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# Unravelling the metabolomic signatures of migrant and non-migrant glass eels (*Anguilla anguilla*) and their response to diazepam exposure

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A B S T R A C T

Understanding the migratory cycle of the European eel is crucial for implementing effective conservation measures. The reasons why some glass eels settle in lower estuaries rather than migrating upriver remain unclear. This study aims to identify metabolomic signatures that distinguish active (migrant) from inactive (non-migrant) glass eels. Using a combination of target and non-target screening (NTS) approaches, the metabolite profile of glass eels was studied, and a PLS-DA classification model was applied to find differences between behavioural phenotypes. This model highlighted methionine, glutaryl-L-carnitine, and palmitoylcarnitine as key metabolites, with methionine being significantly different between groups. Glutaryl-L-carnitine strongly correlated with activity, suggesting it might be a more sensitive indicator of glass eel activity than previously studied parameters such as weight loss and oxygen consumption. The findings suggest that differences between active and inactive eels result from both swimming activity and intrinsic metabolic differences, with methionine linked to both factors. We also explored potential differences in how diazepam affects active and inactive glass eels. However, our metabolomic approach lacked the sensitivity to detect significant variations. Overall, this study provides valuable insights into the metabolomic distinctions between active and inactive glass eels, establishing a foundation for future research in this field.

## 1. Introduction

The migratory cycle of the European eel (*Anguilla anguilla*) remains one of the most complex patterns in the animal kingdom. Adult eels spawn in the Sargasso Sea and the leptocephali (i.e., the larvae stage) drift with the Gulf Stream to reach the European continental shelf, where they metamorphose and turn into the so-called glass eel. In their second migratory phase, glass eels migrate up estuaries to enter rivers, taking advantage of the current during the upstream flood tide and hiding in the estuarine bed during ebb tide (Gascuel, 1986). Along the estuarine migration, glass eels undergo several changes including morphological, physiological, and behavioural alterations (Elie and Rochard, 1994), as well as pigmentation processes (Elie et al., 1982), gut development, osmotic adaptations (Ciccotti et al., 1993), and hormonal modifications (Jegstrup and Rosenkilde, 2003). In addition, previous studies have suggested that glass eels do not use every flood tide to migrate (Gascuel, 1986; Beaulaton and Castelnaud, 2005) and that some individuals may not migrate to rivers but instead complete their life cycle in coastal or estuarine waters (Tsukamoto and Aoyama, 1998; Daverat et al., 2006).

Since the European eel exhibits environmentally determined sex differentiation, these distinct migratory patterns could significantly impact the fate of the population. On the one hand, males are generally observed to dominate in high-density environments, often associated with estuarine or lower river reaches. On the other hand, females tend to become more dominant with increasing distance from the sea (Davey and Jellyman, 2005; Harrison et al., 2014). Therefore, understanding the sexual maturation of glass eels and its role in migration patterns requires further study. Although many studies have aimed to explain the mechanism behind the differentiation of the two migratory phenotypes (i.e., active glass eels that are able to use the tide to migrate efficiently, and inactive ones that settle in estuaries (Liu et al., 2019)), a widely accepted physiological explanation is still lacking (Beaulaton and Castelnaud, 2005).

Some studies have suggested that the energetic condition of the glass eels partially explain the facultative migration (Bureau Du Colombier et al., 2007; Edeline et al., 2007; Liu et al., 2019). However, as described by Claveau et al. (2015), contamination may also affect migratory

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behaviour. Glass eels are particularly vulnerable to contamination since estuaries are frequently impacted by anthropogenic activities. (e.g., wastewater discharge). Therefore, the protection of this endangered species also depends on a broader change of attitude towards pollution reduction, which must be driven by monitoring actions.

In this vein, previous works have attempted to describe the occurrence and fate of contaminants of emerging concern (CECs) in the Adour estuary (Bayonne-Baiona, Basque Country, France), which it is one of the main habitats for glass eels. In 2017, the three main wastewater treatment plants (WWTPs) that release their effluents into the estuary were studied and contaminants included in the Water Framework Directive (WFD) were quantified (Cavalheiro et al., 2017). More recently, the environmental risk assessment conducted in a previous study (Alvarez-Mora et al., 2022), led to the identification of diazepam, irbesartan and propranolol as the most notable CECs released by the WWTP of Bayonne. Glass eels were then exposed to these CECs to study their bioconcentration and biotransformation potential. More recently, Bouchard et al. (2023) conducted a behavioural experiment and discovered that diazepam affected the swimming activity of glass eels, also promoting bolder behaviour. These alterations may have significant ecological consequences and reinforce the previously mentioned observations. In the foundational study that underlies this research, the impact of two drugs, including diazepam, on glass eels was explored from a metabolomics standpoint (Alvarez-Mora et al., 2023). In that work, it was shown that the mixture of both pharmaceuticals led to significant alterations in the metabolomic profile of glass eels. The effectiveness of the approach employed in that study now motivates us to explore potential variations in the metabolome of migrant and non-migrant glass eels.

Metabolomics refers to the study of the small endogenous (<1.5 kDa) metabolites in an organism or in a tissue or biofluid. Alterations in the metabolite profile can provide a clear insight into the health status of an organism and are, thus, often used in toxicology to find the biochemical pathways disturbed by a stressing event (e.g., disease, nutritional imbalance, contaminant exposure …). Metabolomics has been successfully applied in environmental impact assessments to explore the cause-effect mechanisms of CECs in aquatic organisms (Bhagat et al., 2022; Colás-Ruiz et al., 2022; Labine et al., 2022; Xu et al., 2022) and it is one of the key elements in the study of adverse outcome pathways (Brockmeier et al., 2017; Dumas et al., 2022).

The underlying premise is that the study and the analysis of slight modifications in the metabolome would provide key information regarding, for instance, the effect of stressors or intrinsic physiological difference. To ensure the proper application of this approach, we must ensure that most of the remaining factors that might modify the metabolomic profile are kept constant (Villas-Boas et al., 2007). This requires carefully designed experiments taking into account any source of variation (i.e., genotype, phenotype, age, sex, feeding …) and minimizing the effect of all the non-relevant ones. As previously mentioned, sex and feeding are not influential since glass eels are not sexually differentiated and fast until the juvenile stage. However, when working with wild glass eels, controlling the genotype is not possible as it is with laboratory animals with common genetic lines, and the migratory phenotype may also play an important role in the metabolite profile variability.

Given the nature of the metabolomics studies, where the discrimination of different experimental conditions may rely on a small proportion of the total variance, unsupervised multivariate analysis tools such as principal component analysis (PCA) may fall short. In contrast, classification methods such as partial least squares discriminant analysis (PLS-DA) can be useful in identifying differences between targeted groups and discovering the metabolites related to the class separation, especially when combined with variance analysis (i.e., ANOVA) tools.

In this work, we aimed to find the metabolomic signatures distinguishing active (migrant) and inactive (non-migrant) glass eel phenotypes, and to determine whether the differences observed between the two groups were due to swimming activity or intrinsic differences in their metabolome. For this purpose, glass eels were previously sorted according to their migratory behaviour, and their metabolite profiles were analyzed by high-resolution mass spectrometry (HRMS) to find discriminating profiles between the two groups. In addition to this, differences in the sensitivity of the two phenotypes to diazepam exposure were also studied.

## 2. Experimental section

### 2.1. Standards and reagents

Information regarding the metabolite standards used in the targeted analysis is provided in Table SI1. The list includes a wide range of metabolites representative of the main biochemical pathways usually assessed in environmental metabolomics. Diazepam and irbesartan standards were acquired from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol (MeOH), water, acetonitrile (ACN) and 2-propanol (IPA) were obtained from PanReac AppliChem (Barcelona, Spain). HPLC grade tert-butyl methyl ether (MTBE) was obtained from Sigma-Aldrich (St. Louis, MO, USA). MS grade formic acid (98% purity) and ammonium formate salt (≥99% purity) were purchased from Fisher Scientific (Madrid, Spain) and Fluka (Steinheim, Germany) respectively.

### 2.2. Ethical note

Procedures used in this study were validated by the ethics committee N°073 'Aquitaine Poissons-Oiseaux' (ref: APAFIS #32100–2021062317263102 v2). The experiment was carried out in strict accordance with the EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e., Directive, 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2013-118, February 1st, 2013).

### 2.3. Fish collection

The sampling site corresponded to the estuarine mouth of a small river (Courant d'Huchet, France: 43.857822, −1.390253, map available in Bolliet et al. (2017)). The fish were collected in December using a dip-net at night and during flood tide. Once collected, the fish were transferred to the laboratory and maintained at 12 ± 0.5 °C overnight in a tank containing aerated water from the sampling site. During the next week, 1/3 of the water was replaced daily by freshwater previously aerated during 24 h. The room was maintained under a photoperiod of 10h light/14h dark with a very low light intensity during the photophase (0.2–0.3 μW/cm$^2$). After this transition to freshwater, 50 glass eels were anesthetized by batches of 10 individuals (Benzocaine, 0.01 mg L$^{-1}$), individually measured for initial wet weight (±1.0 mg) and length (±0.5 mm) and tagged with Visible Implant Elastomer (VIE Tag). Each individual VIE tag was a unique combination of two or three hypodermic spots of four different colors on the back (Liu et al., 2019). Once tagged and handled, all individuals were released into a bucket to recover overnight prior to the exposure to diazepam.

### 2.4. Exposure conditions and behavioural test for phenotype differentiation

Two groups of 25 glass eels were placed in two tanks supplied with the same water in open circuit with a peristaltic pump (10.0 mL/min). Another peristaltic pump (1 ml/min) supplied one of the tanks with water and the other one with diazepam in order to obtain a concentration of 5 ng/ml in the tank. The choice of concentration used is based on a previous study by the group (Alvarez-Mora et al., 2022), where this compound was found at approximately this concentration and identified as one of the main risk drivers in the Adour estuary (a habitat for this species and an area with high anthropogenic impact). Since that study

demonstrated a close alignment between the nominal and measured concentrations in the water during the experiment, we decided not to analyze the water for this study. Both tanks were equipped with shelters located opposite the arrival of diazepam and they were submitted to the same conditions of light intensity, photoperiod, and temperature as during freshwater acclimatization. Flows of water and diazepam, as well as water temperature were checked daily during the 7 days of exposure.

After one week of exposure, the 25 control and 25 exposed glass eels were transferred together to the activity tank, an annular tank installed in a temperature-controlled room as described by Liu et al. (2019). Two pumps fixed on the opposite ends of the tank allowed the change in the water current direction every 6.2 h to mimic the tides (alternately clockwise and counterclockwise water flow). The swimming behaviour of glass eels was tracked individually during 7 days by a camera programmed to record 15 s every 40 min. This method to distinguish between active and inactive glass eels was originally developed to assess circatidal swimming behavior (i.e., aligned with the tidal schedule). These experiments revealed two distinct groups: active eels, which might correspond to glass eels that migrate up estuaries in wild, and inactive glass eels, which halt their migration to complete their life cycle between marine and brackish waters.

In our facility, glass eels synchronizing their swimming activity to the change in water current direction were considered as having a high probability of migration (active). Individuals remaining permanently or mostly hiding in the substrate, regardless of the water flow direction, were considered as not being prone to migrate (inactive) (Bolliet and Labonne, 2008; Bureau Du Colombier et al., 2007; Liu et al., 2019). The room was maintained under the same conditions of light intensity, photoperiod, and temperature than during exposure and a constant UV light (0.6 μW/cm$^2$) was added to see the VIE Tag.

After 7 days of observation, active and inactive glass eels were removed from the tank and placed in sheltered, flow-free tanks for 5 days before being euthanized so that swimming activity would not influence the results of the metabolomic analysis (i.e., altered metabolite levels due to physical activity). During these 5 days, the glass eels remained in the shelters and no swimming activity was observed. The experiment was conducted without feeding the glass eels, as they do not feed during this stage of development in the wild. This approach also avoided any interference from differences in feeding among the glass eels.

The biometric results of the sampled glass eels can be found in Table SI2. In summary, for each glass eel, its length and weight were measured at the beginning and at the end of the experiment, and its behaviour (active/inactive) was deduced based on the number of observations collected during the video tracking (i.e., number of video sequences in which each glass eel appears). Since the threshold for deciding whether a glass eel is considered active or inactive depends on the conditions of the experiment itself and can be dynamic, it was decided on the basis of the activity results obtained. As shown in Table SI2, 23 of the 50 glass eels showed an activity between 0 and 7 observations, while for 18 of them, more than 17 observations were measured, clearly differentiating two groups. Nine glass eels (5 exposed to diazepam and 4 controls) were discarded from the rest of the experiment because of intermediate activity. Consequently, four experimental groups were used in this study: control active (CA, N = 10), control inactive (CI, N = 9), diazepam-exposed active (DA, N = 8), and diazepam-exposed inactive (DI, N = 14) glass eels.

### 2.5. Metabolite extraction

Since dissecting the glass eels was not feasible and the sample size from individual organs, such as the liver, would not suffice for analysis, the entire body was used. To facilitate sample extraction (~100 mg), the body was first cut using a scalpel, and an unbiased two-stage extraction method was employed. For that, 300 μL MeOH and 100 μL of Milli Q water (UHPLC-MS quality) were added to the samples before being homogenized (6400 rpm–60s x 3–15 s) (Precellys®, Bertin Instruments, Montigny-le-Breonneux, France) at 4 °C using homogenization tubes with ceramic beads (1.0 mm diameter). 900 μL MTBE and 250 μL Milli Q water were then added and the homogenization process was repeated (Chen et al., 2013). The extracts were centrifuged for 5 min (4 °C, 21000G) (Allegra X-30R Centrifuge, Beckman Coulter®), the solid fraction was removed and the liquid centrifuged for 15 min (Ribbenstedt et al., 2018). The liquid phase had two phases, the organic fraction on top and the aqueous fraction at the bottom which were collected separately. The organic phase was evaporated under $N_2$ flow (XcelVap®), the residue was dissolved in 1.5 mL IPA:MeOH:water (4:3:1) mixture (Paglia and Astarita, 2017) and diluted for injection (1:10, v/v). In the same way, the polar phase was collected and diluted directly (1:10 and 1:50) using a mixture of water:methanol (3:1). For quality control purposes, a pool containing small aliquots from all the extracts was also prepared and injected for analysis along the sequence (N = 6).

### 2.6. Instrumentation

Sample analysis was carried out using a Dionex Ultimate 3000 UHPLC (Thermo Scientific) coupled to a Q-Exactive Focus hybrid quadrupole-Orbitrap MS (Thermo Scientific, Waltham, Massachusetts, United States) with a heated electrospray ionization source in positive and negative modes (HESI, Thermo, CA, USA). For the polar metabolome analysis, the separation was achieved using a Kinetex® PS $C_{18}$ (2.6 μm, 100 Å, LC Column 150 × 3 mm; Precolumn SecurityGuard® ULTRA Cartridges UHPLC $C_{18}$ 2.1 mm) column and for the lipidome analysis a Luna® Omega $C_{18}$ (1.6 μm, 100 Å, LC Column 150 × 2.1 mm) was used. Mobile phases in polar analysis were A: HPLC Water (10 mM Ammonium formate in the negative mode and 0.1% Formic acid in the positive mode) and B: HPLC Water:AcN 5:95 (10 mM Ammonium formate in the negative mode and 0.1% Formic acid in the positive mode). The gradient was as follows: 0 min 5% B; 1 min 5% B; 15 min 95% B; 20 min 95% B, 24 min 5% B, with flow rate of 0.3 mL/min, column temperature of 35 °C, and injection volume of 5 μL. On the other hand, separation conditions for the lipidome analysis were adapted from Chen et al. (2013). Mobile phases were A: 60% ACN in water, and B was IPA:AcN (9:1), both containing 10 mM ammonium formate and 0.1% formic acid in both positive and negative modes. The gradient was as follows: 0 min 40% B; 3 min 70% B; 10 min 100% B; 22 min 100% B; 27 min 50% B; 28 min 50% B with a flow rate of 0.2 mL/min, column temperature of 35 °C and injection volume of 5 μL. Data were acquired using the full scan data dependant MS2 (Full-Scan-dd-MS2) mode, where the full scan range was fixed between m/z 50–750 for polar compounds and 70–1000 for non-polar ones with a resolution of 70.000 in the Full MS scan. Three ddMS2-scans were completed for every full scan with a resolution of 17.500. The ionization conditions in the HESI were the followings: The sheath and the auxiliary gas flow rates were established at 55 and 10 units respectively, the ion spray voltage was 3.10 kV for positive ionization and 3.20 kV for negative, and temperatures were kept at 300 °C for the capillary and 290 °C for the auxiliary gas heater.

### 2.7. Data handling

For the targeted analysis of the polar metabolome data were processed using Trace Finder 5.1 (Thermo Scientific). The integration of the chromatographic peak areas was carried out by the automatic function using the Intelligent Component Integration System (ICIS) algorithm. The correct integration was evaluated metabolite by metabolite over the calibration solutions. The NTS of metabolites was performed using Compound Discoverer 3.3 (Thermo-Fisher Scientific, Waltham, MA, USA). Features were detected following the non-targeted metabolomics workflow (Section SI.5.6.3) using a mass tolerance of 5 ppm and a minimum peak intensity of $10^5$. All the metabolomic features found were matched with freely available MS mass lists such as HMDB 5.0

(Wishart et al., 2022), LipidMaps (Fahy et al., 2007), and the endogenous metabolite database from Compound Discoverer and mzCloud MS libraries. Additionally, from the resulting list of candidates, a manual inspection was carried out to remove either noisy peaks or non-meaningful peaks (bad chromatographic shape, poor fragmentation), and only features with peak areas 10 times larger in samples than blanks were further considered. Through the Fish Scoring application, the *in-silico* fragmentation of the final list of candidates was run to include the match level among the quality metrics.

Measured concentrations by the targeted analysis and peak areas from the NTS were uploaded to MetaboAnalyst (v5.0) (Pang et al., 2022) for further statistical evaluation. PLS-DA was followed by statistical tests, including two-way ANOVA to analyze the effects of diazepam treatment, activity, and their interaction, as well as FDR-adjusted t-tests to identify significantly altered metabolites. The robustness of the proposed classification models was assessed by means of the regression coefficients for the training set ($R^2$) and the cross-validation ($Q^2$). In addition, since metabolomics often involves a large number of hypothesis tests, these statistical tests need to be corrected to avoid false positives leading to false findings. Thus, instead of using the common metrics (i.e. p-value) the results are expressed in metrics such as the false discovery rate (FDR or q-value) (Scheubert et al., 2017). Finally, the relations and the capacity to predict the activity of glass eels from metabolite concentrations was also assessed by means of partial least squares (PLS) using the PLS-Toolbox 8.9.1 (2022, Eigenvector Research, Inc., Manson, WA USA) in Matlab (2019b, Mathworks®).

## 3. Results and DISCUSSION

### 3.1. Metabolomic analysis of the behavioural phenotypes

To study the differences between the two glass eel phenotypes, in the first part of the study only control glass eels (CA vs CI) were considered.

#### 3.1.1. Analysis of the polar metabolome

Regarding the polar-metabolome, metabolites were annotated according to the confidence levels proposed by Schymanski et al. (2014). The non-target screening (NTS) complements the list of compounds quantified by targeted analysis with new tentative candidates, but it is important to report the confidence level and treat them as potential candidates rather than confirmed compounds. Unlike the compounds studied by targeted analysis, where the structure is confirmed by a reference standard, compounds annotated at level 2a have probable structures matched in spectral libraries thus obtaining maximum confidence in annotation for the NTS. Those annotated at level 3 are tentative candidates with multiple matches in spectral libraries, often representing isomers of the same feature.

The 40 metabolites quantified by the target screening approach were annotated as level 1, whereas for the NTS approach 44 metabolites were annotated as level 2a and 6 as level 3 (Table SI3). Quality control samples were used to exclude all the metabolites showing an RSD >40%. The data resulting from both screening methods were merged, normalized by the actual weight of the samples and autoscaled to homogenize the scale differences between concentrations and peak areas (from target and non-target screening respectively).

To identify differences between the behavioral phenotypes of glass eels, we initially constructed a PLS-DA classification model to distinguish between active and inactive controls. Two samples from the active control group were excluded from further statistical analysis due to falling outside the 95% confidence ellipse (CA, N = 8). Subsequently, we applied FDR-adjusted t-tests (q-values) to validate whether the metabolites responsible for group separation in the model exhibited significant differences between the groups. In addition, as the main aim of the study is to find inherent metabolomic markers of the difference between phenotypes and not metabolomic differences produced after or related to activity during experimentation, the areas/concentrations of the most important metabolites found were compared with activity to find correlations.

At first glance, the differences between the two groups were noticeable in the PLS-DA scores plot (Fig. 1), since the 95% confidence level ellipses of the two groups were almost completely distinguished. Three metabolites showed relatively high variable importance in projection (VIP) values (≥2), listed in order of importance, the essential amino acid methionine and two acylcarnitines, glutaryl-L-carnitine and palmitoylcarnitine. However, analyzing the robustness of the classification model we can observe that the cross-validation metrics are rather weak ($R^2 = 0.93$; $Q^2 = 0.38$), suggesting that the separation is not as good as the distribution of the scores plot shows. What this model indicates is that the disparities between these two groups represent a minuscule fraction of the overall variability. Thus, even metabolites with deemed high VIP may not exhibit significant differences. This was checked by assessing the FDR (q-values), which confirmed that only one of these metabolites, methionine, is significantly different between the groups (q-value = 0.049).

Even though the glass eels were kept inactive for five days before euthanasia, the correlation between the individual top metabolites and activity, weight loss, and length loss was investigated. This was done to ensure that any group differences observed were not solely due to variations in swimming activity, but rather reflected genuine differences in the two behavioral phenotypes. Fig. 2 shows that both Glutaryl-L-carnitine and methionine exhibit a positive correlation ($R^2 = 0.68$ and 0.7 respectively) with the activity of the glass eels, indicating that these metabolites might be potential markers of the swimming activity. However, no other metabolite exhibited a similar pattern, and notably, methionine (shown earlier to be the only metabolite significantly differing between phenotypes) also demonstrated a weak correlation with weight loss.

Although this was not one of the objectives set at the beginning of the study, we must also highlight the correlation found between glutaryl-L-carnitine concentration and activity. Previously, studies like that of Liu et al. (2019) attempted to connect activity levels in glass eels with standard metabolic rate (measured through oxygen consumption) and weight loss. Despite clear differences in these parameters between glass eels caught early in the season (autumn) versus later (spring), no significant correlations have ever been identified between active and inactive glass eels sampled concurrently. However, in this study, we propose that levels of this carnitine might be more sensitive indicator of glass eel activity, as shown in the correlation graphs (Fig. 2). Still, methionine shows a weak correlation, which could indicate that the differences between the two experimental result from a combination of the effects of swimming activity and intrinsic differences in the metabolome of each phenotype, with this metabolite likely related to both.

In our attempt to find relationships between our set of metabolites and the response in the form of activity, we built up a PLS regression model with the biometric results and the targeted analytical results. Although the main use of this type of model is the prediction of the response through its relationship with the set of predictor variables, its use is also widespread among the different omics for the identification of metabolites, proteins or genes associated with the response to an event (e.g., disease, stressor, explorative behaviour …)(Azizan et al., 2020; Kogelman et al., 2022; Lin and Long, 2023). The PLS model constructed with the concentrations from the targeted analysis does not show a great robustness in terms of root mean square error of calibration (RMSEC) and cross validation (RMSECV), 10.8 and 16.6 respectively. This means the validation prediction error would be ±16.6 observations ($R^2 = 0.8$ & $Q^2 = 0.53$). Although these values again indicate that the relationship between metabolite concentrations and activity is weak, the purpose of this method is to identify other metabolites that contribute to the correlation. Interestingly, among the metabolites that appear to correlate slightly with activity, several acylcarninites stand out (Fig. 3). In the absence of a more in-depth and specific investigation of this family of metabolites, the results presented above point to an interesting

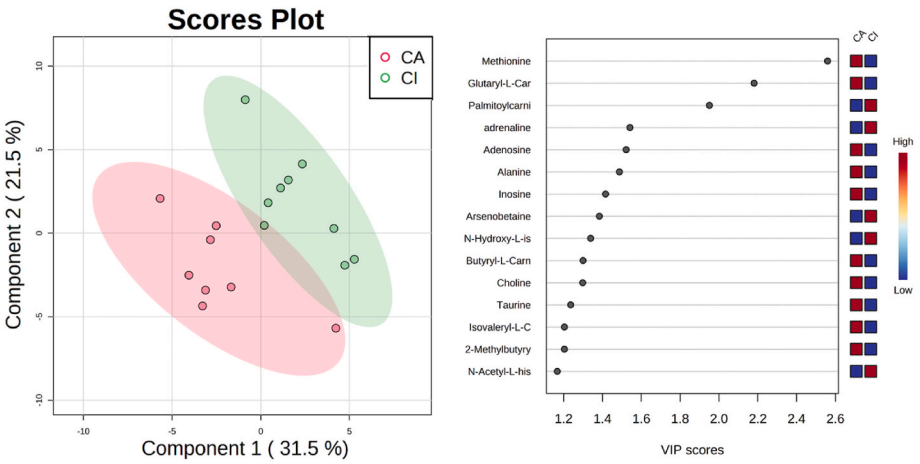


**Fig. 1.** PLS-DA scores plot of the polar metabolites identified in active and inactive control groups (left) and its VIP (Variable Importance in Projection) scores (right). CA refers to the active control group, while CI denotes the inactive control group.

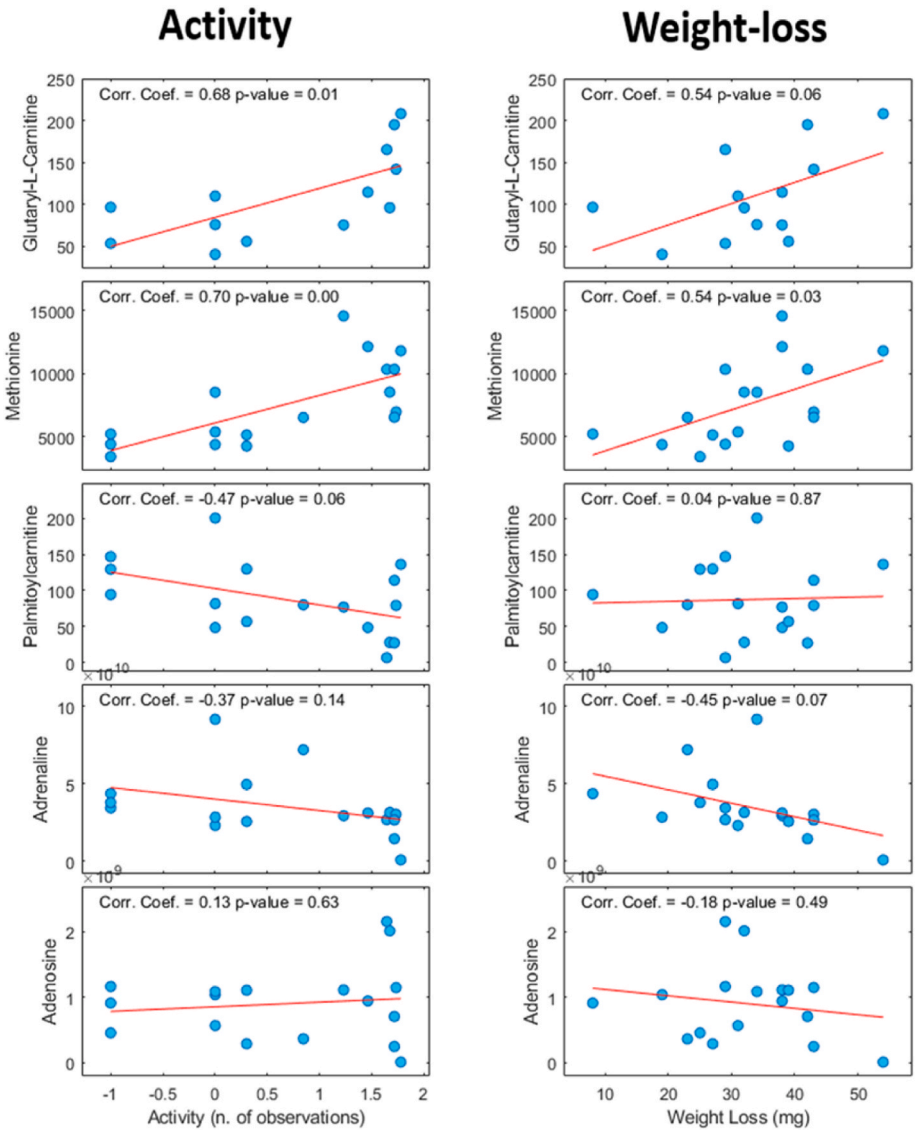


**Fig. 2.** Correlation plots between the concentrations/areas of the most important metabolites found in the PLS-DA and the activity (left column) and weight loss (right column) of glass eels. All control samples (CA + CI, N = 17) were analyzed, except for glutaryl-L-carnitine, which showed no detectable concentration in 4 samples (final N = 13). The results indicate that two metabolites (glutaryl-L-carnitine and methionine) are positively correlated and might be with swimming activity.

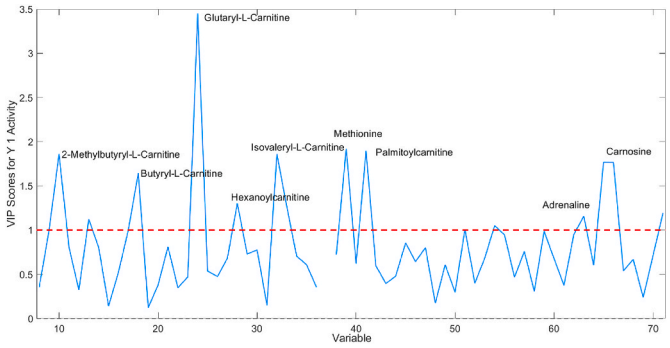


**Fig. 3.** VIP (Variable Importance in Projection) scores on the prediction of the activity from metabolite concentration/areas with the PLS (Partial Least Squares) model. VIP values > 1 indicate a potential relation between the metabolite concentration and the activity.

correlation between this particular family and the activity of the glass-eels, with the strong correlation with glutaryl-L-carnitine being particularly noteworthy. It is also important to note that only short-chain acylcarnitines are included in the targeted analysis carried out and perhaps the study of long-chain acylcarnitines would have provided more relevant information. In fact, altered levels of these fatty acid metabolites may be indicative of problems such as very-long-chain acyl-CoA dehydrogenase deficiency, which is linked to symptoms such as muscular weakness (Glasgow et al., 1983; Hisahara et al., 2015).

Apart from correlations with activity, methionine emerged as the most crucial metabolite for distinguishing between the two phenotypes.

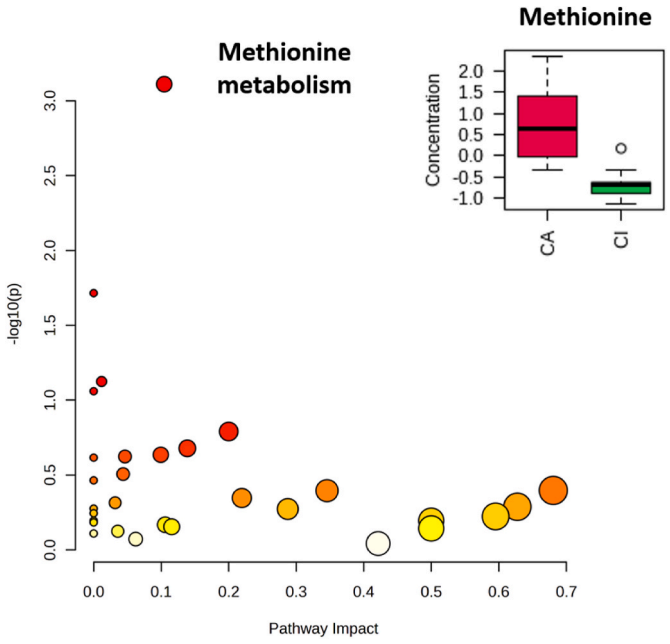


**Fig. 4.** Pathway analysis comparing the main altered metabolic routes in active controls (CA, N = 8) versus inactive controls (CI, N = 9) is depicted, with each circle representing a metabolic pathway. Circle colour intensity corresponds to the relevance of the pathway in differentiating the conditions (y-axis), while circle size indicates the percentage of identified metabolites within each pathway (x-axis, pathway impact of 1 means that all metabolites belonging to the pathway were detected). For interpretation, the greater the number of identified features within a pathway, the further to the right the circle is on the plot and the larger its size. Circles are positioned higher on the plot and display more intense colors when the metabolites within the pathway show greater significance in their differences between groups, as indicated by corrected p-values. The primary altered pathway identified was methionine metabolism. To the right, a box plot illustrates the significant metabolite, methionine.

Additionally, pathway analysis (refer to Fig. 4) indicates that the only significantly altered biochemical pathway is methionine metabolism (FDR = 0.023). As a key component of protein synthesis, methionine plays an important role in supporting growth, development and overall metabolic processes in fish (Wang et al., 2023). Recent work by Sui et al. (2024) demonstrated that methionine deprivation significantly depressed fish growth and reduced the whole-body and muscle protein. At the same time, stored energy plays a major role in migration according to some studies (Bureau Du Colombier et al., 2007). The energy density is the main index for measuring energy storage capacity and different energy-carrying molecules affect this index in different ways (Liu et al., 2022). For example, lipids deplete faster than proteins, having a lower impact on energy density (Penney and Moffitt, 2014). Although protein content was not addressed in this study, the relationship between protein and methionine levels may be behind the mechanism that explains the lack of migration propensity. Further proteomic studies would be necessary to try to explain this relationship.

Methionine is also an essential compound for host defence against oxidative stress (Espe et al., 2023). It assists in the formation of glutathione and taurine, both essential compounds for host defence against oxidative stress, although deficiencies of these metabolites has not been observed (Lu, 2013). The mechanism by which methionine deficiency may be related to migratory behaviour needs further exploration, but the results suggest that this metabolite might be an inherent marker of the glass eel phenotype. This is the first time this relationship has been proposed to understand the migration of glass eels. Still, due to the limited number of glass eels available for the bioassay, this new hypothesis should be tested with a larger experimental group.

### 3.1.2. *Lipidome analysis*

In contrast to the polar metabolome, where analytical standards were accessible, lipidome analysis was conducted solely through non-targeted screening. Therefore, lipid identification was categorized according to Schymanski's confidence levels 2 and 3. A total of 266 lipids were identified, most of them as level 3 (Table SI4). Eighteen compounds that showed RSD >40% in QC samples were excluded from the statistical analysis. The large number of lipids identified complicates the interpretation of the results, so instead, the interpretation was based on lipid families. This approach was feasible because the lipids within the different families were well correlated in our samples (Fig. 5). The

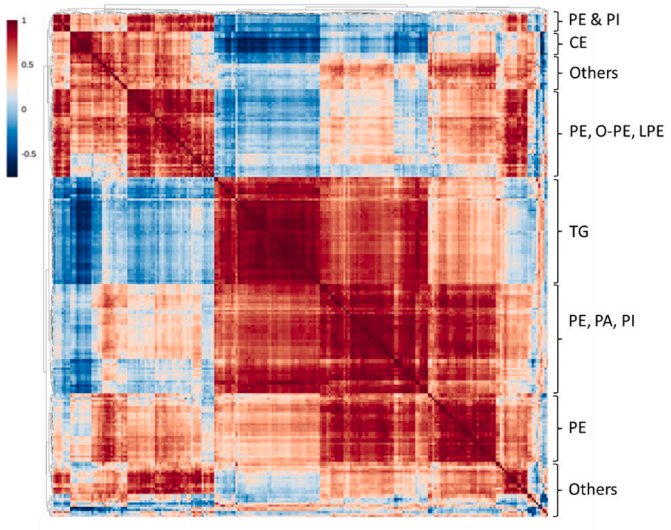


**Fig. 5.** Correlation heatmap of the 266 identified lipids in quality control samples (n = 6). Each pixel shows the correlation between the variables on the axes (symmetrical about the diagonal). Values close to 1 (red) correspond to positive correlation between variables whereas a value close to −1 (blue) correspond to negative correlation.

correlation heatmap was constructed using Pearson's *r* to measure the variable distance. The colour trends observed can differentiate some of the lipid families found in the analysis, with the majority of the lipids belonging to phosphatidylethanolamines (PEs) followed by triglycerides (TGs), cholesterol esters (CEs) and diverse phospholipids such as phosphatidic acids (PAs), phosphatidylinositol (PIs) and their oxidized forms (O-PX).

To explore the differences between the control groups, autoscaled data were used to build the PLS-DA model. The classification model showed poor performance in separating the control groups (not shown). Likewise, none of the lipids were significantly altered according to the FDR adjusted *t*-test (Figure SI5). All results indicate that the lipid profile studied is not useful for the distinction between active and non-active glass eels. Although some studies have linked fat content to migration mechanisms in glass eels (Bureau Du Colombier et al., 2007) and silver eels, with Larson et al. (1990) identifying it as a trigger for the start of spawning season, Liu et al. (2019) found no evidence for this occurring in glass eels at the beginning of the season, which is when our glass eels were caught. However, this evidence is not met at the beginning of the season, the period during which the glass eels used in this experiment were captured. Our study does not contradict the hypothesis that energy storage may affect migration, but suggests that at least during the early season, protein content would be more important than lipid content.

### 3.2. Phenotype-specific response to diazepam

The second aim of this work was to analyze the phenotype-based sensitivity to diazepam, i.e. to study whether groups with different phenotypes are affected differently by diazepam. Although the results of our previous work (Alvarez-Mora et al., 2023) showed that by means of metabolomics we were unable to find out the effect of diazepam (only a noticeable effect was observed when exposed to irbesartan and diazepam mixture), a parallel experiment showed that exposure to this pollutant is able to affect the behaviour of glass eels (Bouchard et al., 2023). That study highlighted the ability of diazepam to induce a boldness effect in glass eels, causing them to come out of their shelter more often during the first few minutes of the experiment, but also its effect in reducing the activity of glass eels. This second effect was observed in our experiment as well, with only 36% of the glass eels exposed to diazepam classified as active, compared to 53% in the control group. However, these percentages should be interpreted cautiously due to the limited sample size. The statistical analysis was performed using two-way ANOVA, a method that evaluates the main effects of two independent variables and their interaction. This approach allows us to simultaneously assess the effect of diazepam, the differences between phenotypes, and the interaction between these factors. As can be seen in Figure SI6., neither diazepam nor the interaction of diazepam and the phenotype have any significant effect in the metabolite profile of glass eels. Furthermore, Fig. 6 illustrates that differences in the concentration of the two significant metabolites between active and inactive glass eels persist at similar levels regardless of diazepam exposure. Therefore, the mechanism through which diazepam may influence the migration tendency of glass eels seems to be independent of the levels of these markers. This supports the hypothesis proposed in the previous study and implies that, according to their metabolomic profile, glass eels not ready to migrate could change their behaviour due to the effect of diazepam. As also highlighted in that study, this would have major ecological consequences for glass eels as it would affect their spatial density and make them more vulnerable to predators. However, the findings of this study should be interpreted with caution due to the limited number of glass eels available. Future research should aim to replicate these results with a larger sample size and, ideally, with multiple independent tanks for each experimental group. Additionally, while the 5-day depuration period is essential to prevent confounding intrinsic differences between phenotypes with those arising from swimming activity, it may limit the detection of diazepam's effects. As a result, it is possible that some acute treatment effects were overlooked due to the experimental design. This potential impact of the depuration period could have been quantified if sufficient replicates had been available, allowing for sampling of glass eels both before and after this phase.

The relationship between metabolism and behaviour in fish is intricate, often challenging to decipher in metabolomic studies due to external factors that can mask results, as well as limitations in the resolution or sensitivity of the analyses. In this case, these external factors and the differentiation of phenotypes seen in the previous section, mask

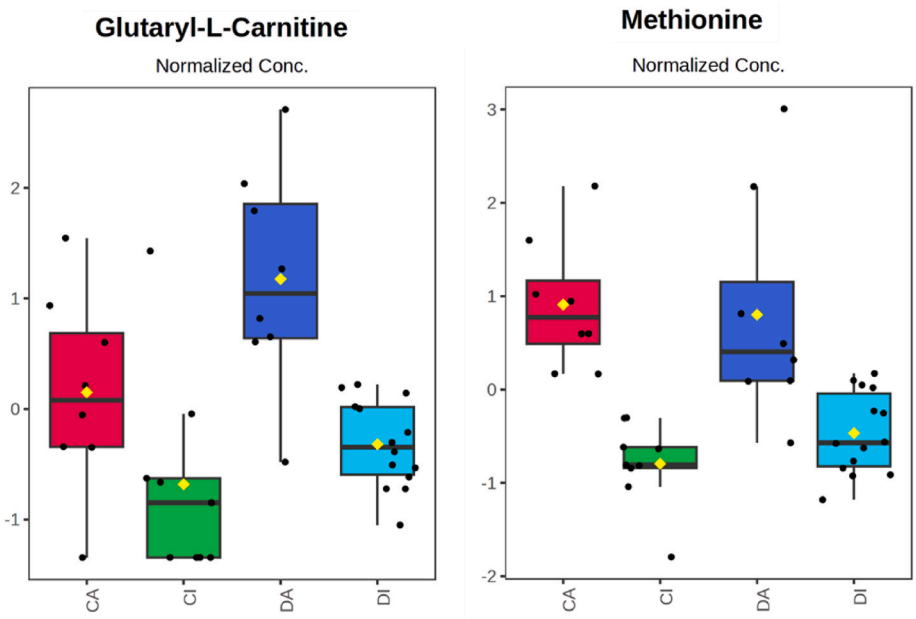


**Fig. 6.** Boxplots showing the normalized concentrations of glutaryl-L-carnitine and methionine across different groups: active controls (CA, N = 8), inactive controls (CI, N = 9), active exposed (DA, N = 8), and inactive exposed (DI, N = 14). The whiskers show the minimum and maximum values, while the box spans from the first quartile (lower limit) to the third quartile (upper limit). The line inside the box represents the median.

the effect of diazepam at the metabolic level. This aligns with findings from the previous study (Alvarez-Mora et al., 2023), where such effects were similarly not observed.

As for the lipid profile results, as in the previous section, the sensitivity of the results of this analysis was not sufficiently high to find significant differences between groups as none of the lipids detected passed the statistical test (FDR >0.05) for CA and DA or CI and DI. Given that there is no evidence that the psychoactive effect of diazepam can have an effect on lipid levels, this is in line with expectations as no differences were seen in section 3.1.2.

## 4. Conclusions

Understanding the European eel's migratory cycle is essential for conservation, and metabolomics can reveal the underlying physiological mechanisms. This study aimed to identify metabolites that distinguish active (migrant) from inactive (non-migrant) glass eels and determine if differences were due to swimming activity or intrinsic metabolic variations. Through target and non-target screening and PLS-DA modeling, we identified three key metabolites: methionine, glutaryl-L-carnitine, and palmitoylcarnitine, with methionine showing significant differences between groups. Glutaryl-L-carnitine's strong correlation with activity suggests it may be a more sensitive indicator than traditional measures. The study indicates that both swimming activity and intrinsic metabolic differences contribute to the observed variations, with methionine involved in both. While our metabolomic approach did not detect significant effects of diazepam on glass eel phenotypes, this research offers insights into the metabolic differences between behavioural phenotypes, and sets the stage for future studies in this field.

## CRediT authorship contribution statement

**Iker Alvarez-Mora:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Valérie Bolliet:** Writing – review & editing, Resources, Methodology, Conceptualization. **Naroa Lopez-Herguedas:** Writing – review & editing, Methodology, Investigation. **Colin Bouchard:** Methodology, Investigation. **Mathilde Monperrus:** Writing – review & editing, Supervision, Conceptualization. **Nestor Etxebarria:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2024.106801.

## Data availability

Data will be made available on request.

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