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Hepatic Mitochondrial Isolation Protocol in the Seahorse *Hippocampus erectus* Using Differential Centrifugation

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Abstract

Mitochondrial isolation is a critical step in respirometry studies that aim to elucidate cellular bioenergetics and mitochondrial function. In aquatic organisms such as fish, measurements of mitochondrial respiration offer valuable insights into how physiological performance is shaped by environmental factors such as temperature changes and oxygen availability. The liver, with its high metabolic activity and mitochondrial density, is an optimal tissue for these analyses. Here, we describe a protocol for isolating hepatic mitochondria from *Hippocampus erectus*, a tropical seahorse species of ecological interest. Approximately 0.2 g of liver tissue was collected from individuals weighing 12.26 ± 0.25 g, resulting in mitochondrial suspensions with protein concentrations of 6 - 10 mg/mL. At these concentrations, oxygen flux can be reliably measured using high-resolution respirometry (HRR) with the Oxygraph^{TM} O2k (Oroboros Instruments, Innsbruck, Austria), which has a minimum detectable flux of approximately 5 pmol $O_2 \cdot s^{-1} \cdot mL^{-1}$. The protocol covers tissue dissection, HRR system calibration, differential centrifugation for mitochondrial isolation, and subsequent measurement of mitochondrial respiration. This work provides essential methodological and physiological insights that advance the growing field of bioenergetic research in non-model marine species.

Guidelines

Measuring mitochondrial respiration

Hepatic mitochondrial respiration was determined using **high-resolution respirometry (HRR)** with an Oxygraph™ O2k (Oroboros Instruments, Innsbruck, AT), which employs a polarographic oxygen sensor to detect oxygen (O_2) flux with a sensitivity of ±1 pmol $O_2 \cdot s^{-1} \cdot mL^{-1}$, following the manufacturer's guidelines and previously established protocols (Gnaiger, 2001; MiPNet06.03; MiPNet22.10).

Before adding the mitochondrial sample, the polarographic sensors were calibrated (*see below:* Equipment Setup).

Equipment setup

Calibration of polarographic oxygen sensors at experimental temperature

1. Add the required volume of mitochondrial respiration buffer (MiR05) to the chamber (this protocol uses 2 mL) and calibrate the O_2 sensors accordingly.

NOTE: Mitochondrial Respiration Buffer MiR05 should be used instead of distilled water for calibration.

2. Wait for an equilibrium with atmospheric oxygen and the required experimental temperature; the system reaches the steady basal consumption state of the system in operation, a point where the O_2 consumption rate is constant.

3. Start recording oxygen consumption. Verify that the recording is stable and that no drifts are apparent. 4. Once a stable signal is obtained and the background oxygen consumption of the system is minimal, proceed with mitochondrial addition.

Materials

Chemicals for *Mitochondrial Isolation Buffer* and *Mitochondrial Respiratory Buffer*

- Sucrose (Sigma, cat. no. S 9378)
- Potassium chloride (KCl, Sigma, cat. no. P 5405)
- Ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA, Sigma, cat. no. E3889)
- 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES, Sigma, cat. no. H 4034)
- Bovine serum albumin (BSA), fatty acid-free (Sigma, cat. no. A 7030)
- Magnesium chloride ($MgCl_2$, Sigma, cat. no. 208337)
- Lactobionic acid (Sigma, cat. no. 15316)
- Taurine (Sigma, cat. no. T0625)
- Potassium phosphate monobasic (KH_2PO_4, Sigma, cat. no. P5379)

Chemicals for respiratory substrates and inhibitors

- Malate (M, Sigma-Aldrich: M1000)
- Glutamate (Sigma-Aldrich: G1626)
- Adenosine 5 ′diphosphate potassium salt (ADP; Sigma, cat. no. A 5285)
- Oligomycin from *Streptomyces diastatochromogenes* (Sigma, cat. no O 4876)
- Antimycin A (from *Streptomyces* sp., Sigma, cat. no A 8674)
- Rotenone ($C_{23}H_{22}O_6$, Sigma, cat. no. 45656)

Other chemicals

- Ethanol (70 %)
- Bi-distilled water
- 5 M Potassium hydroxide (KOH, Sigma, cat. no 484016)

Preparation of mitochondrial isolation buffers

***Mitochondrial Isolation Buffer A*:** Mix all the reagents from Table 1.
***Mitochondrial Isolation Buffer B*:** Mix all the reagents in Table 1 except for the BSA-free fatty acids.

Table 1. Modified isolation buffer from Lin *et al.* (2016)

	A	B	C	D	E	F
	Component	**MW g/mol**	**Molarity (mM)**	**Osmoles**	**Osmolarity**	**Amount for 100 mL final volume [g]**
	Sucrose	342.30	310	1	310	10.61
	KCl	74.55	52	2	104	0.38

	A	B	C	D	E	F
	EGTA	380.35	2	1	2	0.076
	HEPES	238.30	25	1	25	0.59
	BSA	0.5%	5 g/L			0.20
					441 mOsmoles	

1. Once dissolved, the pH of the buffer is adjusted at 7.4. The pH adjustment is done with 5 M KOH.
2. Both mitochondrial isolation buffers are stored at -80 °C for up to six months.

Important: When using both mitochondrial isolation buffers, thaw at room temperature or in a 36 °C water bath. They are kept refrigerated until use. Once opened, they can be refrozen at -20 °C and used for the next six days.

Preparation of MiR05 Mitochondrial Respiratory Buffer

The MiR05 mitochondrial respiration buffer was prepared according to the standardized protocol described in Gnaiger *et al.* (2018). See Table 2 for buffer components and final concentrations.

Table 2. Mitochondrial respiration buffer MiR05 (Gnaiger et al. 2018)

	A	B	C	D
	Component	**MW g/mol**	**Final concentration [mM]**	**Amount for 250 mL final volume [g]**
	EGTA	380.4	0.5	0.047
	MgCl2	95.2	3	0.071
	Lactobionic acid	358.3	60	5.375
	Taurine	125.1	20	0.625
	KH2PO4	136.1	10	0.340

	A	B	C	D
	HEPES	238.3	20	1.191
	Sucrose	342.3	110	9.413
	BSA	0.1%	1g/L	0.250

Use on the day of experiment:

1. Thaw at room temperature or in a water bath at 36 ° C.
2. Once thawed add 0.05 g of BSA in 50 mL of MiR05.
3. Keep cold until use.

Preparation of substrates and inhibitors for high-resolution respirometry

All substrates and inhibitors used in this procedure were prepared following the manufacturer's guidelines available on the Oroboros Instruments website. Detailed preparation steps are provided below; for further reference, consult **https://wiki.oroboros.at/index.php/**

- **400 mM Malate stock solutinon:**
  Dissolve 268.2 mg of L-malic acid in 3 ml of bio-distilled water, neutralize with 5 M KOH (~ 900 µl. Adjust the final volume to 5 ml. Prepare 200 µl aliquots and store at -20 °C.

- **2000 mM Glutamate stock solution:**
  Dissolve 1691 mg of L-glutamic acid monosodium salt hydrate in 4 mL of bio-distilled water. Check the pH and adjust to 7.0 if necessary, using 5 M KOH (usually no adjustment is needed). Adjust the final volume to 5 ml. Prepare 500 µl aliquots and store at -20°C.

- **500 mM ADP stock solution:**
  Dissolve 501.3 mg of ADP in 1.2 mL of bio-distilled water (ADP does not dissolve at this stage), neutralize with 5 M KOH (~ 450 µl) and check pH 7. Adjust the final volume to 2 ml. Prepare 200 µl aliquots and store at -80 °C.

- **5 mM Oligomycin stock solution:**
  Dissolve 4 mg of oligomycin in 1 ml of ethanol 70%. Prepare 200 µl aliquots and store at -20 °C.

- **1 mM Rotenone stock solution:**
  Dissolve 0.39 mg of rotenone in 1 ml of ethanol 70%. Prepare 200µl aliquots and store at -20 ° C.

- **5 mM Antimycin A stock solution:**
  Dissolve 5.4 mg of antimycin A in 2 mL ml of 70% ethanol solution. Prepare 200 µl aliquots and store at -20 °C.

Troubleshooting

Safety warnings

Note on nomenclature: This protocol adopts the updated functional terminology proposed by Gnaiger et al. (2020), in which mitochondrial respiratory states are defined based on their physiological function (e.g., **LEAK, OXPHOS, ROX**), rather than using the classical State 3/State 4 framework introduced by Chance and Williams (1955), which categorized respiration according to discrete transitions in ADP and substrate availability. For instance, $LEAK_n$ refers to leak respiration in the presence of NADH-linked substrates (malate and glutamate) in the absence of ADP, replacing what was traditionally referred to as State 2.

Ethics statement

Euthanize performed following the guidelines of the Ethics and Scientific Responsibility Committee of the institution (Official Letter No. CEARC/Bioethics/230123).

Before start

Before starting, pre-chill all materials to be used in the procedure.

Procedure: Isolation of Mitochondria from the Liver of Adult Seahorse (*Hippocampus erectus*)

1 **Starve the seahorse overnight** before the isolation experiment.
Note: The metabolic properties of liver mitochondria are strongly influenced by the metabolic state of the liver (fasted vs. postprandial; Panov 2015).

2 **Euthanize an adult *Hippocampus erectus* specimen** (12.26 ± 0.25 g) following the guidelines of the Ethics and Scientific Responsibility Committee of the institution (Official Letter No. CEARC/Bioethics/230123) and quickly remove the liver (**Figure 1a**).
CRITICAL STEP: A minimum of **0.2 g of liver tissue** from *H. erectus* is required for mitochondrial isolation.

3 **Place the liver immediately on a Petri dish on ice** and rinse the tissue with 1 mL of *Mitochondrial Isolation Buffer A* to remove blood and clean the organ.

4 **Mince the liver** into small pieces with a scalpel, keeping the Petri dish on ice throughout the process (**Figure 1b**).

5 **Transfer the minced liver** pieces into a homogenization tube containing 2 mL of ice-cold Mitochondrial Isolation Buffer A.

***NOTE*:** Homogenization and all subsequent steps must be performed at 4 °C.

6 **Homogenize the liver tissue** in a Potter-Elvehjem PTFE pestle and glass tube homogenizer (Sigma-Aldrich P7859-1EA) mounted on a drill set at 350 rpm. Perform 3–4 gentle strokes to fully homogenize the minced tissue, always keeping the tube on ice (**Figure 1c**).
CRITICAL STEP: Insert the drill-mounted pestle into the homogenization tube while it is rotating to prevent bubble formation and surface tension, which could cause the isolated mitochondria to rupture.

7 **Transfer the homogenate by rapid decantation** into a pre-cooled 2-mL Eppendorf ® tube.

8 **Centrifuge at 450 rcf, 4 °C, for up to 5 minutes,** keeping it on ice as much as possible (**Figure 1d)**.

9 **Transfer the supernatant** from the previous step through a filter into a new pre-cooled 2-mL Eppendorf® tube using a micropipette and keep it on ice (**Figure 1e, f)**.
CRITICAL STEP: Hold the Eppendorf® tube by the top of the cap to prevent warming and keep it on ice.

10 **Centrifuge the transferred supernatant** at 7,939 rcf for 15 min at 4 °C to pellet the mitochondria (**Figure 1h)**.

11 **Discard the supernatant by rapid decantation.** While keeping the tube on ice, quickly and carefully add 1 mL of cold Mitochondrial Isolation Buffer B and gently resuspend the pellet using a soft-bristle brush (no dyes).
CRITICAL STEP: Keep the tube on ice and resuspend without lifting it.

12 **Add 1 mL of cold *Mitochondrial Isolation Buffer B*.** Subsequently, shake gently and quickly to homogenize and keep on ice.

13 **Centrifuge at 7,939 rcf at 4 °C for 15 min** (**Figure 1i)**.

14 **Discard the supernatant by rapid decantation and retain the pellet**.
CRITICAL STEP: Keep the tube on ice and decant quickly.

15 **Add 160 µL of cold Mitochondrial Isolation Buffer B** to concentrate the sample and gently resuspend the pellet as in steps 10 and 11. Keep on ice.

16 **Measure mitochondrial protein concentration using the Bradford method** (Bradford, 1976). Based on our experimental results, mitochondrial suspensions from the liver of adult *Hippocampus erectus* contain approximately **6-10 mg protein/mL per 0.2 g of minced tissue**.
CRITICAL STEP: Use the mitochondrial preparation for respirometry experiments within 1–3 h to ensure optimal functional responses.

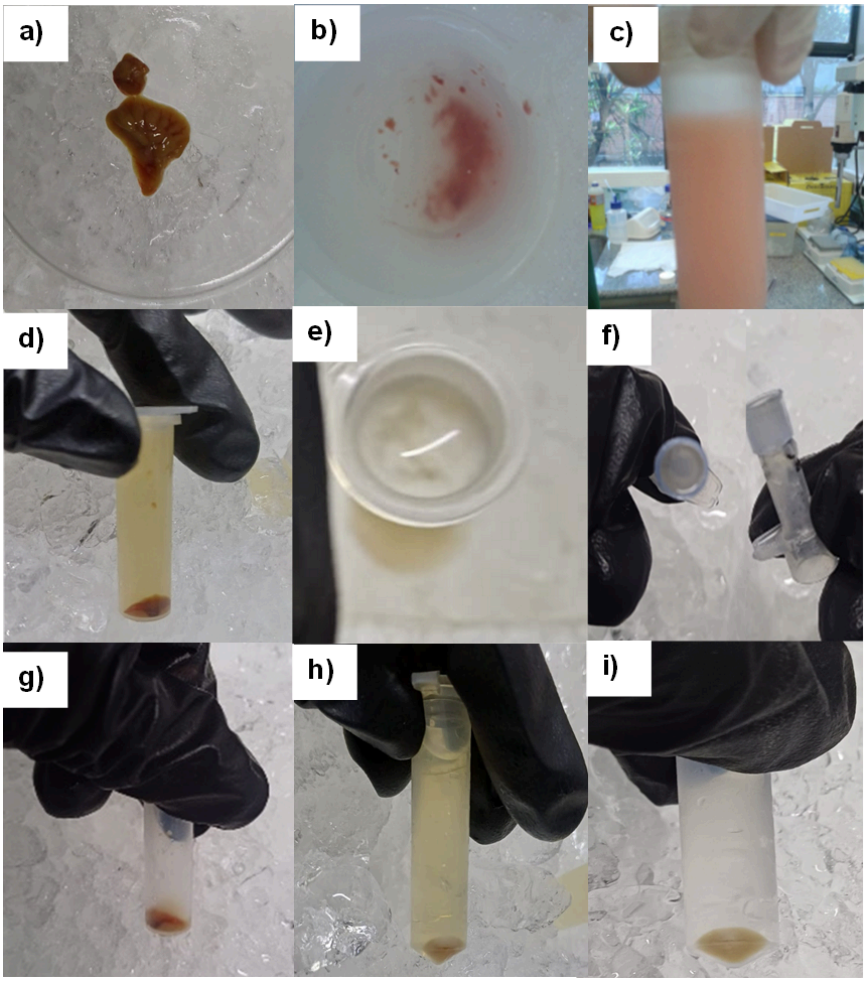

Figure 1. Isolation of mitochondria from the liver of *Hippocampus erectus*. **(a)** Freshly dissected liver placed on ice. **(b)** Liver cut into small pieces in a Petri dish with isolation buffer A. **(c)** Homogenization of liver tissue using a Potter-Elvehjem pestle mounted on a drill at 350 rpm. **(d)** First centrifugation at 450 rcf, producing a pellet and a supernatant. **(e, f)** Supernatant with lipid layer and appropriate filters. **(g)** Residual pellet after supernatant removal. **(h)** Second centrifugation at 7,939 rcf to pellet mitochondria (first wash). **(i)** Third centrifugation at 7,939 rcf to pellet mitochondria (final, second wash).

Addition of Seahorse Liver Mitochondria

17 Using a Hamilton microsyringe (Oroboros Instruments), add 600 µg of mitochondria isolated from seahorse liver to a 2 mL respiration chamber (final concentration 300 µg/mL).

Note: The sample concentration should be high enough to provide a reliable respiratory flux per chamber volume, particularly when mitochondria have low activity. This concentration corresponds to a minimum detectable flux of ~5 pmol $O_2 \cdot s^{-1} \cdot mL^{-1}$.

Addition of Malate (M) and Glutamate (G)

18 After adding mitochondria, add 10 µL of 400 mM malate (M) and 10 µL of 2000 mM glutamate (G) to a 2 mL respiration chamber (final concentrations: 2 mM malate, 10 mM glutamate).

Purpose / Effect:

- Feeds electrons into Complex I (glutamate–malate pathway) for NADH-linked respiration.
- Establishes the **LEAK*n*$_{MG}$'** state, representing basal respiration driven by proton leak in the absence of adenylates (i.e., before ADP addition).

Addition of ADP

19 Add 10 µL of 500 mM ADP stock solution to the 2 mL respiration chamber (final concentration: 2.5 mM ADP).

Purpose / Effect:

- Stimulates active ATP synthesis, significantly increasing oxygen consumption.
- Establishes the **OXPHOS$_{MG}$'** state, where oxygen flux reflects ADP-driven oxidative phosphorylation via Complex I.

***CRITICAL STEP*:** The oxygen consumption rate must increase substantially compared to the leak state (**LEAK*n*$_{MG}$'**). This confirms that the mitochondria are well-coupled and functionally intact.

Addition of Oligomycin A

20 Add 1 µL of 5 mM oligomycin stock solution to the 2 mL respiration chamber (final concentration: 0.0025 mM).

Purpose / Effect:

- Inhibits ATP synthase, effectively blocking oxidative phosphorylation (OXPHOS).
- Induces the **LEAK$_{OMY\,MG}$'** state, where oxygen consumption rapidly decreases and stabilizes at a level reflecting proton leak across the inner mitochondrial membrane and other non-phosphorylating processes.

Addition of Rotenone and Antimycin A

21 Add rotenone (final concentration: 0.0025 mM) and antimycin A (final concentration: 0.0125 mM) to the 2 mL respiration chamber.

Purpose / Effect:

- Rotenone (Complex I inhibitor) and antimycin A (Complex III inhibitor) block electron flow through the electron transport system (ETS), fully inhibiting mitochondrial respiration.
- Establishes the ROX state, where residual oxygen consumption reflects non-mitochondrial processes (e.g., oxidative side reactions or background chemical consumption).

Calculations and Corrections

22 Correct the respiratory states for non-mitochondrial respiration (ROX):

$OXPHOS_{GM} = OXPHOS_{MG}' - ROX$

$LEAK_{OMY\,MG} = LEAK_{OMY\,MG}' - ROX$

Respiratory parameters

23 Respiratory Control Ratio (RCR): $OXPHOS_{MG} / LEAK_{OMY\,MG}$

Phosphorylation capacity (*P*): $OXPHOS_{MG} - LEAK_{OMY\,MG}$

Final note: Ensure that a steady-state rate is reached before proceeding to the next titration. Re-oxygenate the chamber if oxygen levels drop below 20 µM to prevent hypoxic conditions, which may alter mitochondrial function.

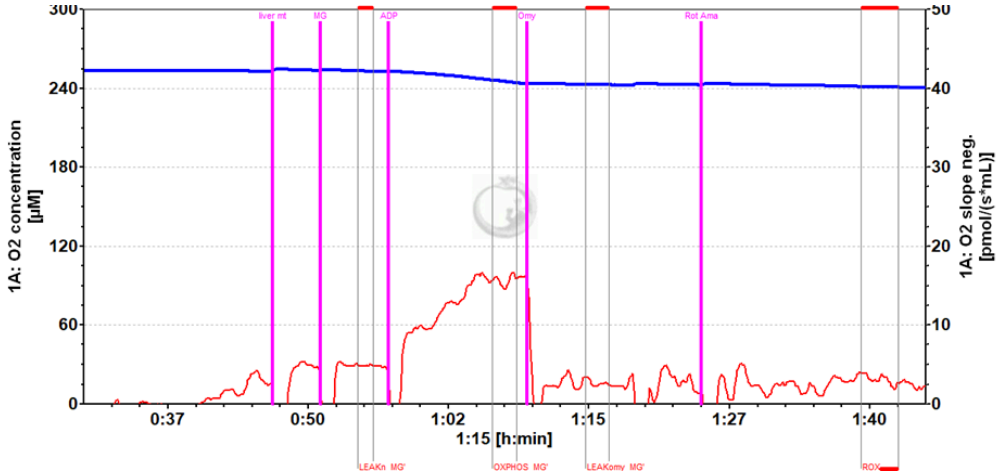

Figure 2. Schematic representation of the protocol used to determine oxygen consumption rates in different mitochondrial respiratory states (LEAK*n* $_{MG}$ ', OXPHOS $_{MG}$', LEAK$_{OMYMG}$', and ROX). The blue line represents dissolved oxygen concentration (µM), while the red line indicates the oxygen consumption rate (pmol O_2·s^{-1}·mL^{-1}). *Liver mt*: liver mitochondria; *MG*: malate and glutamate; *ADP*: adenosine diphosphate; Omy: oligomycin; Rot/Ant: rotenone and antimycin A. The respiratory states were defined as follows: LEAK*n*$_{MG}$' refers to leak respiration in the presence of malate and glutamate; OXPHOS$_{MG}$' corresponds to ADP-stimulated oxidative phosphorylation; LEAK$_{OMY\ MG}$' represents oligomycin-induced leak respiration; and ROX indicates residual non-mitochondrial respiration.

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