22.7). Furthermore, improved ingestion was detected at higher light intensities, suggesting that these larvae are visual feeders, which probably explains their large eye globules. However, the observed chemo-attraction and successful food intake with and without light, indicates that this species is able, besides using visual cues, to utilize other stimuli (olfaction, taste) to detect prey (Butts et al., 2016). Although the diet did not enable larval survival and growth, the study significantly enhanced insights in the eel larval feeding biology and provided a benchmark diet for future feeding trials.

Molecular ontogeny of first-feeding European eel larvae

Based on the first-feeding trial, we focused on larval nutritional condition examining key physiological mechanisms that regulate ingestion, digestion, and growth (Figure 22.8). As such, eel larvae were reared with or without the presence of algae (*Nannochloropsis*, *Pavlova*, and

Figure 22.6 Captive-bred, feeding European eel larva (~15 dph) targeting small (<100 µm) food particles. Photograph: Sune Riis Sørensen, DTU Aqua.

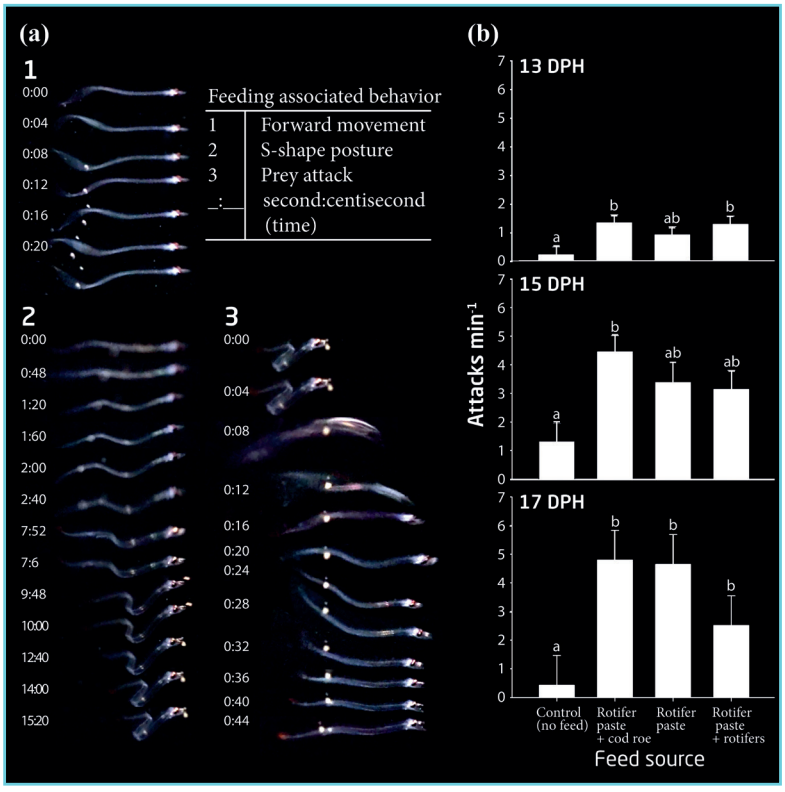

Figure 22.7
European eel larval feeding associated behaviours. (a) Illustrations of *S*-bending attacks: larva remains stationary, using frequent caudal beats, before exhibiting an s-shape posture and executing an explosive lunge. (b) Effect of feed source on the frequency of larval attacks at 13, 15 and 17 dph. Feed sources with different letters are significantly different ($p < 0.05$, least square means, ANOVA). Error bars represent least square means standard error. Source: Butts et al. (2016).

Tetraselmis) from 0 to 14 dph and with or without the presence of food (rotifer paste) from 15 to 24 dph (Politis et al., 2018a). Genes encoding appetite stimulators (*ghrelin*) and inhibitors (*cholecystokinin*) were expressed at basic levels on 4 dph, while their expression increased on 12 dph, indicating the beginning of the first-feeding window. Moreover, expression patterns of genes encoding important digestive enzymes relating to hydrolysis of protein (*trypsin*), lipid (*triclyceride lipase*) and carbohydrate (*amylase*) revealed that essential digestive ontogenetic processes occur from 14 to 20 dph (Figure 22.8). These ontogenetic molecular mechanisms were not affected by algal presence (green-water) and occurred irrespective of exogenous food ingestion signifying that they are linked to internal rhythms, which are under endocrine control.

The larvae that ingested a paste consisting of enriched rotifers as described in Butts et al. (2016) showed a molecular response to initiation of exogenous feeding in the expression pattern of genes relating to energy metabolism (*atp6*, *cox1*), food intake (*pomc*), growth (*igf1*) and thyroid metabolism (*thrαA*, *thrβB*). Moreover, the transcript levels of protein (*try*) digestion enzymes were higher than those of carbohydrate (*amyl*) and lipid (*tgl*) digestion enzymes, similar to the findings for pre-leptocephali and leptocephali larvae of Japanese eel (Hsu et al., 2015), indicating a nutritional predisposition for proteins during those life stages. This would be in accordance with their natural feeding regime, as they are assumed to feed on marine snow, primarily consisting of protein detritus and/or gelatinous plankton (Ayala et al., 2018; Miller et al., 2012; Tomoda et al., 2018). Likewise, as with Japanese eel (Hsu et al., 2015),

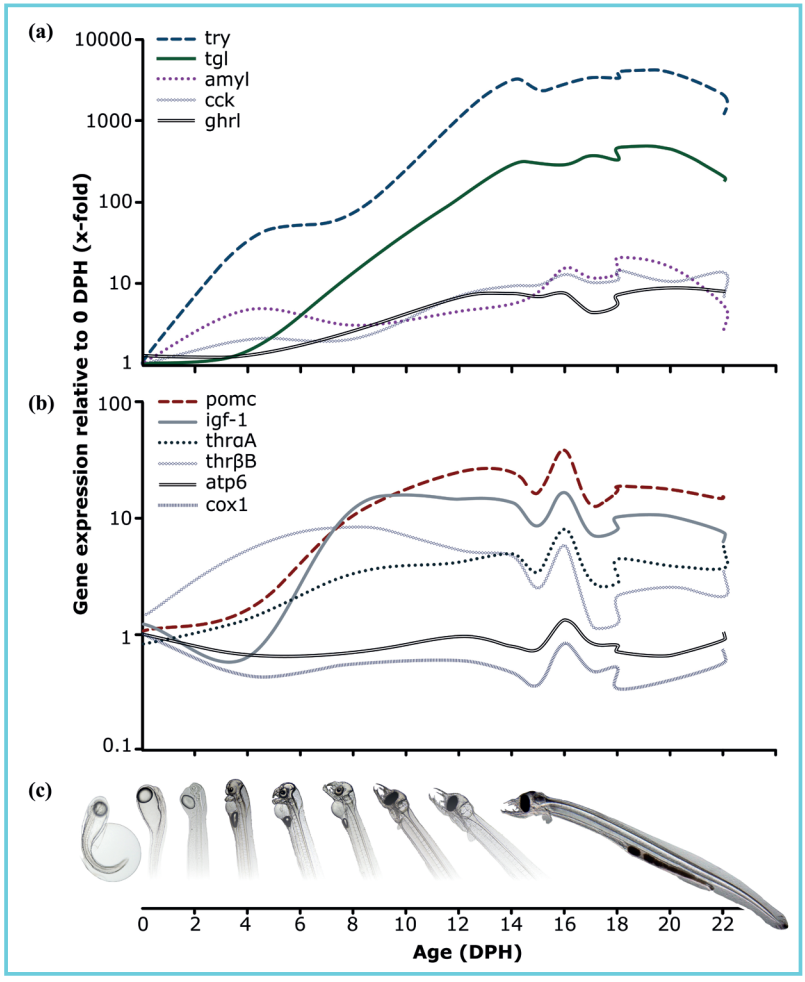

Figure 22.8
Conceptual overview of European eel larval gene expression in relation to age in days post hatch. Expression ($2^{-\Delta\Delta ct}$) calculated in relation to the average expression of each gene on day 0. (a) Relative expression for trypsin (*try*), triglyceride lipase (*tgl*), amylase (*amyl*), cholecystokinin (*cck*) and ghrelin (*ghrl*). (b) Relative expression for proopiomelanocortin (*pomc*), insulin-like growth factor (*igf1*), thyroid hormone receptors (*thrαA*, *thrβB*), ATP synthase F0 subunit 6 (*atp6*) and cytochrome-c-oxidase (*cox1*). (c) Comparable larval development from hatch into the feeding stage. Source: Politis et al. (2018a).

elevated expression levels of *amyl* (carbohydrate hydrolysis) were detected within the first-feeding window (Figure 22.8), which may reflect a primary mode of digestion (Zambonino-Infante and Cahu, 2001). Furthermore, higher RNA content in feeding compared to non-feeding larvae was observed, which indicates increased metabolic activity associated to protein synthesis. Additionally, increased DNA content in feeding larvae compared to non-feeding larvae was observed, which in combination with increased RNA content and greater body area, indicated a better growth pattern in the feeding larvae. However, RNA : DNA ratios still decreased from 12 dph onwards, indicating a decreasing larval condition, probably leading to the 'point-of-no-return' and subsequent mortality.

Thus, the study molecularly identified the first-feeding window in European eel, revealing that exogenous feeding success occurs concurrently with the onset of a broad array of genetically pre-programmed molecular factors, which regulate physiological functions of feeding. Here, feeding eel larvae sustained growth and condition at a higher level than non-feeding larvae. These results add to our understanding of the physiological changes occurring during the transition

from newly hatched endogenous feeding pre-leptocephalus larvae to the exogenous leptocephalus stage. This new insight regarding a still undisclosed phase in the European eel life cycle may help developing efficient feeds and feeding strategies for European eel larvae.

Conclusion and perspectives

European aquaculture production has a substantial potential for expansion through species diversification and domestication, while at the same time reducing pressure on natural populations. In this regard, closing the life cycle of European eel in captivity would enable a sustainable and profitable aquaculture sector along with supporting conservation measures by replacing wild-caught glass eels with hatchery propagation of fry. Here, we have outlined a wide range of challenges that need to be overcome in order to develop closed-cycle propagation and mass production of glass eels. We also described the recent progress in establishing the scientific knowledge base needed to overcome these barriers for anguillid eel targeted for aquaculture. This research, encompassing the complex physiology, molecular adaptations and life history traits of eels, has since the 1990s, rapidly substantiated insights throughout ontogeny. At the same time, it has led to significant progress in advancing methodologies for captive breeding as well as larval culture technology leading to glass eel production for the Japanese eel and first-feeding larval trials for the European eel, bringing new attention to eel as a candidate species for commercial hatchery development.

Overall, this rapid substantiation of insights on eel reproduction and early life history, originate from combining extensive experimental work and field collection with advanced analytical methods and techniques. Particularly, the assembly and annotation of the European and Japanese eel genomes have accelerated acquisition of new knowledge, thereby providing a source of data for the discovery of genetic markers, and the comprehensive characterization of genes and their expression in different developmental stages and environments. Moreover, these advancements will allow the fast detection of biomarkers associated with eel physiological status that can guide future research in breeding for domestication. The application of these tools, with certainty, will increase our understanding of the endocrine reproductive control mechanisms and viable offspring production, uncover further aspects of parental influences on embryonic and larval developmental competence, and assist in identifying effective diets and establishing suitable culture conditions for growth of European eel leptocephali. Another aspect relates to integrating knowledge gained from experimental and field research in order to enhance our understanding of the European eel biology and ecology. For instance, the experimental identification of the optimal thermal environment for embryonic and early larval development may support new hypotheses regarding the spawning location in nature, while defining temperature tolerance limits may help predict environmental impacts of rising ocean temperatures. On the other hand, sampling during surveys enables analysis of larval biochemical composition and identification of the natural larval diet, which can help optimizing hatchery feeds for eel larvae. In future scenarios, integrating such multi- and interdisciplinary research may mutually benefit hatchery development and conservation of the European eel, and help preserving a fascinating, catadromous species of high ecological and economical importance.

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