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"INSECT CELL LINES: A SUSTAINABLE SOURCE OF NOVEL FOOD"

SSD

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ABSTRACT

This thesis explores insect cell lines as a foundation for sustainable protein production, focusing on cultivated meat and circular bioeconomy pathways. The work focuses on cellular physiology, differentiation pathways, and tissue organization relevant to muscle and adipose development for cultivated meat. The thesis reviews the technological and socio-regulatory context for *Hermetia illucens* (black soldier fly, BSF) across food, feed, and pet nutrition, highlighting consumer acceptance factors, risk management along processing chains, and the European "novel foods" framework. These considerations inform product design and regulatory readiness for insect-derived ingredients and for insect-based cellular agriculture. Empirically, the thesis advances practical routes to muscle-oriented insect primary cultures in two insect species. In BSF, it identifies a developmentally informed window to obtain heterogeneous yet myogenesis-prone primary cultures and links controllable culture levers—such as adhesion kinetics and calcium chelation—to shifts in myogenic organization, supported by molecular and imaging readouts. These findings establish a tractable benchmark for downstream efforts in serum reduction, scalability, and scaffold integration for cultivated meat. As concern the second insect species treated, *Tenebrio molitor* (yellow mealworm), the thesis establishes embryo-derived primary cultures with sustained viability and spontaneous contractile features and evaluates selective-adhesion and time- dependent pre-plating as routes to myogenic enrichment, while also delineating constraints encountered in neonate-larvae based approaches. Collectively, the thesis contributes (i) a cross-species conceptual framing for insect cells in cellular agriculture, (ii) practical, species-tailored workflows for initiating and biasing myogenic phenotypes, and (iii) a translational lens spanning safety, acceptance, and regulation.

ABSTRACT

Questa tesi esplora le linee cellulari di insetto come base per la produzione sostenibile di proteine, con particolare attenzione alla carne coltivata e ai percorsi di bioeconomia circolare. Il lavoro si concentra sulla fisiologia cellulare, sui percorsi di differenziamento e sull'organizzazione tissutale rilevanti per lo sviluppo muscolare e adiposo in ambito carne coltivata. La tesi esamina il contesto tecnologico e socio-regolatorio relativo a *Hermetia illucens* (black soldier fly, BSF) nei settori alimentazione umana, mangimistica e pet food, mettendo in evidenza i fattori che influenzano l'accettazione da parte dei consumatori, la gestione del rischio lungo le filiere di trasformazione e il quadro europeo dei "novel foods". Queste considerazioni orientano la progettazione del prodotto e la preparazione regolatoria per ingredienti derivati da insetti e per applicazioni di agricoltura cellulare basata su cellule di insetto. Dal punto di vista empirico, la tesi sviluppa protocolli pratici verso colture primarie di insetto a orientamento muscolare in due specie di insetti. Nel caso di BSF, viene individuata una finestra temporale definita dallo stadio di sviluppo che consente di ottenere colture primarie eterogenee ma predisposte alla miogenesi e mette in relazione specifiche leve di coltura controllabili—come la cinetica di adesione e la chelazione del calcio—con cambiamenti nell'organizzazione miogenica, supportati da analisi molecolari e di imaging. Questi risultati stabiliscono un riferimento sperimentale gestibile per sviluppi successivi in termini di riduzione del siero, scalabilità e integrazione con scaffold per carne coltivata. Per quanto riguarda la seconda specie di insetto considerata, *Tenebrio molitor* (yellow mealworm), la tesi istituisce colture primarie derivate da embrioni con vitalità sostenuta e caratteristiche contrattile spontanee, e valuta l'adesione selettiva come strategia per ottenere un arricchimento miogenico, delineando al contempo i limiti riscontrati negli approcci basati su larve neonate.

Nel complesso, la tesi contribuisce (i) un inquadramento concettuale comparativo per l'impiego delle cellule di insetto nell'agricoltura cellulare, (ii) workflow pratici e specie-specifici per l'avvio e l'indirizzamento di fenotipi miogenici e (iii) una prospettiva traslazionale che integra aspetti di sicurezza, accettazione e regolamentazione.

RESEARCH AIMS

This doctoral research was conducted at the Insect physiology and molecular biology Laboratory, Department of Basic and Applied Sciences (DiSBA), University of Basilicata (UNIBAS), under the supervision of Prof. Patrizia Falabella and the co-supervision of Dr. Ambrogio Laginestra and Dr. Carmen Scieuzo. Selected activities were performed in collaboration with the Tufts University Center for Cellular Agriculture (TUCCA), Medford, USA, under the supervision of Prof. David L. Kaplan. This thesis aims to establish insect-derived cell-culture pipelines as a viable foundation for cultivated meat by integrating a cross-species conceptual frame with species-tailored experimental workflows for *Hermetia illucens* and *Tenebrio molitor.* It aims to identify developmental stages optimal for isolation and to standardize primary-culture methods that yield myogenesis-prone *H. illucens* populations, and deploys enrichment levers—EGTA- mediated Ca^{2+} chelation and time-dependent adhesion—to bias early myogenic organization, validated through morphological, molecular, and functional readouts (e.g., MHC labeling, intermittent contractility). In parallel, it establishes embryo- derived *T. molitor* cultures and applies the same enrichment logic to map adhesion kinetics and selective retention of myogenic cells, codifying antibody-screening to support cross-species immunolabeling. Collectively, the research enhances reproducibility, and scalable media design accelerating the progression from insect primary cultures toward application-ready muscle tissues for cellular agriculture.

CHAPTER 1

1. A glance into the near future: cultivated meat from mammalian and insect cells

This chapter frames the thesis by articulating the conceptual and translational rationale for cultivated meat across mammalian and insect systems and clarifies how process constraints motivate insect-cell platforms. This chapter is based on the following publication:

Giglio, F., Scieuzo, C., Ouazri, S., Pucciarelli, V., Ianniciello, D., Letcher, S., Salvia, R., Laginestra, A., Kaplan, D. L., & Falabella, P. (2024). A glance into the near future: Cultivated meat from mammalian and insect cells. Small Science. https://doi.org/10.1002/smsc.202400122

Global meat production is anticipated to increase by almost 44 Mt by 2030, reaching 373 Mt because of rising production as meat prices resume following COVID-19, according to the publication "OECD-FAO Agricultural Outlook 2021-2030" (OECD/FAO,2021). Meat consumption is influenced by a variety of factors, including prices, tradition, environmental concerns, animal welfare and health. Population growth is the primary driver of rising meat consumption, with a projected 14% increase in global meat intake due to an anticipated 11% global rise in population by 2030. Specifically, this increase will be 12% in Latin America, 18% in Asia-Pacific, 30% in Africa, 0.4% in Europe, and 9% in North America. Economic growth and its structural changes encourage increased meat consumption. According to empirical studies about consumer behavior, a higher income drives consumption of more high-value foods, such as animal proteins, and fewer low-value products, such as carbohydrates. All these factors have contributed to a dramatic increase in livestock production over the past decade, with growing demand for animal-based foods among a significant portion of the global population, represented by developing countries (Delgado *et al.,* 1999). This rising demand is problematic since current large-scale animal farming techniques (generating more than 50% of the world's meat supply) are associated with public health risks, environmental degradation, and animal

welfare concerns (Rao *et al.,* 2005). For example, 75% of new infectious diseases in humans are caused by animal sources (zoonotic), primarily as a result of increased human- animal interactions caused by animal husbandry, loss of natural habitats, and the increasing global population (Jones *et al.,* 2013; Wolk, 2017). Animal husbandry, which accounts for 80% of antibiotics used in the United States and 73% of antibiotics sold globally, exacerbates antibiotic resistance, causing increasing risk to human health. Based on the 2020 report by the United Nations Environment Programme on the prevention of future pandemics, the escalation in the worldwide requirement for animal protein products and the unsustainable intensification of agriculture, including the surge of intensive animal agriculture, are two of the seven significant anthropogenic factors that contribute to the emergence of zoonotic diseases. Beyond the dangers to human health, livestock are responsible for 14.5% of all anthropogenic greenhouse gas emissions measured in CO_2 equivalents. In addition, the production of animal feed has a substantial impact on the environment in terms of land and water use (Godfray *et al.,* 2018). In response to growing concern about the sustainability of large-scale agriculture, new technologies are emerging for more efficient protein production. One such solution is cultivated meat (CM; also known as cell-based or cultured meat), which involves the production of meat through *in vitro* cultivation of animal stem cells, mimicking the natural process of cell growth and division in animals, resulting in a product like traditional meat in terms of nutrition and taste. This is intended to address environmental and animal welfare issues while meeting the needs of a growing global population. Research into cultivated meat dates to 2002, when it was observed that the utilization of cultured fish cells could potentially aid in the development of a goldfish muscle explant (Brnjaminson *et*

al., 2002). The first official taste of CM was in 2013, when Dr. Mark Post's team created a highly publicized hamburger from bovine muscle cells. A growing number of organizations are currently commercializing and scaling CM (at least 70 were reported in mid-2021) (Post, 2012). For these reasons, in November 2022, the Food and Drug Administration (FDA) assessed the safety of "Cultured *Gallus gallus* cell material" provided by UPSIDE. The FDA determined that this was safe and found no evidence that its production process could introduce harmful substances or microorganisms into the food (US Food Drug Adm, 2022). Similarly, in March 2023, the FDA evaluated cultured *Gallus gallus* cell material from GOOD Meat,[10] a company that already sells CM in Singapore. Singapore was the first country to approve cultured meat production in December 2020, specifically the cultivated chicken bites produced by the US start-up Eat Just consisting of 70% cultivated chicken cells and 30% plant-based components (US Food Drug Adm, 2023; Singapore Food Agency, 2021; Failla *et al.,* 2023). Food safety regulations vary between countries and regions. In the USA, the FDA oversees food safety, except for meat and poultry, which fall under the jurisdiction of the US Department of Agriculture Food Safety and Inspection Service (USDA– FSIS) under the Federal Meat Inspection Act (FMIA) (Post *et al.,* 2020). Regarding regulations for cell-based meat for human consumption, the USDA and FDA issued a joint statement in 2018 (Grossman, 2019). Under this agreement, the FDA oversees early stages of cell-based meat development, including cell collection development, differentiation, and proliferation processes (US Food and Drug Adm, 2024). This oversight applies to products derived from cell lines of USDA- amenable species and requires a USDA mark of inspection (Broucke *et al.,* 2023). Once cells or tissues are ready for harvest, regulatory oversight shifts from the FDA to USDA–FSIS, which

ensures the safety, labeling, and overall quality of cell-based meats. Both agencies inspect production facilities, with USDA–FSIS focusing on final production stages (US Food and Drug Adm, 2024). Unlike the United States, CM in Europe falls under either the EU Novel Foods Regulation, which pertains to foods and ingredients not significantly consumed in the EU before May 15, 1997 (Broucke *et al.,* 2023; EU 2015/2283) or the GMO legislation (embodied by the GMO Directive (European Commision, 2001) and GMO Regulation (European Commision,2013), when genetic modification techniques falling within the scope of GMO legislation are used to generate or modify the cell lines (for example in some induced pluripotent stem cell (iPSCs)–based systems) (Stephens *et al.,* 2018; US Food and Drug Adm, 2024). Member States conduct consultations to determine whether a particular food falls under which regulation, with safety assessments conducted by the European Food Safety Authority (EFSA). The main objective of this review is to assess the potential of insect cells as a sustainable and efficient source of CM. This involves a comprehensive comparison of their characteristics with those of mammalian cells, identifying their respective strengths and weaknesses. In exploring both muscle and adipose tissues, it is essential to recognize the distinct differences between mammals and insects in terms of cellular origins, molecular regulatory pathways, and physiological functions. These comparisons not only illuminate the biological complexity and diversity of these systems in both groups but also highlight their potential biotechnological applications, including the cultivation of meat.

1.2 Structure of muscle tissue

In the production of CM, three fundamental elements play pivotal roles: cells,

signals (present in the culture medium), and scaffolds. Cells are the key element, while the culture media provides essential nutrients and small molecules to support cell growth and functions. Scaffolds, made of biocompatible materials, serve as a support to which cells are anchored, facilitating their proliferation and differentiation (Figure 1). The aim of the *in vitro* CM process is to recreate the tissue structure of animals from different cell sources, primarily focusing on muscle and fat. Myoblastic cells, crucial for muscle tissue formation, can be obtained through various methods. The most common approach involves performing a tissue biopsy of the desired animal or utilizing post-mortem tissues. The alternative approach utilizes a source of pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) or iPSCs. In the first scenario, primary cell cultures can be used directly. In the second, the cells undergo differentiation into mesodermal cells before becoming muscle progenitor cells (Reiss *et al.,* 2021). Myoblast cells fuse naturally in a process known as myogenesis, i.e., the formation of muscle tissue that occurs particularly during embryonic development. The architecture of skeletal muscle is a well-organized distribution of multinucleated contractile muscle cells (also known as muscle fibers) and related connective tissue (Mukund and Subramaniam 2020). Muscle development occurs *in vivo* during embryogenesis with the multiplication of mononucleated myoblasts, which eventually fuse and divide to produce muscle fibers (Chal and Pourquie 2017). Muscle fibers are functional units surrounded by connective tissue, intramuscular fat, blood vessels, and nerves. The muscle fibers are organized into bundles, and the surrounding connective tissue is composed of endomysium, perimysium, and epimysium. The vessels ensure the transfer of oxygen and nutrients (Listrat *et al.,* 2016). The nutritional value of meat derives mostly from high-quality protein from muscle that contains all

essential amino acids, essential fatty acids, and a variety of vitamins and minerals. Red muscle tissue has more myoglobin and, consequently, more heme iron than white muscle tissue (Listrat *et al.,* 2016), making it a more nutritious source of bioavailable iron. Intramuscular fats contribute to the texture, nutrition, and species-specific flavor of meat (Kerry *et al.,* 2002). The predominant composition of intramuscular fat consists of adipocytes, which are situated within the interstitial spaces of muscle fibers and fascicles. Intramuscular adipose tissue is composed of various lipid components, including structural lipids, phospholipids, and intracellular lipid droplets located within muscle fibers. In addition, lipids found in fat contain crucial lipophilic vitamins, including A, D, K, and E, alongside essential omega-3 polyunsaturated fatty acids (Fish *et al.,* 2020).

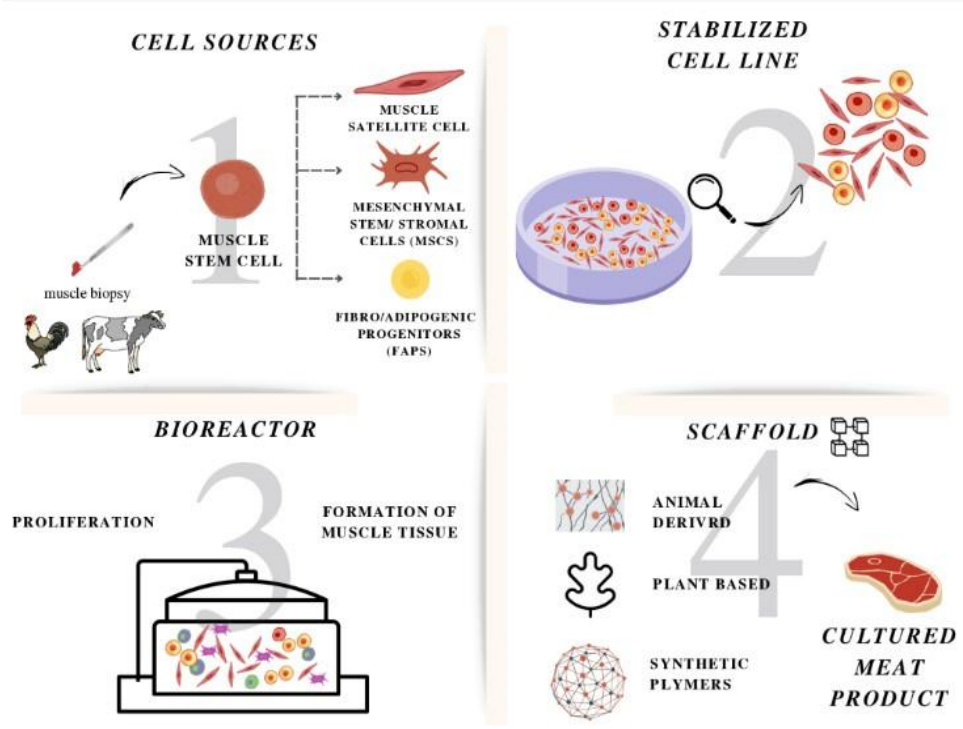


Fig 1. *Overview of in vitro cultivated meat production.* The first step involves harvesting cells from a live animal and obtaining stem cells. The primary cells are then cultivated in an appropriate, nutrient-rich medium and a cell line is established. A co-culture of muscle and adipocyte cells are introduced into a

bioreactor, a sterile food production facility, to grow and differentiate until muscle tissue is formed. The cells will organize on scaffolds of various origins to form the edible product (Image Created with BioRender.com).

1.3 Cell Sources

The composition of meat typically comprises approximately 90% muscle fibers, 10% fat and connective tissue (Listrat *et al.,* 2016; Warris and Rhodes, 1977), although this varies depending on specific cut of meat and the species it is derived from. Skeletal myocytes are the most numerous cell types in meat, with adipocytes, fibroblasts, chondrocytes, and hematopoietic cells also present and often providing support (Figure 2a). Stem cells sourced from a living animal biopsy can be cultivated *in vitro* to yield substantial cell numbers. These versatile stem cells possess the capability to differentiate into either muscle or fat cells, determined by their specific type (Post, 2014). Primary cell types for CM production must be capable of adequate self-renewal and differentiation into the mature cell types that characterize meat. Stem cells are the best option for use as a source of starting cells to satisfy these needs. Adult stem cells and PSCs are the two types of stem cells with the proliferative capacity and differentiation potential necessary for the generation of CM. Traditionally, tissue- specific stem cells have been the preferred cell source for CM production. They are undifferentiated progenitor cells present in the organs and tissues of animals. Tissue-specific stem cells are multipotent, meaning they may differentiate into several cell types, the majority of which are relevant to the organ or tissue in which they reside. Within the microenvironment of muscle tissue, the three most frequently encountered types of progenitor/stem cells are muscle satellite cells, mesenchymal stem/stromal cells (MSCs), and fibro/adipogenic progenitors (FAPs). The progenitor cells possess the ability to undergo differentiation and give rise to various mature cell types, including but not limited to skeletal

myocytes, adipocytes, chondrocytes, and fibroblasts. Muscle satellite cells are a type of stem cell that can be located beneath the basement membrane of muscle fibers. These cells can differentiate into myocytes, which then form multinucleated myotubes that are densely packed into myofibers. Muscle satellite cells are one of the most prevalent forms of tissue-resident adult stem cells (Bentzinger *et al.,* 2012), and their extraction from animals and maintenance *in vitro* are well-described (Ding *et al*, 2018; Li *et al.,* 2015). Muscle satellite cells are commonly found in the bone marrow, but they can also be found in other anatomical locations, such as skeletal muscles and play a crucial role in muscle regeneration following damage (Yin *et al.,* 2013). MSCs possess the ability to undergo differentiation into adipocytes, chondrocytes, and fibroblasts (Warriss and Rhodes, 1977; Ding *et al*, 2018). Mosa Meat, the pioneer of the first cultivated hamburger, has laid the groundwork for cleaner meat alternatives through the use of mesenchymal stem cells (MSCs) (Post, 2014; Mosa Meat, 2024). Various startups, such as BioTech Foods (Bio Tech Food, 2024), harness skeletal muscle cells from cattle and/or pigs to create CM products, whereas companies like Meatable (Meatable, 2024) use pluripotent stem cells (e.g. iPSCs) that are subsequently differentiated into skeletal muscle cells. These products range from minced meat alternatives to delectable and nutritious items like nuggets, hamburgers, and sausages. To achieve the ideal amplification level for processed CM, muscle satellite cells must undergo cell fusion and transition into multinucleated, postmitotic muscle fibers. The differentiation process to myotubes *in vitro* begins upon MSCs' exposure to a differentiation medium, typically spanning three to five days (Danoviz and Yablobka-Reuveni, 2012). Controlling MSCs activity often involves manipulating extracellular signaling molecules present in the culture medium. Growth factors (GFs) such as insulin-

like GFs (IGF-1 and -2), fibroblast GF, hepatocyte GF, and cytokines like TNF-α and LIF (leukemia inhibitory factor) play pivotal roles in driving MSCs activation and proliferation (Bertizinger *et al.,* 2010; Spangenburg and Booth, 2002). FAPs are a separate population of mesenchymal cells found in the interstitial space of skeletal muscle. FAPs can differentiate into both fibroblasts and adipocytes, which are the connective and fatty tissues found in meat and play a crucial role in myogenic development and organization (Li *et al.,* 2015; Maqsood et. al., 2013). Dedifferentiated fat cells (DFAT) have been identified as a plausible cellular source for the cultivation of adipose tissue. These cells are obtained through the process of dedifferentiation of mature adipocytes. Several commercially accessible immortalized preadipose cell lines exist, such as 3T3-L1, 3T3-F442A, and OP9. These exhibit distinct attributes, conducive to the generation of cell culture fat for human consumption. These include a notable capacity for cellular proliferation, resilient differentiation into adipocytes, uniformity in cell populations, uncomplicated maintenance procedures, and comprehensive characterization. Nonetheless, most of these cellular lineages are derived from murine origins, thereby restricting their efficacy in the context of investigating and advancing the production of CM (Fish *et al.,* 2020). When satellite cells, MSCs, and FAPs are combined, they have the potential to generate all the cell types present in meat. While tissue-specific stem cells are readily available and capable of differentiating into the necessary mature cell types found in meat, their proliferation and maintenance *in vitro* are restricted. Pluripotent stem cells have potential as a second cell source for CM production, even though primary tissue-specific stem cells are a popular cell source. PSCs, such as ESCs and iPSCs, are highly proliferative in culture and can differentiate into every cell type seen in the three primary germ layers (i.e., mesoderm,

endoderm, ectoderm). ESCs are sourced from the inner cell mass of the blastocyst, a developmental stage that takes place during the initial phases of mammalian growth. iPSCs are generated by triggering pluripotency genes in somatic cells (Figure 2b) (Listrat *et al.,* 2016; Ben- Arye and Levenberg, 2019). PSCs derived from non-muscle sources, can be isolated from various domestic animals and harnessed as myogenic cell reservoirs for CM production. Recent advancements include the chemical and genetic modification of pig pluripotent stem cells to prompt their differentiation into myogenic cells capable of forming embryonic muscle fibers (Genovese *et al.,* 2017; Shaikh *et al.,* 2021). Gourmey utilizes PSCs to craft cultured foie gras (Gourmey, 2024). It's crucial to note that in the EU, any CM product – whether derived from PSCs or adult stem cells – must undergo a detailed pre-market safety assessment, typically within the framework of the Novel Foods Regulation (Regulation (EU) 2015/2283). GM labelling is required only when the product meets the legal definition of a genetically modified food, i.e. when it contains, consists of, or is produced from GMOs; the mere use of pluripotent stem cells does not automatically trigger GM labelling, which instead depends on whether genetic modification is applied to the cell line or during the production process (Regulation (EC) No 1829/2003; Regulation (EC) No 1830/2003; Shaikeh *et al.,* 2021).

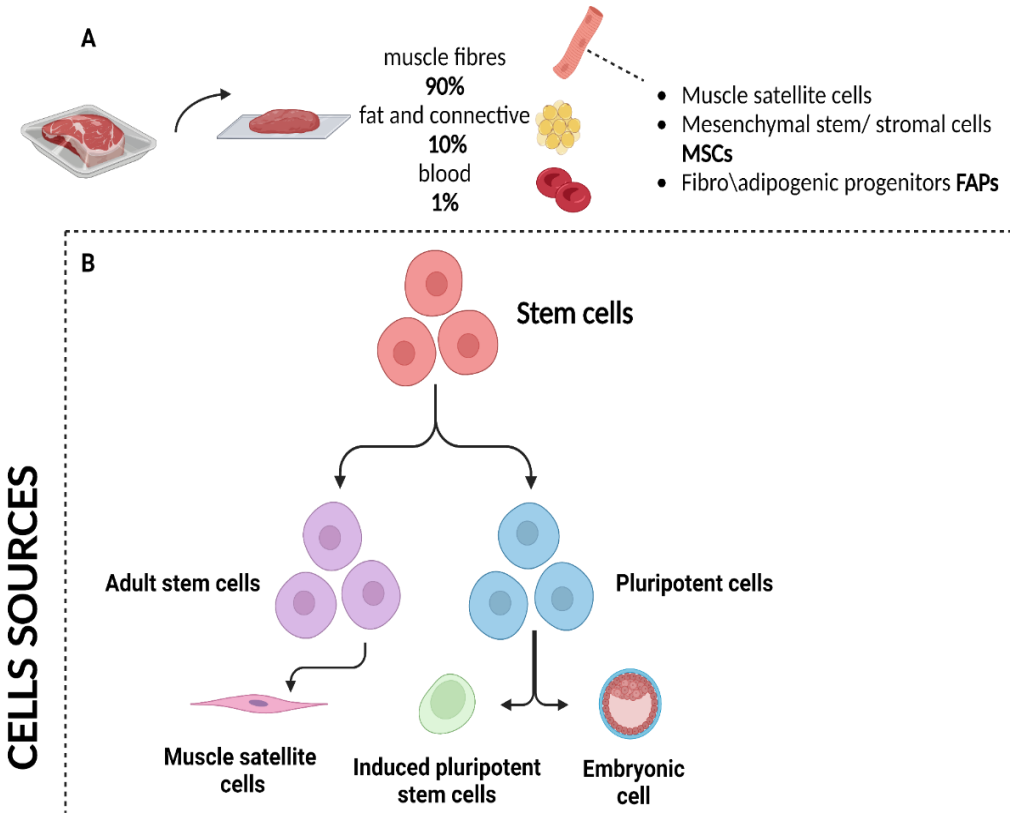


Fig 2 A. *The main components of meat.* In the microenvironment of muscle fibers, there are progenitor/stem cells such as muscle satellite cells, mesenchymal stem/stromal cells (MSCs) and fibro/adipogenic progenitors (FAPs). **B.** *Schematic representation of the main sources of stem cells capable of self-renewal and differentiation into the cells that characterize meat, i.e. skeletal myocytes, adipocytes, chondrocytes and fibroblasts* (Image Created with BioRender.com).

1.4 Cell Immortalization and Differentiation

1.4.1 Rationale for immortalization of cultivated meat cells

Although primary cell cultures have the benefit of being able to be employed relatively quickly for meat production, they have the disadvantage of being limited in the number of cell divisions they can undergo before senescence or cell cycle arrest. This makes long-term and commercial production difficult. The use of primary cell cultures necessitates the breeding animals providing

biopsies on a regular basis, as well as the testing and approval of these biopsies for use in food production (Maqsood *et al.,* 2013). Unlike primary cell cultures, immortalized cell lines are not susceptible to senescence and can undergo an endless number of cell divisions. The production of immortalized cell lines is a crucial requirement in the field of cell culture. Even though work on cell lines began more than 50 years ago, there are few cell lines suitable for the cultivation of meat. In fact, they must conform to particular requirements such as the capacity to proliferate and differentiate (e.g., form mature muscle from muscle cells and accumulate lipids as fat cells) efficiently on an industrial scale, be authorized as safe for ingestion as food, and have the desired properties in terms of flavor, consistency, and nutrition (Gormey 2024). The primary culinary components of animal meat consist of skeletal muscle and adipose tissue. Pertinent cellular entities include satellite cells and stem cells derived from fat tissue, alongside mesenchymal stem cells, versatile fibroblasts, and various types of stem cells (Stout *et al.,* 2023; Yun *et al.,* 2023). Myoblast cell lines from model animals are the closest existing cell lines. In addition to consumer impressions, current cell lines lack the flavor, nutrients, and texture associated with meat (Astruc, 2014). Therefore, immortalized cell lines for CM should be derived from cells that are well-known to consumers and are pleasant, nutritious, and safe for food (Bryant and Barnett, 2020). Only recently have attempts been made to establish banks for collecting cell lines appropriate to the development of CM. For example, the Good Food Institute (GFI) and Kerafast (Boston, Massachusetts) are working together to maintain a bank of terrestrial and aquatic cell lines that can be used for research on CM; however, the number of useful cells remains relatively low (Blog Kerafast, 2023).

1.4.2 Methods to immortalize cultivated meat cells

Currently, there are three methods to obtain immortalized cell lines: spontaneous immortalization, the development of the telomerase catalytic subunit (TERT), and introduction of viral oncogenes (e.g. SV40 large T antigen or HPV E6/E7) that functionally inactivate the p53\p14\Rb tumour-suppressor pathways. Each technique employs telomerase expression, cell cycle inactivation/bypass, or both (Harada *et al.,* 2003; Maqsood *et al.,* 2013). Mammalian cells rarely spontaneously immortalize, and spontaneous immortalization is typically associated with malignancy. However, recent work in the cellular agriculture field has shown that fibroblasts from poultry and cattle can undergo spontaneous immortalization during long-term culture while retaining a non-transformed phenotype, providing a relevant cell source for CM production (Pasitka *et al.,* 2024; Pasitka *et al.,* 2025). Historically, immortal cell lines were first obtained either by spontaneous immortalization of long-term cultured fibroblasts (e.g. mouse fibroblasts in the 1940s) or by direct derivation from malignant tumours, such as the *HeLa* cell line established from cervical cancer cells taken from Henrietta Lacks. Immortalization can also be triggered by mutagenesis via radiation or chemical carcinogens (Jedrzejczak-Silicka, 2017; Yao and Asayama, 2017). It is also possible to identify clones with immortalization markers, significant TERT expression, or low p15/p16/Rb expression by serial passage of a cell line. The biotechnological company “Future Meat Technologies” has generated a cell line that has spontaneously been immortalized. The present cell line was obtained through the cultivation of fibroblasts that were extracted from a chick embryo, followed by the isolation, concentration, and expansion of colonies of cells that exhibited superior growth characteristics, also known as foci. The colonies underwent expansion to attain a uniform morphology culture,

which exhibited the ability to sustain itself beyond 20 to 30 divisions, exceeding the growth potential of unaltered somatic cells. Each time a non-immortal cell divides, the telomeres become shorter, up to a point called the Hayflick limit. This makes the telomeres vulnerable to damage and causes senescence. Infection of human fibroblasts and keratinocytes with a retrovirus encoding human TERT results in the immortalization of the cell lines. Ectopic expression of TERT in human endothelial cells was also immortalized using plasmid transfection (Harada *et al.,* 2003; Xu *et al.,* 2004). Repressing the p53/p16/Rb pathways, which bypass the stress response system, is an additional strategy for immortalizing cells. Under normal conditions, p53 is activated in response to DNA damage or other stresses, resulting in cell cycle arrest and apoptosis (Chen, 2016). Rb and p16 activation inhibit the activation of DNA replication by other proteins, resulting in cellular senescence (Chen, 2016; Takahashi *et al.,* 2007). Because p16 and Rb are blocked or altered, DNA replication can continue, resulting in cell division (Maqsood *et al.,* 2013). TERT expression or p15/p16/Rb inactivation alone is frequently inadequate to immortalize a cell line, indicating that both telomere shortening and the p53/p16 stress response must be avoided. As previously demonstrated, myoblasts must avoid both senescence-triggering events to attain immortality (Ryu, 2016). Upside Foods, a producer of CM, submitted a patent application in 2016 to immortalize cell lines by overexpressing TERT and utilizing CRISPR-Cas to suppress the expression of p15 and p16 in chicken skeletal muscle cells.[58] TERT overexpression by an ectopic TERT gene enhanced cell proliferative capacity indefinitely, but the deletion of p15 and p16 alone increased cell proliferative capacity. Myogenic cell lines can also be made immortal by expressing genes in a way that skips the shortening of the telomeres and the p16 stress pathway. Other approaches to

immortalizing myogenic cell lines can avoid both telomere shortening and stress pathway p16 by ectopically expressing TERT and inhibitors of Rb kinase 4 cyclin dependent (CDK4) and cyclin D1 (Harada *et al.,* 2003; Stadler *et al.,* 2013; Genovese *et al.,* 2023).

1.4.3 Differentiation in the context of cultivated meat

Muscle cell differentiation occurs *in vivo* when satellite muscle cells transition from a quiescent to a proliferative state, culminating in myoblast formation (Chal, 2017). From the multiplication of myoblasts, adequate quantities are produced for muscle regeneration, and a portion of these cells also revert to a quiescent state. Because proliferation and differentiation are mutually incompatible processes cells grown *in vitro*, cells are typically expanded first and then triggered to differentiate. Differentiation is often achieved by eliminating growth factors or introducing differentiation-promoting proteins. For example, eliminating serum from the culture medium stimulates *in vitro* differentiation of muscle stem cells, and further maturation can be induced by mechanical and electrical stimulation. By acting as a support for propagation and differentiation, scaffolds play a crucial function in terms of mechanical stimulation; their application for cell differentiation mimics the extracellular matrix-cell interactions, generally found *in vivo* via activation of integrin receptors. In addition to mechanical stimulation, electrical stimulation can be utilized to promote cell proliferation and differentiation. The combined effects of electrical and mechanical stimulation increase the earliest phases of cell proliferation in the absence of a scaffold structure. The effects of electrical stimulation on rat L6 myoblasts were demonstrated using a commercial cell culture stimulation device (Je *et al.,* 2019). Electrical stimulation controls myogenic differentiation by reducing the expression of small GTPases (Schmidt *et al.,* 2019) (Figure 3).

When generating CM products, it is likely that muscle cells will be differentiated by the simplest method possible to recapitulate the texture and nutrition of animal-derived meat.

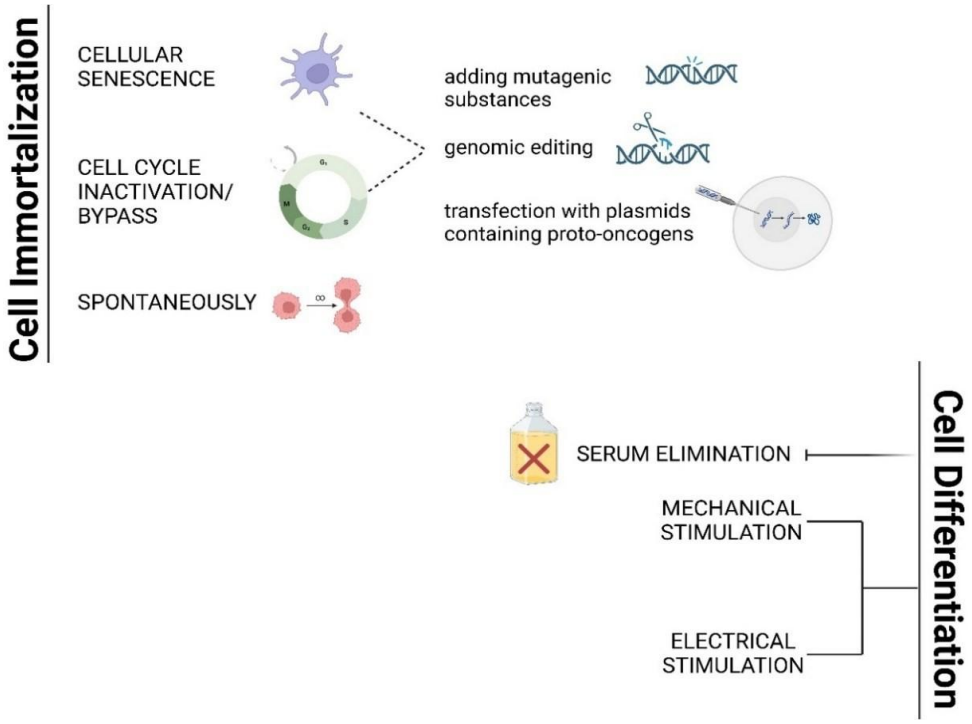


Fig 3. *Three methods to obtain immortal cell lines.* (i) development of the telomerase catalytic subunit (TERT) to avoid cellular senescence; (ii) inactivation, or loss of cell cycle checkpoint control by acting on the p53/p14/Rb pathway; (iii) spontaneous immortalization (Image Created with BioRender.com).

Adipose tissue is responsible for the regulation and homeostasis of energy metabolism. It is mostly composed of adipocytes surrounded by fibroblasts, fibroblast- preadipocyte cells, endothelial cells, nerve cells, and immune cells. For CM production, effective differentiation of adipocytes (i.e., lipid accumulation) is essential. More research is required to create expandable adipogenic stem/progenitor cell lines from meat animal species, food-grade culture conditions for mature adipocytes, and scalable protocols for creating

edible fat tissue, even though the molecular functions and mechanisms of adipocytes have been relatively well studied.

Several approaches have been developed for distinguishing preadipocytes from cells capable of lipid droplet accumulation and exhibiting the morphological and biochemical characteristics of mature white adipocytes (Niemela *et al.,* 2008). Differentiation strategies for preadipose cell lines and primary cultures of fat precursor cells have been created, and the responsiveness of preadipocytes to inducing stimuli can vary greatly. In the presence of fetal bovine serum, preadipocytes spontaneously differentiate into fat cell groups to some extent. The quantity of lipid production may be dose-dependently regulated by altering the serum concentration in the growth medium. Inducing substances such as dexamethasone, used to activate the glucocorticoid receptor and 3-isobutyl-1-methylxanthine receptor pathways (IBMX) (or 1-methyl-3-isobutylxanthine, MIX), which is used to stimulate the cAMP-protein-dependent kinase, can enhance differentiation (Yu *et al.,* 2011). In addition, high quantities of insulin have been combined with these inductors. It has been established that insulin/IGF-1, glucocorticoid, and field signaling pathways are involved in the adipocyte differentiation process (Zagury *et al.,* 2022).

1.5 Culture media for cultivated meat

How to develop and maintain concurrently muscle satellite cells (or, more broadly, muscle precursor cells [MPC]), myoblasts, myocytes (also known as myotubes or myofibers), adipose-derived stem cells (ADSC), adipocytes, and fibroblasts, is a significant unresolved technical challenge to produce lab-grown meat (O'Neill *et al.,* 2021). Cell proliferation and maintenance depend on a variety of components, such as hormones and growth factors. These signals, which are produced *in vivo* by endocrine glands, bind to specific receptors on the

cell membrane or in the cytoplasm and activate the pathways that control cell division and proliferation. Cell culture media contain all the components necessary for cellular survival as well as to stimulate responses such as adhesion, proliferation, and differentiation. In general, basal media consist of carbon and nitrogen sources like glucose, glutamine, and other amino acids; inorganic vitamins and salts; and signaling molecules like growth factors. [67,53] Formulas for culture media vary according to the application. Recently, several commercially available basal media have become available and usually contain most, but not all, of the ingredients needed for cells to grow, such as glucose, amino acids, and vitamins. Conventionally, basal media formulations are supplemented with complex components of animal origin (i.e., serum) that supply additional nutrients and signaling molecules (Yao and Asayama, 2017). Eagle minimal essential medium (MEM) (Eagle, 1959), Dulbecco modified Eagle medium (DMEM) (Dulbecco and Freeman, 1959), and Ham F-12 (HAM, 1965) are some examples. Serum is the blood fluid collected following blood coagulation; it is rich in proteins, nutrients, growth factors, and hormones. Although serum is traditionally used for muscle cell culture (Shahini *et al.,* 2018), it is antithetical to CM production because it is derived from animals. Indeed, a food-grade medium that is inexpensive, can control large-scale cell proliferation and differentiation, have acceptable sensory properties, and contain no animal products, is necessary for the effective production of cultivated meat (O'Neill *et al.,* 2021). Recently, serum-free media have been created and treated with growth factors (particularly insulin, FGF2, and TGF) extracted from animal serum or recombinantly produced (Shiozuka and Kimura, 2000). Essential 8™ medium and Fibroblast Growth Medium™ are two serum-free media formulations that are commonly used in research to promote stem cell growth.

These two formulations have recently shown promise in CM applications, as they support the proliferation of primary bovine myoblasts for at least six days (Kolkmann *et al.,* 2020; Stout *et al*, 2022). Many ingredients, including hormones and growth factors, are essential for the proliferation and maintenance of cells. *In vivo*, these signals are generated by endocrine glands and bind to specific receptors on the cell membrane or in the cytoplasm, activating the pathways involved in cell proliferation and differentiation. Insulin and IGFs can promote the development of pluripotent, or adipose-derived, stem cells into adipocytes when required in a CM application (Dohmen *et al.,* 2022). FGF2 is a growth factor commonly added to muscle cell proliferation media that has a trophic impact on myoblasts by suppressing differentiation (Groux-Muscatelli *et al.,* 1990). Insulin is a commonly used hormone for *in vitro* maintenance of stem cells. In rat cell cultures, insulin addition stimulated the development of myoblasts (Mandek and Pearson, 1974). The control of catabolism by glucocorticoids influences the proliferation of distinct cell types. *In vitro*, dexamethasone reduces myoblast doubling time of muscle stem cells, increasing their proliferation potential (Guerriero and Florini, 1980). In addition, dexamethasone treatment promotes satellite cell myogenic differentiation, as evidenced by increased sarcomere formation and enhanced contraction in the resulting myotubes (Syverud *et al.,* 2016). The traditional adipogenic differentiation cocktail includes insulin, dexamethasone, indomethacin, isobutylmethylxanthine and rosiglitazone (Song *et al.,* 2023). Moreover, it has been shown that only two inducers, rosiglitazone and insulin, are needed for serum-free adipogenesis (Mitic *et al.,* 2023). Tissue development requires many growth factors, including TGF-β, differentiation factors (GDFs), and bone morphogenetic proteins (BMPs). *In vitro*-cultivated myoblasts treated with TGF-

β are inhibited from developing into myotubes, but their diminished differentiation capacity may be restored once TGF- β treatment is stopped (Massague *et al.,* 1986). *In vitro*-cultivated animal cells may become contaminated with bacteria, fungi, and yeast. Penicillin, streptomycin, amphotericin B, and gentamicin are some of the antibiotics and antimycotic drugs that have historically been used to treat these contaminations (Menkem *et al.,* 2019). However, antibiotics should generally be avoided during CM production due to their negative side effects on consumers' sensitivities and they may also contribute to the spread of antibiotic-resistant bacteria. Both issues could make it more challenging for consumers to accept CM products. Thus, the procedure for producing CM aims to avoid the use of antibiotic media supplements (O'Neill *et al.,* 2021), which are otherwise crucial in the initial phases of primary cell culture development. For *in vitro* cell cultivation, it is essential to replicate the animals in an *in vivo* environment. Incubators are primarily used to maintain a constant temperature, humidity, and pH for cellular homeostasis. The inclusion of sodium bicarbonate buffer in the cell culture medium enables the carbon dioxide (CO_2) content in the incubator to be regulated, hence preserving the medium pH. The normal incubator settings for culturing stem cells, including muscle cells, are 36.5 to 37.5°C like normal physiological temperature and 5 to 10% CO_2 for pH regulation (Yao and Asayama, 2017). Cellular characteristics such as proliferation and differentiation are influenced by variations in cellular respiration and mitochondrial activity due to differences between physiological (1 to 6%) and atmospheric (20%) oxygen concentrations. In addition, several recent studies have shown that hypoxia impacts the stemness of muscle stem cells (Absollahi *et al.,* 2011). In contrast to atmospheric oxygen concentrations, mouse satellite cells can grow under

hypoxic conditions (2% O_2), proliferating twice as rapidly. When transplanted into cardiotoxin-damaged muscle, cells grown under hypoxia generated more new muscular fibers than cells cultivated in normoxia (Urbani *et al.,* 2012). Similarly, the myogenic potential of pig satellite cells was improved at low oxygen levels (Redshaw and Loughna, 2012). To achieve successful CM production, several key factors must be considered: access to cost-effective food-grade facilities and inputs the capacity to manage cell growth and differentiation on a large scale, desirable sensory characteristics, and the exclusion of animal-derived components. Valuable insights for developing suitable culture media can be gained from understanding traditional applications of culture media and the metabolic pathways involved in muscle development and protein synthesis. Additionally, strategies employed to enhance media for large-scale microbial fermentation processes, which yield fundamental chemicals and less valuable food components, can offer valuable guidance. It's crucial to acknowledge that the culture media used can significantly impact the sensory properties of harvested muscle or meat. Residues from the media present within or on cells could influence flavor, texture, or color. For example, certain amino acids like glutamic acid and asparagine, which contribute to the umami taste in meat, are found in some cell culture media, introducing an additional layer of complexity to media formulation considerations. Recent studies, including preliminary sensory assessments, suggest that laboratory-scale CM prototypes demonstrate acceptable organoleptic qualities. The process of developing new formulations for CM media begins by identifying key components through experimental or theoretical analysis, followed by optimizing their concentrations. This task is complex due to the large number of components involved, exemplified by DMEM containing between 30 to 52 components,

which can lead to intricate interaction effects among them (Chen *et al.,* 2011). Furthermore, physiological variability of cell lines necessitates reoptimization as processes evolve and new (Chen *et al.,* 2021) components are identified, a common scenario in an industry working with diverse cell lines (Zhang and Block, 2009). Therefore, efficient methods for identifying and adjusting concentrations are crucial. Traditionally, media design begins with a one-factor-at-a-time (OFAT) approach, where each component is assessed individually for its effect on cell response. However, this method overlooks interaction effects, potentially resulting in suboptimal media designs (Gupte and Kulkarni, 2003). To overcome this limitation, Design of Experiment (DOE) techniques like Factorial, Plackett-Burman, and Central Composite designs are employed. These methods involve simultaneously changing multiple nutrient concentrations, enabling faster optimization and have been successfully applied in various industries to characterize and optimize production processes (Fisher, 1992; Horvath *et al*, 2010; Zhao *et al.,* 2017; Zhang *et al*, 2009; Zhang *et al.,* 2007). DOE experiments are conducted at the extreme ends of the design space to estimate first-order effects of each media component without interference from others. These experiments are complemented by response surface models (e.g., linear or polynomial models) to predict optimal concentrations and sequentially improve mixtures (Chambers and Swalley, 2009). While effective, these methods can still be experimentally intensive, particularly when optimizing formulations with numerous variables. Alternatively, stochastic optimization methods like genetic algorithms treat media combinations as evolving chromosomes under selection pressures, aiming to maximize the fitness (e.g., biomass) of nutrient combinations (Weuster-Botz, 2000). Modern stochastic methods integrate mathematical surrogate models (e.g., neural networks) to aid

in prediction and store information about component effects and interactions, enhancing optimization efficiency over time (Chen, 2007). These advanced optimization techniques have been successful in designing complex microbial media with fewer experiments compared to traditional DOE, demonstrating promising efficiency improvements in media formulation. Continued advancements aim to make these methods more accessible to practitioners, reducing reliance on specialized expertise in artificial intelligence and numerical optimization methodologies (Cosenza and Block, 2021).

1.6 Scaffolding

In most cell culture applications for biotechnology, tissue culture flasks and Petri plates are used to establish two-dimensional (2D) cell cultures. 2D cell culture is the most common approach for studying cell morphology and the effects of prospective therapies on cell functions. When cells are transplanted from their original tissue into a 2D environment, they typically lose their normal shape, resulting in alterations of the metabolism and gene expression (Kapalczynska *et al.,* 2018). 2D cell culture techniques do not adequately recreate the *in vivo* environment of the native tissue complex of skeletal muscle, with the absence of cellular connections and communication between cells resulting in slower cell proliferation, less differentiation, and an inability to create epithelial tissue characteristics such as tubular and cystic structures (Kapalczynska *et al.,* 2018). To control the *in vitro* formation of muscle tissue, scaffolds are often used to simulate the extracellular matrix (ECM) generated by cells and support cell adherence, proliferation, and differentiation. A suitable scaffold for the growth of muscle cells must be edible and cytocompatible, and if utilized in a 3D format must facilitate the exchange of gases, nutrients, and waste to avoid necrosis of the cells. Mimicking the rigidity and protein composition of native ECM helps

to replicate the natural microenvironment. This mimicry promotes cell-cell and cell-matrix communication, facilitating cell proliferation and differentiation (Fernandez-Costa *et al.,* 2021). The scaffolds for CM will likely be modeled in line with tissue engineering (TE) scaffolds, based on biocompatibility, biodegradability and mechanical properties, while pore size, architecture, and manufacturing methodologies must also be considered (O'Brien, 2011). Scaffold architecture usually should be porous in order to allow continuous media perfusion, mimicking natural vascularized tissue. Given that muscle tissue (myocytes) constitutes the predominant component of meat, the goal of TE for CM is to produce muscle tissue using cell culture and proper scaffolding. Scaffold requirements for fat cells are less stringent compared with muscle cell culture, but growth of fat cells within a 3D matrix will likely improve mouthfeel of final products. However, they must still serve as viable substitutes for the typical role played by the extracellular matrix. Currently, a variety of scaffolds, including those of synthetic and natural origin, are extensively employed. Polyglycolic acid (PGA) and poly lactic glycolic acid (PLGA) are synthetic materials that are utilized as scaffolds for muscle cells. It is noteworthy that within the category of natural scaffolds, there exist various types such as collagen, collagen-chitosan hydrogels crosslinked with glutaraldehyde, fibrin, and HYAFF®, which is a polymer derived from hyaluronic acid (Flynn and Woodhouse, 2008). Such scaffolds can be constructed with both synthetic or natural polymers, including those obtained from plants and animals, depending on the origin of the material (Seah *et al.,* 2022).

1.6.1 Natural Polymers

Frequently used scaffolds for skeletal tissue engineering are composed of three main groups of natural polymers including proteins (silk, collagen, gelatin,

fibrinogen, elastin, keratin, actin, myosin), polysaccharides (cellulose, amylose, dextran, chitin/chitosan, glycosaminoglycans), and polynucleotides (DNA, RNA) (Stevens *et al.,* 2008). Natural edible and food-safe polymers are commonly used in the production of CM, with plant protein-based scaffolds being particularly desirable because of the low cost, nutritional value, and cytocompatibility (Reddy and Yang, 2011; Huang *et al.,* 2018).

1.6.2 Animal-derived

Avoiding the use of animal-derived scaffolds in CM production is an ethical choice that aims to minimize negative impacts on the lives of animals and promote sustainable and compassionate practices in the food industry. However, it is important to discuss their characteristics by presenting some examples. Collagen is considered an optimal material for scaffolds that resemble the ECM for human skeletal muscle engineering, and the majority of bio-artificial muscles (BAM) are grown on collagen scaffolds (Hinds *et al.,* 2011; Bomkamp *et al.,* 2022; Snymn *et al.,* 2013; Brand and Bursac, 2009). Gelatin, a natural component of meat generated when collagen is denatured by processing and heating, has been used to manufacture CM, although it is generally preferred to avoid animal-derived materials in the process (Ahmed *et al.,* 2008). Cultured bovine aorta smooth muscle cells and rabbit skeletal muscle myoblasts replicated several morphological and mechanical properties of natural meat but lacked the contractile architecture because the gelatin fibers used as the substrate had been cross-linked to prevent deterioration (MacQueen *et al.,* 2019). Fibrin scaffolds, a naturally occurring fibrous protein that forms blood clots at injury sites, have been used to maximize BAM vascularization. According to the findings, fibrin gel is sufficient for the generation of vascularized BAMs (Ahmed *et al.,* 2008; Noori *et al.,* 2017; Spicer, 2020). Hyaluronic acid (HA) is commonly used in TE

because it promotes rapid wound healing and regulates adipogenesis, angiogenesis, and tissue organization in cells. In addition, attempts have been made to replace animal sourced HA with endotoxin-free microorganism-generated HA via genetic engineering (Halbleib, 2003; Davidenko, 2010). Another biopolymer of animal-origin used in skeletal muscle scaffolds is chitosan. Chitosan is the main derivative of chitin, a biopolymer found in the exoskeleton of crustacea and insects and in the cell walls of fungi (Muxika *et al.,* 2017). Currently, the main source of chitin is from crustaceans, but because of limitations linked to seasonality and the poor sustainability of crustacea farming, alternatives are needed. Insects, particularly bioconverters, represent a new alternative and more sustainable source of chitin and chitosan. Indeed, bioconverter insect farms, aimed at the production of animal feed and organic by- product management (using as insect feed), have spread worldwide, generating huge amounts of insect chitin, mainly derived from pupal exuviae and dead adults. The first characterization of insect chitin and chitosan showed a high degree of similarity with crustacean counterparts, providing a good starting point to use insect biopolymers in the same applications already tested using crustacea sources, including scaffolds (Hahn, 2020; Triunfo *et al.,*2022).

1.6.3 Plant-derived

Plant-derived scaffolds (e.g., zein, soy protein, wheat gluten) are of interest for CM researchers because of their biodegradability and edibility. Occasionally, these scaffolds may also impart nutritional value and texture to CM (Gershlak *et al.,* 2017). One limitation of plant-derived scaffolds is their lack of mechanical properties; however, this can be remedied by crosslinking. Physical crosslinking, such as UV or thermal processes, are commonly employed. Otherwise, ingestible or FDA-approved enzymatic or chemical crosslinkers like citric acid,

sodium hydroxide, sodium phosphates, or transglutaminase can be utilized to alter the properties of plant-derived scaffolds, enhancing their mechanical strength to support cell growth. The choice of crosslinkers depends on factors such as the base polymeric material, the scaffold architecture, the synthetic process, and cell culture conditions (Jahangirian *et al.,* 2019; Caliari and Burdick, 2016).

Proteins derived from plants can be converted into fibers, films, and hydrogels. In addition, they are readily accessible and reasonably priced. Soy and zein-derived proteins are commonly used in the production of scaffolds. Soy protein is beneficial for tissue engineering since it is biocompatible and shares biochemical properties with the extracellular matrix (Percival, 2002). Textured soy protein is favorable for cell adhesion, proliferation, and differentiation of bovine cells, and has been used as an edible scaffold to generate cow muscle tissue (Ben-Arye *et al.,* 2020). Soy protein has also been combined with other natural polymers such as chitosan and cellulose and demonstrated favorable adherence and proliferation of multiple cell types (L929, Schwann cells, and human mesenchymal stem cells) (Chien and Shah, 2012; Gan *et al*, 2016; Luo *et al.,* 2010). Corn zein protein is being investigated for medical applications due to its adaptability and biocompatibility (Liu *et al.,* 2005; Jiang *et al.,* 2013). It is soluble in ethanol, which facilitates electrospinning and the formation of nanofibers, and nontoxic crosslinking enables fibroblast cell adhesion and growth on electrospun scaffolds (Jinag *et al.,* 2013). Zein scaffolds have also been shown to increase the adhesion, proliferation, and differentiation of human mesenchymal stem cells (Qu *et al.,* 2008).

1.6.4 Polysaccharides

In tissue engineering applications, biocompatible polysaccharides derived from

plants, such as cellulose and starch, have been used. Cellulose, a linear polysaccharide, is considered as the most sustainable material due to the inexhaustible supply from plant cell walls. Cell culture research has employed a range of cellulose fibers (Rodriguez, 2011; Courtnay, 2017). However, cellulose is non-degradable in the human body. Pectin, a natural polysaccharide derived from plant cell walls, provides useful properties as an artificial extracellular matrix (Iravani and Varma, 2019). Pectin/carboxymethyl cellulose/microfibrillated cellulose (pectin/CMC/MFC) scaffolds with different concentrations of MFC (0- 0.4%) support NIHT3 fibroblast cell survival (Ninan *et al.,* 2013). Starch is typically blended with synthetic polymers to increase its mechanical and structural qualities as a scaffolding material (Iravani and Varma, 2019). Alginate, derived from brown seaweeds like *Laminaria hyperborea* and Lessonia, is widely present in coastal waters globally and holds promise as a scaffold material. It is a biocompatible, non-toxic, non-immunogenic, and biodegradable biopolymer that is economically viable and easily manufacturable. Alginate can be converted into a hydrogel by cross-linking with bivalent cations such as calcium ions (Ca^{2+}), making it suitable for applications in various fields including food due to its safety profile. However, its negative charge impedes natural cell adhesion, limiting its use in specific applications. To address this, RGD-modified alginate gels are commonly used as *in vitro* cell culture platforms, allowing control over myoblast phenotypes. Myoblast adhesion and proliferation on RGD-modified alginate gels surpass those on unmodified gels. Moreover, the delivery of VEGF and IGF-1 from alginate gels regulates angiogenesis and myogenesis, facilitating muscle regeneration. Despite its suitability for cell-cultivated meat scaffolds, alginate's poor cell adhesion remains a challenge, limiting its use to specific applications. Addressing this,

researchers achieved 82% cell adhesion coverage by controlling the structure during alginate's ionic crosslinking. After an 11-day culture period, they evaluated cell adhesion, differentiation, and network formation, observing a 12.7% increase in cell growth. Lastly, a hybrid cell-cultivated meat product was created by blending mycelium-derived single-cell protein with cell-cultivated meat, yielding an edible, cost-effective product with desirable texture (Seo *et al.,* 2023). Agarose, derived from marine red algae, is a natural polysaccharide highly valued in biomedical applications due to its unique ability to form thermo-reversible gels. However, unmodified agarose lacks the optimal cell adhesion properties required for effective tissue engineering and cell culture applications (Zarrintaj *et al*, 2018). To overcome this limitation, researchers have implemented chemical modifications, such as carboxylation via TEMPO-mediated oxidation under alkaline conditions (Kuo *et al*, 2005). This approach introduces carboxyl groups onto the agarose backbone, significantly enhancing cell adhesion and bioactivity. The introduction of carboxyl groups transforms the surface properties of agarose, promoting cell adhesion, proliferation, and differentiation through improved protein absorption. Additionally, the conjugation of dopamine to carboxylated agarose further enhances cell adhesion, leveraging dopamine's adhesive characteristics inspired by marine mussel proteins. The chemical modifications of agarose were meticulously characterized using advanced analytical techniques including FT-IR, 13C NMR, and gel permeation chromatography (GPC), confirming the successful integration of carboxyl and dopamine functionalities. *In vitro* cell culture experiments have demonstrated that these modifications substantially enhance the bioactivity of agarose, making it a promising scaffold material specifically tailored for cultured meat production (Kuperkar *et al.,* 2024). In summary, the strategic modification

of agarose with carboxyl and dopamine functionalities represents a significant advancement in scaffold design, particularly crucial for cultured meat production. This innovative approach supports the development of tissue engineering scaffolds that facilitate robust cellular adhesion, growth, and differentiation, thus advancing the field of alternative protein production.

1.6.5 Decellularized plant scaffolds

As an alternative to synthetic polymers or animal-derived scaffolds, the cellulose skeleton of plant tissue can be employed as an affordable scaffold for mammalian cells following decellularization (Bilirgen *et al.,* 2021). Cellular content is removed from the natural plant material to create an acellular, three-dimensional scaffold that preserves its structural, chemical, and mechanical cues via chemical, physical, or enzymatic methods (trypsin, nucleases, hypo/hypertonic solutions, detergents, solvents). After that, this scaffold may be repopulated with animal cells to create tissue-engineered constructions for a variety of uses (Harris *et al.,* 2021). Natural topographies in decellularized plant tissue scaffolds are capable of simulating some of the *in vivo* features of matrices. However, decellularized plant scaffolds lack a variety of metabolic signals, found in the natural environment, that are necessary to mammalian development. In order to customise these scaffolds for certain cell types, biofunctionalization or coating with functional surface proteins may be required (Modulvsky *et al.,* 2016). Decellularized plant scaffolds, such as those comprised of jackfruit, spinach leaves and broccoli, have been examined as prospective scaffolds for the regeneration of vascularized tissue mass, utilizing the existing structure to provide perfusion during cell culture (Campuzano and Pelling, 2019; Thyden *et al.,* 2022). If the decellularization procedure is non-toxic, it could be used to manufacture CM with structure and lend texture to the final product (Gershlak *et*

al., 2017).

1.6.6 Synthetic polymers

To develop a scaffold for CM, the components must either be edible or biodegradable without creating toxic by-products (Gershlak *et al.,* 2017). Otherwise, the cells must be separated from the scaffold. Synthetic polymers most often used in tissue engineering are copolymers of polylactic acid (PLA), polyglycolic acid (PGA), and polylactic glycolic acid (PLGA). These are polymers that can be absorbed by living organisms or break down hydrolytically (Ma, 2004; Langelaan *et al.,* 2010). Polylactic acid (PLA) has emerged as a crucial material in tissue engineering due to its ability to replicate the physical characteristics of the human extracellular matrix. PLA nanofiber nonwovens, particularly those created through electrospinning, have garnered significant interest for their potential as tissue engineering scaffolds. However, recent studies have shifted focus towards melt blown PLA fabrics as alternative scaffolds. These fabrics can be tailored with varying crystallinities, tensile moduli, and pore diameters to mimic specific tissue properties. In a recent study, melt blown PLA nonwovens were engineered to resemble human dermis, showing promising outcomes when tested with human dermal fibroblasts over various time frames. Results demonstrated robust cellular attachment, proliferation, and migration, along with cellular penetration through the scaffold thickness. These findings suggest that melt blown nanofiber nonwovens hold substantial promise as tissue engineering scaffolds, potentially opening new avenues for research in this dynamic field. In another advancement, three-dimensional (3D) printing technology has been employed to create patient-specific scaffolds using PLA-based materials like PLA-Baghdadite (Bgh). Following fabrication, these scaffolds were treated and coated with chitosan

(Cs)-vascular endothelial growth factor (VEGF) or lyophilized Cs-VEGF to enhance their properties. The coated scaffolds exhibited superior porosity, compressive strength, and elastic modulus compared to traditional PLA samples. Importantly, these scaffolds were found to promote osteogenic differentiation when cultured with rat bone marrow-derived mesenchymal stem cells (rMSCs), showcasing their potential for bone healing applications. Moreover, innovative scaffold design strategies have been explored using PLA, such as directional porous structures fabricated via ice templating and phase inversion techniques. These scaffolds were engineered to accelerate bone repair by facilitating the growth and proliferation of bone cells. The study showcased the scaffold's biocompatibility, mechanical properties, and efficacy in promoting bone regeneration in animal models with large-sized defects. Previously, edible films formed of PLA have been obtained from dairy waste (via use in fermentation to generate lactic acid that is subsequently polymerized) (Gazzola *et al.,* 2019; Salehi *et al.,* 2023). However, these materials should have a minimal environmental effect in line with the objectives of CM, and, above all, it could not be a good model for CM scaffold due to its animal origin (Wong *et al.,* 2016; Toong *et al.,* 2020). Synthetic polymers often lack biological activity when compared to natural polymers. Hybrid natural- synthetic scaffolds may be useful to satisfy the criteria for CM scaffolding (Carletti *et al.,* 2011). If the scaffold is included in the final food product, the fabrication process and outcomes must be safe for ingestion. The texture, digestion, cooking, water-binding capacity, and flavor of scaffolds for CM must be considered, particularly in ways that are different than for medical scaffold designs. Ensuring suitability for human consumption as a food ingredient involves a comprehensive approach. This includes not only nutritional analysis, but also mechanical testing to assess

texture (such as Warner–Bratzler shear force, water-holding capacity, and cooking loss). The morphology of a three-dimensional scaffold may be optimized, including fiber size, surface topology, porosity, and pore alignment (Garg and Goyal, 2014).

1.7 Bioreactors

Bioreactors are critical for cell expansion and provide stimulation and capacity to scale up cell sources to produce CM. A bioreactor is a container that provides a controlled environment for the growth and development of its cellular contents. A bioreactor maintains the proper biological conditions for cells and culture media, including aiding nutrient transport and cell expansion and differentiation by stirring or stimulating the cells. The classification of bioreactor types is based on the method of medium input into the bioreactor main vessel: batch, fed-batch, and continuous (Antolli and Liu, 2012). A batch bioreactor is a chamber that contains a predetermined volume of growth medium and operates by cultivating cells until they reach their maximum density, at which point they are harvested for utilization or transferred to a larger bioreactor (Liu, 2020). A fed-batch bioreactor, also known as a semi-continuous bioreactor, has an inlet channel for providing fresh media to the cells at predetermined time intervals chosen to maximize growth. In the absence of a connector to remove conditioned media and cellular products that collect during culture, a fed-batch bioreactor can also increase volume over time (Jagschies *et al.,* 2017). This distinguishes fed-batch bioreactors from the last major category, continuous. In the production of CM, the preference is often given to fed-batch or continuous medium introduction. This approach supports the handling of substantial media volumes, is amenable to automation, and facilitates the recycling of conditioned medium (Hanga *et al.,* 2020). In addition to classification based on medium intake and removal,

bioreactors may also be classified based on mixing of internal contents. The bioreactor mixes contents to promote growth and development of the cells. Mechanical bioreactors achieve mixing by agitators or impellers, and these are the most frequently used bioreactors for bioprocess development. The most common mechanical bioreactors are stirred tank bioreactors, which employ an impeller to induce convective flow and facilitate nutrient circulation and diffusion inside the vessel. For bioprocess scaling, stirred tank bioreactors have been the most widely used and since they are well-established and scalable, they may be the most suitable bioreactor type for scaling the production of CM (Martin *et al.,* 2004). Spinner flasks may create turbulent flow that is not favorable to cell multiplication, and the direct contact of cells with the propeller may cause damage. For mammalian cell growth, a continuous stirred tank reactor, which combines continuous medium input with a stirred tank bioreactor system, has been extensively used. Another example of mechanical bioreactor is the rotating-wall vessel bioreactor that rotates the bioreactor primary vessel around its central axis to dynamically cultivate the vessel contents in suspension (Meyer *et al.,* 2016). Rotating-wall vessel systems have the advantage of minimum shear stress and may facilitate the formation of three-dimensional (3D) aggregates. Nonetheless, several cell types have elevated apoptotic rates early in culture using these systems. Rotating-wall vessel systems employ batch culture, but perfusion can be incorporated to automate the operation (Hammond and Hammond, 2001). A mechanically active bioreactor system is the last prevalent mechanical bioreactor type. The bioreactor employs a regulated application of mechanical force to cells or tissue scaffolds, specifically using dynamic compression. This stimulation promotes cellular growth by simulating the natural developmental environment and can strengthen and align cells or scaffold

structures (Meinert *et al.,* 2017). This form of agitation may be advantageous for the development of CM, as myofiber alignment and mechanical strength are essential characteristics. A hollow fiber bioreactor has been used to promote the proliferation of skeletal muscle cells (Battahlli *et al.,* 2011; Yamamoto *et al.,* 2012). The classification of hollow fiber bioreactors as hydraulic bioreactors indicates that mixing is accomplished by liquid flow rather than mechanical mixing. This entails seeding the cells in a matrix of porous hollow fibers so that they adhere to the surface of the fibers, where the medium can also circulate. A hollow fiber system has the advantage of producing minimal shear stress and allowing for a greater variety of nutrients to be carried, making it excellent for highly metabolic cell types. Another bioreactor type is air lift, which achieves mixing using gas purging and may be useful for meat production (Hosoyama *et al.,* 2014). However, it lacks the record of accomplishment of other bioreactor designs that have been improved for several large-scale bioprocesses. Several cellular parameters must be evaluated when designing a bioreactor system to produce CM. Several kinds of meat cells, including myocytes, are anchorage-dependent and must attach to a surface to proliferate and differentiate appropriately. Before differentiating into specialized cell types that require anchoring, it may be possible to grow the initial cell source in suspension. Alternately, growth methods employing non-adherent free-floating spherical aggregates may be beneficial to avoid the potential requirement for a substrate during bioreactor development (Hosoyoma *et al.,* 2014). This culture method would be more applicable to sources of pluripotent stem cells that can be cultivated as free-floating aggregates. Other adult stem cell sources, such as MSCs and muscle satellite cells, necessitate attachment substrates. There is also a risk of necrotic core development if the aggregates become too large and limit

nutrition and oxygen passage. Considerations should also be given to the idea of co-cultivating muscle and fat cells to obtain CM. Today, however, it is still challenging to perfect the growth media that can support both cell types; it is likely that the two cell types will be cultivated separately soon. Perfusion bioreactors, which combine continuous medium input with perfusion flow, are a method for producing meat products of a certain size (Datar and Betti, 2010 and Specht *et al.,* 2018). This is due to perfusion flow rate in these bioreactors that can be adjusted to the shape and size of the cultivated tissue. However, it should be noted that an increase in perfusion flow rate in proportion to the size and scale of the scaffold may lead to elevated shear stress and reduced pressure, potentially causing cellular harm. Certain bioreactor systems may be excellent for producing one form of CM, but they may not be suited for the development of other types and sizes of meat. As the field expands and seeks to meet a wide variety of CM products, bioreactor systems for large-scale production will need to be continually optimized.

1.8 Insect Cells as a Source of Cells for Cultivated Meat

Cells sourced from a variety of species, principally bovine, porcine, and avian, have been targeted for the development of CM. Cells derived from less common non-mammalian species (e.g. insects and fish) may be useful in overcoming current technological challenges that prevent the development and extension of cell cultures, such as the need for adherent cells and the high cost of media. Mammalian cells require a set of specific growth conditions and tight process control to maintain their functions: pH range of approximately 6.8–7.8, temperature range of 30–39°C, carbon dioxide, specific antibiotics, expensive growth factors, animal-derived serum and adhesion for growth. Although some media formulations exclude serum, mammalian cells are difficult to adapt or

thrive in serum-free conditions (Baust *et al.,* 2017; Rubio *et al.,* 2019b). Furthermore, most CM-relevant cell types require adherent cultures, constraining growth by surface area. These limitations render large-scale production of mammalian-based cell culture systems difficult and inefficient and motivate the exploration of alternative cell sources. Similar physicochemical and nutritional constraints also apply to many fish cell lines developed for cultured seafood (CSF), which are typically derived from muscle, mesenchymal or embryonic progenitors. These cells are often maintained at species-appropriate temperatures below those used for mammalian cultures and, in the case of marine species, at higher osmolarities than mammalian media (Gardell *et al.,* 2014; Lakra *et al.,* 2011), yet they generally remain dependent on adherent growth and complex media formulations. In practice, proliferation and long-term maintenance of several CSF-relevant fish lines require combinations of FBS, species-specific fish serum and/or embryo or tissue extracts (e.g. from bone, muscle or fry), which act as sources of growth factors and hormones in addition to basal media such as Leibovitz's L-15, F10 or DMEM/F12 (Chen *et al.,* 2003). Because fish and bovine growth factors share only ~77% amino acid identity, bovine-derived factors in FBS interact less efficiently with fish receptors. As a result, FBS is a suboptimal universal mitogenic supplement for fish cells, and researchers often resort to species-matched sera or tissue extracts, which, however, still suffer from the same limitations as FBS, including limited availability, batch-to-batch variability and ethical concerns (Yuan & Hong, 2017; Bejar *et al.,* 2002; Chen *et al.,* 2003). Attempts to adapt fish lines to low- or serum-free conditions via gradual weaning have so far achieved only partial reductions in serum content and frequently result in impaired proliferation or loss of viability upon complete serum withdrawal (Liu *et al.,* 2017). In contrast, insect cells have properties that

indicate suitability for large-scale production in a more cost-efficient manner and, in several respects, appear closer to an industrially feasible, serum-free platform than many currently available fish or mammalian cell systems. The use of insect cell culture for food applications has been summarised recently (Rubio *et al.,* 2019b) and will be briefly discussed here. Insect cells can tolerate a wide range of environmental variables, including pH (6.0-7.0), and temperature (20-32°C), and are typically grown without CO2. The immortalization process can also occur spontaneously, as demonstrated by several insect cell lines in the Cellosaurus database and one cell line derived from *Manduca sexta* and have been explored for food purposes (Letcher *et al.,* 2022; Expasy-Cellosaurus, 2023). Insect cells have the flexibility to grow either in suspension or attached to surfaces because they are not affected by contact inhibition. This means they can be cultivated in suspension bioreactors, where factors like surface-to-volume ratio and cellular biomass concentration can be finely tuned within a confined space (Jahangirian *et al.,* 2019; Caliari and Burdick, 2016). A further difficulty with mammalian cell culture is the amount of culture medium required to support cells because of the high glucose consumption rates and toxic by-product accumulation during cell expansion. In the context of cellular agriculture, this is a focal point of ongoing research as cost-effectiveness is crucial for consumer adoption of CM products, and media is a large contributor to production costs. In contrast, insect cells produce fewer toxic by-products like lactic acid due to their metabolic processes, they exhibit lower sensitivity to toxic compounds (e.g., ammonia, a by-product of catabolism), and they require less glucose for growth, thereby reducing the cost of materials and the volume of culture media required. These characteristics result in reduced material costs and lower volumes of culture media necessary, simplifying and making production scalability more

economical. Rubio *et al.* investigated the differences between mammalian and insect cell cultures, focusing on cost, maintenance, and adaptability. These characteristics are crucial for advancements in tissue engineering, particularly in applications such as bio-fabrication, biobots, and cultured meat. Table 1 provides a detailed comparison between these two types of cultures, highlighting their respective advantages and limitations (Rubio *et al.,* 2019a). Currently, finding a reliable and scalable source of insect muscle and fat cells is a significant obstacle to the production of insect tissues for food. To appreciate how insect cell cultures may be utilized to make CM, it is essential to understand the physiology of the cell types of interest. The mechanisms described below are advantageous because they provide a method to explore *in vitro* the key biological properties of insect muscle and fat cells. Nonetheless, establishing stable lines of muscle and adipogenic progenitor cells capable of protracted proliferation and possible differentiation are essential for the manufacturing process and its scalability (Kadim *et al.,* 2015).

Table 1: *Comparative Analysis of Mammalian and Insect Cell Cultures for CM.* The table outlines key differences between mammalian and insect cell cultures, focusing on growth conditions, cost-effectiveness, and adaptability, while highlighting the advantages and limitations of both sources.

	Mammalian Cell Cultures	**Insect Cell Cultures**
Advantages	-Widely used in medical and biotechnological research -Better consumer acceptance	-They are more adaptable to serum-free media -They grow in the absence of CO_2 -They can withstand adverse environmental conditions -They grow at room temperature (20-32°C) -Require less nutrients -Less frequent replacement of medium -Lower costs -Easier transition between adherent and suspension cultures
Limitations	-More glucose consumption -More lactic acid production and need for replacement of acidified culture medium -They are not easily adaptable in serum-free media - Due to contact inhibition, they can only grow in adhesion, therefore needing large spaces -More susceptibility to environmental conditions -Necessity of controlled levels of CO_2 -Need to maintain a temperature of 37°C -Higher costs and Complexity of scalability	-Further studies are needed - Complexity of scalability -Low consumer acceptance

1.9 Muscle cells

Key differences between mammalian and insect muscle development include the origin and types of muscle cells, the molecular pathways that regulate them, and the function of muscle in the body. Table 2 offers an intricate comparative analysis concerning cell types, development, and molecular pathways. For an extensive review of primary myogenic insect cell culture attempts, readers are referred to Rubio *et al.* (Rubio *et al.,* 2019a). It has been suggested that CM production can help with environmental and animal welfare issues. While establishing bioproduction methods from mammals has been the focus of academic research on cell-cultured meat, it would be preferable to begin with relevant animal species like insects. More study is required to determine whether the pattern observed in mammalian cell types, in which cell multiplication decreases as animals age, is also reflected in the relationship between the age of insects and their proliferative ability. The demands that have given rise to the establishment of this new frontier may be addressed through understanding of the features of muscle and adipogenic growth. Additionally, controlling the creation of muscle or fat *in vitro* would require knowledge of the signals that govern these processes *in vivo*, and mammalian indications are not suitable for insect cells.

1.9.1 Muscle types

Several cell types that are all specialized for contraction are referred to as "muscle". Despite their other differences, all muscles share the same metabolic processes that drive contractions. This process involves the interaction of actin and myosin proteins within the muscle fibres, with ATP serving as the primary energy source (Alberts *et al.,* 1991). In vertebrates, the broadest classification of muscle is based on the presence or absence of regular cross striations. In

mammalian systems, there are three main types of muscle: skeletal, cardiac, and smooth, which has no striations. Skeletal muscle cells can be enormous (up to half a meter long with a diameter of 100 micrometers in adult humans) and are often called muscle fibers because of their elongated shape. Each cell constitutes a syncytium containing many nuclei immersed in the same cytoplasm. Other types of muscle cells are more conventional in that they possess a single nucleus (Alberts *et al.,* 1991). The walls of numerous organs and tubes in the body are lined with layers of smooth muscle cells, and smooth muscle does not contract voluntarily. The smooth muscle cells shorten when forced to contract, driving the organ's luminal contents, or the cell shortening changes the diameter of a tube to control the flow of its contents. Smooth muscle cells lack the striated banding pattern present in cardiac and skeletal muscle and are neurally innervated by the autonomic nervous system. In addition, hormones, autocrine/paracrine substances, and other regional chemical signals regulate the contractile state of smooth muscle (Webb, 2003). The thick central layer of the heart is made up of cardiac muscle (also known as myocardium). The individual cells that make up the heart muscle are known as cardiomyocytes. Cardiomyocytes' main function is to contract in order to create the pressure required to pump blood through the circulatory system (Tran and Weber, 2022). Each cardiac muscle cell, or cardiomyocyte, is a tubular structure made up of chains of myofibrils, which are rod-like components inside the cell. Sarcomeres, the primary contractile units of muscle cells, are repeated in sections to form the myofibrils. Long proteins that form myofilaments, or thick and thin filaments, make up sarcomeres. Actin is a protein found in thin myofilaments, whereas myosin is a protein found in thick myofilaments. As the muscle contracts and relaxes, the myofilaments move past one another. When seen under a microscope, the arrangement of thin and thick

myofilaments overlapping within the cell's sarcomere gives the illusion of being striated, similar to that of skeletal muscle (Saxton *et al.,* 2019). All insect muscles follow a similar structure, with elongated cells holding the contractile components and frequently inserting into the integument at each end. But various muscles have diverse internal arrangements of the muscle cells, and wing muscles frequently have distinctive shapes (Kachwaha, 2011; Iwamoto, 2011). Insect muscles are almost all striated and are divided into two groups: skeletal muscles and visceral muscles, only a small part of which are of the smooth type, lining the walls of internal organs. Skeletal muscles can be attached to the exoskeleton's internal surface area in significantly greater numbers than can fit on the skeletal framework of vertebrates. Elongated contractile fibers that are parallel to or converge at the point of insertion form the skeletal muscles. A consistent network of longitudinal and circular fibers can develop in visceral muscles (Wigglesworth, 1967). Somatic muscles (or of the body wall) of insects like *Drosophila melanogaster* do not have many muscular fibers like those found in mammals. Additionally, the 30 segmentally repeated muscle fibers that make up somatic muscles are arranged in a clear pattern. The body wall muscles of many insects have from 4 to 24 nuclei, compared to up to 1,000 nuclei in mammalian muscles. The visceral muscles that surround the gut, in addition to the somatic muscles, are syncytial. Circular binucleate muscles with partial fusion and multinucleate longitudinal muscles make up the larval mid-gut muscles. In the flight muscles, quiescent satellite cells have been identified that, as in mammals, can be activated by injury (Rout *et al.,* 2022).

1.9.2 Muscle development

As already mentioned, prototypes of CM products have focused on the differentiation of stem cells, such as muscle satellite cells, to produce skeletal

muscle, which is the main component of traditional meat. Consequently, our focus will be on the development of skeletal muscle. Myogenesis, the process of muscle formation, is substantially conserved in both invertebrate and vertebrate species. The embryogenic myogenesis of *Drosophila melanogaster* is a well-recognized model for studying the genes and mechanisms that drive muscle development (Dohmen *et al.,* 2022). Myoblasts, precursors of skeletal muscle cells, fuse to form multinucleated cells after a proliferation period. Like somatic musculature, the muscles of the visceral mesoderm (circular and longitudinal muscles) of many insects are composed of founder cells and fusion-competent myoblasts. The founder cells responsible for the development of the circular and longitudinal muscles originate from distinct regions within the mesoderm. The founder cells of the visceral circular muscles (cFC) and the myoblasts competent for fusion (cFCM) originate from the mesoderm of the visceral trunk, abbreviated as TVM. Founder cells and fusion-competent myoblasts are the two groups of myoblasts that fuse in a variety of insects. Founder cells express a particular combination of identity transcription factors that facilitate the identification of muscle fibers and define their orientation, shape, size, and attachment site. Fusion-competent myoblasts, on the other hand, have a generic identity. They express the transcription factor Lame Duck (Lmd), but it is not yet clear how reprogramming occurs (Rout *et al.,* 2022). During fusion, they undergo a profound change in phenotype that depends on the coordinated activation of a set of specific genes (see section *3.1.3 Molecular pathway*). The regulatory protein is myoD1, normally expressed only in myoblasts and muscle cells. The experimental induction of myoD1 is also capable of transforming a fibroblast into a myoblast. The skeletal muscle cell, once formed, is generally preserved throughout the animal's life. Some myoblasts persist in the adult muscle and

appear as small, flattened, quiescent cells in close contact with mature cells within their basal lamina envelope. These satellite cells are activated to proliferate when the tissue is damaged or, for example, by artificially treating them with fibroblast growth factor (FGF). Myoblasts maintained in culture for up to two years retain the ability to differentiate and fuse to form muscle cells in response to appropriate changes in culture conditions. FGF is essential in keeping myoblasts in a proliferative state and preventing them from differentiating (Alberts *et al.,* 1991). Since there is evidence that insect myoblasts require direct interaction with neurons to fully develop, the absence of support cell types in the initial cultures may be the source of restricted differentiation in these isolated insect muscle cells that have been immortalized. For CM large-scale production, the ability to control the proliferation and differentiation of cultured muscle cells is critical.

1.9.3 Molecular pathway

In mammalian systems, myoblasts are actively growing muscle precursor cells that are produced once a quiescent satellite cell is activated. Its proliferation is fueled by the myogenic regulatory factors (MRFs) MyoD and Myf5. Proliferation is aided by fibroblast growth factor (FGF), inhibited by myostatin and transformed growth factor (TGF). IGFs, or insulin-like growth factors, promote both proliferation and differentiation. Myoblast fusion into primary myofibers is fueled by IGFs and the MRF myogenin. IGFs and MRF4 encourage further fusion and differentiation, resulting in secondary fibers that eventually mature into myotubes with associated quiescent satellite cells. A second pair of MRFs, MRF4 and Myogenin, are increased during differentiation, promoting differentiation and fusion as well as assisting in maintaining the mature muscle structure (O'Neill et al., 2021). It has been demonstrated that CTNNB1 (β -catenin) and GSK3B (glycogen synthase

kinase-3) control the direction of skeletal myogenesis in animals like pigs from the earliest stages of embryonic development through terminal differentiation (Je et al., 2019). The two types of myoblasts found in insects originate from mesodermal regions that exhibit elevated levels of the bHLH transcription factor Twist. The high Twist domain exhibits a distinct mechanism whereby a muscle progenitor cell is selectively identified through a crosstalk between the Notch and Ras/MAPK signaling pathway. The progenitor myoblasts undergo asymmetric division, resulting in the emergence of either two founder cells or a founder cell and an adult precursor cell (AMP). The latter, while in a state of quiescence and undifferentiation during embryonic development, undergoes reactivation in the second larval instar, ultimately leading to the generation of adult fly muscles. The myogenic cluster's residual cells that exhibit Notch expression undergo differentiation into myoblasts that are capable of fusion (O'Neill et al., 2021).

1.9.4 Hormonal regulation

In *Drosophila melanogaster*, as in many other holometabolous insects, muscle formation occurs twice: during embryogenesis and metamorphosis. These insects show critical morphological differences between larval and adult stages. As a result, the muscles that develop during the embryonic stage of an organism are eliminated during the pupal phase of metamorphosis and substituted with adult muscles (Rout *et al.,* 2022). Without the insertion of additional myoblasts or nuclear division within the muscle syncytium, muscles in the larval stage enter a degree of hypertrophy. The arrangement of adult muscles differs significantly from that of larval muscles. The embryonic mesoderm engages the precursors of the adult muscles and delays their differentiation. The myoblasts for the head and thorax muscles are retained in the imaginal discs until pupal development, when the muscles develop. The imaginal disc is one of the components of a

holometabolous insect larva that will change during pupal metamorphosis into a section of the adult insect's exterior. There are disc pairs that may be used to create various structures, including wings, legs, antennae, and other parts (Beira and Paro, 2016). Most larval muscles are histolyzed to create adult muscles during metamorphosis, while adult muscles are de novo formed through the migration and fusion of adult muscle precursor cells (Morris *et al.,* 2011). Insect muscle cells undergo profound changes during the animal's life cycle. In this dynamism during growth, molting, pupation, and metamorphosis, certain hormones play an important role. In several species, the molt is stimulated by a hormone called ecdysone (also known as molting hormone). This hormone is secreted by two prothoracic glands, situated in the insect's thorax, and it is responsible for the growth and differentiation of adult structures. The production of ecdysone is in turn stimulated by a brain hormone, namely the prothoracicotropic hormone (PTTH) (Schmidt-Nielsen, 1997). In holometabolous insects, complex signals control both the timing and developmental stage as the animal undergoes metamorphosis. The endocrine function that controls these stages has been extensively studied in several moths, including the silk moth (*Bombyx mori*) and the tobacco hornworm (*Manduca sexta*). Whether a molt leads to larva, pupa, or an adult depends on the presence or absence of juvenile hormone. The juvenile hormone is present in the earlier larval stages, and the larval molt leads to a bigger larva. In the last larval instar, the level of juvenile hormone falls sharply, and pupa is formed. The final molt, when the pupa develops into an adult, depends on the absence of juvenile hormone (Schmidt- Nielsen, 1997). Despite a less comprehensive understanding of hormonal pathways in insect muscle development compared to humans and other animals, significant advancements have been achieved in differentiation

and proliferation. Low doses of 20-hydroxyecdysone stimulate myoblast proliferation in *M. sexta*, but concentrations beyond the critical threshold inhibit myoblast growth. Methoprene, an analogue of juvenile hormone, inhibits the capacity of high doses of ecdysteroid to induce proliferative arrest and differentiation (Champlin *et al.,* 1999). The hormonal regulators of the differentiation process can be employed to regulate cell growth throughout production (Klowden, 2004). In mammals, primary muscle-resident progenitor cells isolated from skeletal muscle differentiate into smooth and skeletal muscle, whereas satellite cells only differentiate to skeletal muscle. Differentiation in culture is based on using biological or chemical substances in cell culture media (Chodkowska *et al.,* 2022). Proliferation, differentiation and fusion processes are associated with the activity of several known hormones, growth factors, and transcription factors. The pathways in which they are involved play a crucial role in controlling muscle growth, energy metabolism, and repair of damaged muscle tissue. Some of the most important hormones involved in muscle development include testosterone, insulin, growth hormone (GH), IGF-1, cortisol and other thyroid hormones. The molecular control mechanisms that direct skeletal muscle development have significant implications for medicine, agriculture and food technology (Je *et al.,* 2019). In addition to their biological functions, the genes involved serve as important markers for monitoring and optimization in cultivated meat applications (O'Neill *et al.,* 2021).

1.10 Fat Cells

In cellular agriculture, fat is essential for flavor and nutrition. To provide healthy and tasty food, both muscle and adipose tissue are needed. Many differences can be detected between the adipose tissue of mammals and that of insects, which vary in terms of their cellular composition, regulation, function, and anatomical

location. Table 3 summarizes numerous distinctions observed.

1.10.1 Mammalian fat tissue composition

In mammals, adipose tissue is a connective tissue that primarily serves as a lipid storage of food and energy, as well as providing a significant amount of heat, water, and thermal insulation. Adipose tissue derives from mesenchymal cells that form during embryonic development from the mesoderm (Berry *et al.,* 2013). Adipose tissue also plays a role in the body's metabolism through the production of hormones, cytokines, proteins, and peptides. In mammals, adipose tissue is composed of white adipocytes (the primary site for energy storage) and brown adipocytes (specialized in thermogenesis). The predominant lipid-containing tissue in mammals is white adipose tissue (WAT), also known as unilocular adipose tissue. The location of WAT tissue is predominantly in the subcutaneous and abdominal region, and prominent deposits are also observed in skin and bone (Leitner *et al.,* 2017). WAT is predominantly found in the mesentery and intraperitoneal, with a lesser presence in the bone marrow and surrounding the visceral organs. The subcutaneous adipose tissue, apart from serving as a reservoir of energy, functions as a thermal insulator against low temperatures. WAT adipocytes are circular in shape and possess a significant size, measuring over 100 μm in diameter. They are characterized by a substantial lipid droplet that occupies most of their internal space. Adipocytes that are unilocular in nature are segregated by slender strata of lax connective tissue, which are replete with reticular fibers that are secreted by the adipocytes themselves. Furthermore, it should be noted that every adipocyte is enveloped by a slender coating of extracellular matrix, which is situated near the plasma membrane. The outer lamina, which bears resemblance to the basal lamina of the epithelium, and is distinct from the adjacent connective tissue, is referred to as the

sheath. In addition to mature white adipocytes and septa, various other cell types, including mast cells, macrophages, leukocytes, dispersed fibroblasts, and undifferentiated adipocytes, can also be observed. The dermal deposit and subcutaneous deposit are distinct entities that are physically segregated. Brown adipose tissue (BAT), also known as multilocular adipose tissue, is due to the presence of adipocytes that possess numerous small lipid droplets within their cytoplasm. It is prevalent in hibernating species, developing fetuses, and mammals during the perinatal period. BAT emerges prior to WAT during the developmental process. Brown adipocytes are smaller than white adipocytes and possess a rounded nucleus that is situated in the central regions of the cytoplasm. The abundant presence of cytochrome oxidase within the mitochondria of adipocytes is responsible for the brown hue exhibited by fresh brown adipose tissue. The high concentration of blood vessels within the tissue is also a contributing factor to the brown hue. BAT adipocytes are distinguished from WAT adipocytes by the presence of the UCP1 protein, which serves to disengage the chain of electron transporters from ATP synthesis. This results in the utilization of the proton gradient's energy for the purpose of heat generation (Leitner *et al.,* 2017).

1.10.2 Insect fat body composition

Numerous insect species have high quantities of essential fatty acids such as omega- 3 and omega-6 (Oonincx *et al.,* 2020). In addition to lipids, the fat body tissue of insects contains proteins and carbohydrates (Skowronek *et al.,* 2021). The insect fat body is essential to metabolic processes. It is situated in the hemocoel, where its constituent cells are near the haemolymph, allowing the exchange of metabolites. The fat layer just below the body wall is typically peripheral or parietal, while the layer that surrounds the feeding channel is

frequently perivisceral. Although parts of the fat body also extend into the chest and head, the majority is in the abdomen. Fat body shape can vary widely between orders and species. In hemimetabolous insects, the larval fat body remains mostly unchanged in the adult form. In holometabolous insects, the fat body goes through a remarkable metamorphosis in which the tissue separates into individual cells. The adult adipose cells in the majority of holometabolous insects come from the larval adipose cells, but the adult fat cells in the Hymenoptera and higher Dipterans are created entirely de novo (Chapman *et al.,* 2013). The storage of body fat plays a fundamental role in the life cycle of holometabolous insects. Throughout the larval feeding stages, energy stores accumulate to facilitate the metamorphic process and create reserves for the emerging adult organism (Mirth and Riddiford, 2007). Furthermore, the quantity of nutrients accumulated in the larvae has significant implications for their adult life, as diminished larval fat body size leads to decreased reproductive capacity (Briegel, 1990). Mature arthropods that exhibit a non-feeding lifestyle depend on these endogenous reserves to sustain their vital functions and reproductive activities. The process of egg development necessitates a significant transfer of resources from the adipose tissue to the ovaries. The significance of fat body reserves transferred from larval stages for oogenesis is evident in autogenic mosquitoes. In these mosquitoes, the activation of the target of the rapamycin signaling pathway and the subsequent maturation of eggs following a blood meal depend on the accumulation of sufficient nutritional reserves during larval development (Arrese and Soulages, 2010). In insects, the fat body is composed of five distinct cellular subtypes, exhibiting heterogeneity in their composition, dimensions, and functions, and physiological roles across various developmental phases. Trophocytes are the most abundant cells. These cells are primarily

responsible for the retention, excretion, and elimination of organic compounds. The cells exhibit variability in both size and quantity. Alterations in size are attributed to the accumulation of numbers and the expansion of the vacuoles. Four distinct types of vacuoles can be identified: digestive vacuoles, which facilitate metabolism and nutrient release during energetic or diapause expenditure; and storage vacuoles, which are responsible for the storage of reserve substances. Condensation vacuoles are associated with the Golgi apparatus and lysosomes and typically harbor proteins and surface vacuoles that arise from the fusion of vesicles (as observed in cellular specimens). The quantity of trophocytes is subject to variation, in addition to variations in their structural composition. Male insects exhibit a lower count of trophocytes in comparison to their female counterparts. Additional cells are also observed during the process of molting (Roma *et al.,* 2010). Enocytes are a type of cell that exhibits a circular or oval shape and are commonly found in association with the epidermal layer of the cuticle. These cells may also be present alongside the predominantly parietal adipose body. Enocytes possess a nucleus that is situated centrally, along with mitochondria, smooth endoplasmic reticulum, and vacuoles that contain lipid, protein, and glycogen droplets and granules. These cells are capable of synthesizing carbohydrates that are transported between the hemolymph and body fat (Gutierrez *et al.,* 2007). Mycetocytes are cellular entities primarily composed of symbiotic prokaryotic microorganisms. They coexist in a perpetual state of symbiosis with insects in a certain quantity. Mycetocytes are observed at the level of cytoplasmic fat and glycogen granules. Mycetocytes are present in nutrient-deficient and imbalanced environments and are responsible for the biosynthesis of certain essential nutrients, including amino acids and B-group vitamins (Willot *et al.,* 1988). Chromatocytes are thin cells

that show a central nucleus and a clear cuticle. These cells are situated in the thinnest layers of the fat body and accumulate lipids to support metamorphosis. Chromatocytes are present in select species of aquatic insects. Finally, urocytes exhibit distinctive features such as a diminished endoplasmic reticulum and a vacuole containing urate granules. Urate is derived from either the metabolic breakdown of nucleic acids or the degradation of proteins. The primary function of these cells is to accumulate and retain urate granules (Li and Yu, 2019). The fat body was one of the earliest forms of insect tissue to be cultivated *in vitro* for the study of protein production (Raikhel *et al.,* 1997). Important proteins, such as vitellogenin, the precursor protein of the egg yolk, and growth hormones that bind to proteins, are generated by fat body cells (Nowock *et al.,* 1975; Sun and Zhang, 2015; Wyatt, 1988). Fat-tissue-specific cells grow slowly at first but can be formed into continuous lines (Mitsuhashi, 1981). Other insect cells can be grown *in vitro* using fat body cells: the accumulation and release of nutrients by fat cells can extend the survival and contraction of muscle cells *in vitro* for months without altering the medium (Baryshyan *et al.,* 2012). Similarly, the development of embryos *in vitro* can be improved through the fat cells.

1.10.3 Fat tissue function

Adipose tissue in mammals serves various functions, including acting as a crucial mediator of metabolic control and communication, regulating thermoregulation, providing protection against cold and trauma, and controlling reproduction and satiety (Berry *et al.,* 2013). The insect's fat body, instead, is a versatile organ that performs a variety of physiological functions, including metabolic regulation, signal integration, regulation of molting and metamorphosis, and synthesis of hormones that modulate systemic function and immune protein synthesis. The substrates and products of numerous pathways in

fat cells include lipids, carbohydrates, and proteins, which can serve as sources of energy production, reserves, and mobilization during various stages of life such as diapause, metamorphosis, and flight. The adipose tissue also acts as the primary site for the integration of innate and adaptive humoral immune responses, as it is primarily responsible for the synthesis of antimicrobial peptides. Throughout the insect's life cycle, the adipose tissue undergoes a sequence of modifications, including development, expansion, and restructuring in the embryonic, larval, and pupal stages, while also governing reproductive processes in the adult stage. These alterations and regulatory mechanisms are regulated by hormonal and nutritional signals (Li and Yu, 2019).

1.10.4 Fat tissue regulation

In mammals, the regulation of metabolism in adipose tissue is primarily governed by insulin, which serves as the catalyst for the absorption and storage of energy (Honka *et al.,* 2018). Leptin is an additional hormone that does cause feelings of satiety. However, decreased levels of leptin serve as an indicator of reduced energy reserves, leading to an increase in appetite and the desire to consume food (Zhang *et al.,* 1994). The regulation of glucose and lipid metabolism within mammalian fat tissue is attributed to Adiponectin, which also facilitates a metabolic profile that exhibits antiatherogenic, anti-inflammatory, and insulin sensitizing properties (Iwabu *et al.,* 2019). The identification of molecules that have effects has contributed to the progress in comprehending adipose tissue as an endocrine organ. Bioactive lipids, including 12,13-dihydroxy9Z-octadecenoic acid (12,13-diHOME) and 12-Hydroxyeicosapentaenoic acid (12-HEPE), are secreted by brown adipose tissue (BAT) and promote the uptake of glucose and fatty acids in both BAT and skeletal muscles, thereby facilitating sustained thermogenesis (Leiria and Tseng,

2020). The metabolism of the fat body in insects is governed by a multitude of compounds, enzymes, and substances, primarily through the influence of hormones that modulate the activity of metabolic processes within the adipose tissue. Hormonal activity plays a crucial role in the process of insect metamorphosis, including the development and timing of molting. The hormones that commonly regulate various processes include adiponectin AKH, ecdysteroid (Ecd), juvenile hormone (JH), neuropeptide activating the diapause-pheromone hormone biosynthesis (DH- PBAN), corazonin (crz), leucochine (Lk), CCHa2, allanostatin-A (Ast -A), tachykinin (Tk), limostatin (Lst), cytokines, short neuropeptide F (sNPF) and neuropeptide F (NPF). The neurosecretory cells in the heart bodies create the peptide known as AKH (Gade and Auerswald, 2003). Additionally, AKH is expressed in the middle intestine, muscle, body fat, and ovaries. It is comparable to glucagon and has 8–10 amino acids. Numerous insects have several AKHs, and migrating locusts have three variations with distinct bioactivities (Arrese *et al.,* 2001). The hormone is originally present as a prohormone that splits off as AKH from the peptide associated with the adipokinetic hormone precursor (APRP) when it is activated. Due to the management of energy stores and their mobilization in insects' bodies during mutation and metamorphosis, the activity of AKH is present at the most crucial developmental phases (Toprak, 2020). It largely affects how enzymes such as glycogen phosphorylase, which converts glucose into sugars, and triglyceride lipase, which is involved in lipid metabolism, function. A common reaction to abrupt changes in lipid levels is the formation of AKH. The transduction signal AKHR (i.e. AKHR) activates the hormone. After that, phospholipase C, which converts membrane lipids into inositol 1,4,5-trisphosphate and diacylglycerol, is activated by AKHR. AKHR influences inositol triphosphate (IP3) concurrently,

which elevates calcium ions in the endoplasmic reticulum and transfers them to the cytoplasm. In addition, the activation of the hormone impacts the commencement of the activity of adenylate cyclase and hence the generation of cAMP. As a result, AKH activation controls the amount of TG in the fat body (Alves-Bezerra *et al.,* 2016). The hormone raises heart rate, motility, and neural signaling; it improves muscular tone; and it protects against oxidative stress. It also affects CREB, calcium homeostasis, and the expression of genes related to fat degradation (Alves-Bezerra *et al.,* 2016; Strachecka *et al.,* 2017). Juvenile hormone (JH) regulates various processes that affect the larva's growth and appearance and promotes the production of vitellogenin, a crucial precursor to the bull protein that is delivered into the ovocytes. It has been demonstrated that JH regulates protein granule existence and that its absence signals metamorphosis by causing the cytolysis of the larval body fat and the synthesis of a new one. The counterpart JH- 1 also results in the vacuolization of aged trophocytes. This hormone's low content induces the formation of vitellogenin in the fat body (Stratchecka *et al.,* 2017; Liu *et al.,* 2009). Ecdysteroid functions in opposition to and concurrently with JH. By promoting tissue dissociation (the metamorphosing stage), tissue remodeling, and the emergence of autophagic structures, Ecdysone controls the timing of metamorphosis. JH prevents premature aging and transformation. Serious deformities, issues with mutation, and a lack of transformation are brought on by a deficiency of any of these hormones (Liu *et al.,* 2009). The intricate nature of hormonal regulation in insects suggests that hormonal signals may have an impact on the storage of lipids in fat cells, and therefore, it is recommended that *in vitro* fat body culture include supplementation with such signals. While the application of these systems in mammals is well understood and can be readily applied in the

cultivated meat industry, the understanding of these systems in insects is comparatively limited, necessitating further research. Currently, most of the experimental research on cellular agriculture has prioritized the production of muscle cells, given their prominence in the biomass of meat products. However, recent industrial developments and proof-of-concept studies increasingly focus on cultivated fat, often combining it with plant proteins, as this strategy more effectively reproduces the sensory properties of conventional meat than muscle cells formulated with vegetable fats alone (Rubio *et al.,* 2020b; Fish *et al.,* 2020; Song *et al.,* 2022). In line with this, fat content is now widely recognised as a central driver of taste, texture, nutritional value, and consumer acceptability of cellular meat. The optimal source to produce cultivated fat (CF) remains uncertain. However, in mammals, several cell types exhibit the ability to undergo adipogenic differentiation *in vitro* (Yuen *et al.,* 2022). These include mesenchymal stromal/stem cells from adipose tissue, bone marrow or umbilical cord, as well as pluripotent stem cells. PSCs comprising both ESCs and iPSCs, represent a versatile source for the generation of mature adipocytes through stepwise differentiation protocols. ESCs have been derived from blastocysts of several species, including cattle and pigs (Bogliotti *et al.,* 2018; Hou *et al.,* 2016; Ezashi *et al.,* 2009), and, more recently, authentic ESCs have also been established across multiple avian species (Chen *et al.,* 2025). (Bogliotti *et al.,* Ezashi *et al.,* 2009). Adipocytes may be derived from mesenchymal stem cells (MSC), which are typically extracted from adipose tissue and bone marrow. Several studies have demonstrated the feasibility of extracting adipocytes from the larval phases of various insects. This involves dissecting the fat body from the abdomen and subsequently mincing it in a suitable culture medium (Rubio *et al.,* 2019b). However, further research is necessary into methods for large-scale

production and control of lipid accumulation in insect fat body cells. Several studies have demonstrated the possibility of co-culturing muscle cells and insect fat cells. Particularly, the functions performed by fat cells, such as nutrient storage and release, have been found to enhance the survival and contraction of muscle cells. Additionally, it has been observed that both cell types can be cultivated using the same culture medium, which is a challenging task for mammalian cells due to the distinct media formulations required by the two cell types (Baryshyan *et al.,* 2012; Schatzlein and Blaeser, 2022). To create edible and nourishing food items, it will be crucial to cultivate both fat cells and muscle cells (Rubio *et al.,* 2019b).

Table 2: *Comparison of muscle cells in mammals and insects.* The table compares the characteristics of muscle cells in mammals and insects, highlighting differences in cell types, development, and molecular pathways, analyzing the unique structural and functional distinctions in their muscle systems.

TYPES OF MUSCLE		
Characteristics	**Mammals**	**Insects**
Classification	Divided into skeletal, cardiac, and smooth muscle.	Divided into skeletal and visceral; primarily striated muscle, only a small part lining the internal organs is smooth type.
Cell Structure	Skeletal and cardiac muscles have striations, while smooth muscle does not. Skeletal muscles can be large and multinucleated.	Similar striated structure, with skeletal muscles attached to the exoskeleton; visceral muscle less numerous.
Contractile Units	Actin and myosin in sarcomeres.	Like mammals, actin and myosin are organized in sarcomeres.

Number of nuclei	From mononucleate to multinucleate depending on the type of muscle.	From binucleate to multinucleate, often fewer nuclei compared to mammals.
MUSCLE DEVELOPMENT		
Development Process	**Mammals**	**Insects**
Differentiation	Differentiation from satellite stem cells to myofibers, using growth factors such as FGF.	Formation from myoblasts (skeletal muscle precursors), which divide into founder cells and fusion-competent myoblasts.
Regeneration	Presence of satellite cells for the regeneration of skeletal muscles.	Limited regeneration; some insects regenerate muscles during metamorphosis.
Interaction with other tissues	Interactions with the nervous system for innervation and regulation.	Essential interactions with neurons for complete development.

MOLECULAR PATHWAYS		
Molecular Regulation	**Mammals**	**Insects**
Regulatory Factors	MyoD, Myf5, myogenin, MRF4, CTNNB1 and GSK3B are crucial for muscle development. FGF, IGFs, Myostatin, TGF regulate proliferation and differentiation.	Twist, a class of bHLH factors, regulates the distinction of myoblasts. Notch and Ras/MAPK pathways for the selection of muscle precursors.
Hormonal regulation	Hormones such as IGF, GH, sex hormones are important for muscle growth.	Ecdysone, induced by PTTH, and juvenile hormones regulate growth and metamorphosis.

Table 3: *Comparison of Mammalian and Insect Fat Cells.* The table delineates a comparative analysis of fat cells in mammals and insects, elucidating their unique characteristics, functional roles, and potential applications in cellular agriculture for food production.

COMPOSITION AND DEVELOPMENT		
Aspect	**Mammalian Fat Cells**	**Insect Fat Cells**
Origin	Derived from mesenchymal cells in the mesoderm during embryonic development.	Located in the hemocoel, with various origins specific to insect physiology.
Types and functions	White Adipocytes (WAT) for energy storage, primarily in subcutaneous and abdominal areas; and Brown Adipocytes (BAT) are specialized for thermogenesis.	Trophocytes for nutrient storage and metabolism; Enocytes for carbohydrate synthesis; Mycetocytes, symbiotic prokaryotic microorganisms for nutrient synthesis; Chromatocytes for lipid storage to support metamorphosis; Urocytes for storing urate granules.
Appearance and Features	WAT has a large, circular shape, adipocytes unilocular; BAT, adipocytes multilocular, rich in mitochondria and with a high concentration of blood vessels, which contribute to the brown hue.	Cells are versatile in function, not specifically color-coded but distinguished by their specific roles and content (such as lipid, protein, and carbohydrate).
FUNCTION AND REGULATION		
Aspect	**Mammalian Fat Cells**	**Insect Fat Cells**
Regulatory Mechanisms	Governed by hormones such as insulin (energy storage), leptin (appetite control), and adiponectin (glucose and lipid metabolism). Bioactive lipids from BAT promote glucose uptake and thermogenesis.	Regulated by a range of hormones like AKH (energy mobilization), ecdysteroid (metamorphosis), juvenile hormone (growth and development), which impact a wide array of physiological

		processes from growth to systemic immunity.
RESEARCH AND APPLICATIONS		
Aspect	**Mammalian Fat Cells**	**Insect Fat Cells**
Cultivation for Food	Studies focus on deriving adipocytes from pluripotent and mesenchymal stem cells, and dedifferentiated fat cells for cultivated meat applications. Commonly used cell lines include 3T3-L1 and other murine lines for research and food production. Continued exploration into efficient and scalable methods to cultivate mammalian adipose tissue *in vitro*, aiming for texture and taste that mimic natural meat.	Emerging research into cultivating insect fat cells, notably for their roles in nutrient storage and release which can enhance muscle cell co-culture systems. Specific culture conditions are being developed to optimize lipid accumulation and usage in sustainable food production systems.

1.11 Economic and environmental sustainability of CM

Using tissue engineering techniques, *in vitro* CM allows the meat production without the use of animals. *In vitro* CM may be more advantageous than traditional meat production in terms of costs, health, animal welfare, and the environmental impact (Bhat *et al.,* 2017). On 5th August 2013, in London, an *in vitro* beef burger was first publicized and tasted. Since then, the media has presented cell-based meat as a novel approach to generate meat with enormous opportunity (Schatzlein and Blaeser, 2022). Insects, as a potential alternative source of protein, also play a role in discussions about sustainable nutrition. It was possible to make a comparison between the nutritional profile of edible

insects and mammalian meat. The Orkusz study compares the nutritional value of insects with that of meat from slaughtered animals, highlighting that both are rich in proteins, essential amino acids, unsaturated fatty acids, vitamins, and minerals (Orkusz, 2021). Although it is not possible to definitively state that insects are nutritionally superior to meat, some insect species show higher energy and specific nutrient contents, such as proteins and polyunsaturated fatty acids, compared to meat. Insects also have higher levels of certain minerals and vitamins and are a source of vitamin C and fiber, which are not present in meat. These nutritional characteristics make insects a potentially valuable resource for CM production, even though not all insect-derived components (e.g. fibre or chitinous fractions) would necessarily be present in the final cultivated products. Nevertheless, insect-based CM could help enrich diets and improve health, contributing to the fight against global malnutrition. A significant benefit of CM production is improved control over flavor, fatty acid composition, fat content, and the ratio of saturated to polyunsaturated fatty acids by modifying the culture medium composition or co-culturing with other cell types. *In vitro* CM does not require killing animals, and animal suffering as well as the number of animals used in meat production are projected to decrease because of *in vitro* CM; in theory, the supply of meat for the entire planet could be produced by a small farm that provides occasional biopsies (Bhat and Bhat, 2011). Ten stem cells could produce 50,000 metric tonnes of beef if they divide and differentiate continuously for two months. Although in practice much optimization is needed to get to this level of efficiency (Bhat and Fayaz, 2011). Furthermore, strict quality control regulations, which are impossible to implement in contemporary animal husbandry, slaughterhouses, or meat packing plants, could significantly reduce the likelihood of meat contamination and the incidence of zoonotic

diseases in large-scale CM facilities. Additionally, traditional meat exposure to hazards like pesticides, arsenic, dioxins, and hormones should be significantly reduced (Wigglesworth, 1967). The *in vitro* cultivation of meat allows faster production of the final product compared to livestock, focusing on key meat components such as muscles while avoiding the production of unnecessary tissues like bones, respiratory organs, digestive organs, skin, and nervous system. In traditional meat production systems, a significant portion of the food consumed by animals does not effectively transform into meat due to metabolism and the formation of inedible parts like bones and brain tissue. In contrast, lab-grown meat is time and energy-efficient, taking only a few weeks instead of months (for chickens) or years (for pigs and cows) to be ready. Moreover, producing meat *in vitro* from insect cells has many environmental advantages linked to the differences from mammalian cells. *In vitro* CM will also significantly minimize land needs. The carbon footprint of meat products should also be reduced through *in vitro* manufacturing, which can also reduce greenhouse gas emissions from raising livestock for meat by up to 90% and land and water resources for raising meat by up to 80%, although further research and development is necessary to support these estimates during large-scale CM production (Fox, 2009). The substantial decrease in land use projected creates opportunities for other uses of the land, such as reforestation, which may help in the recovery of many endangered species. The scientific, environmental, and animal rights sectors are also supportive of *in vitro* meat production because it is a more environmentally friendly method of manufacturing meat with fewer negative impacts on human health. By cultivating cells from rare or endangered animals held in captivity, or even cells retrieved from samples of extinct creatures, it would be possible to create new types of meat and meat-based

products for future markets, effectively enabling their consumption without impacting current populations. In many instances, such as those involving space missions, polar stations, troop encampments in remote theatres of war, and bunkers intended for long-term personnel survival after nuclear or biological attacks, it may be more effective to produce food on-site. *In vitro* meat production is a potential solution in these circumstances. In particular, the European Space Agency (ESA) is looking for ideas into how cellular agriculture could be used to grow food on long-term space missions. This will reduce the quantity of perishable food that must be transported, give an alternate source of nourishment, and provide fresh food. Such a novel food production system for space should be included in a closed-loop arrangement so that resources, especially the growth medium, may be recycled or regenerated, thereby minimizing dependence on supplies from Earth (ESA, 2022). *In vitro* meat production has many supporters but also raises concerns. The unnatural nature of *in vitro* meat is an issue with adoption by the public and appears to be a factor in opposition to new food technology, at least in Europe (Hopkins and Dacey, 2008). Novel foods are supposed to be essential to the shift to sustainable food systems. Nevertheless, whether and how much they are adopted into the diets of the public will determine their success. Frameworks for the production and distribution of CM are starting to be developed by governments and regulatory agencies. At EU level, CM falls under the Novel Foods Regulation (EU) 2015/2283, which requires pre-market authorisation based on EFSA's risk assessment, while countries such as Singapore, the United States and Israel have already granted limited approvals for specific cultivated meat products (Lanzoni *et al.,* 2024). In parallel, some national legislators have adopted markedly restrictive approaches. In Italy, Law No. 172/2023 prohibits the production and

placing on the market of foods and feeds constituted, isolated or produced from cell cultures or tissues derived from vertebrate animals and restricts the use of the legal denomination “meat” for plant-based products; on its literal wording, the ban does not extend to cell-based foods obtained from invertebrates, so insect-derived CM remain in principle outside the scope of this national prohibition, although they would still require authorisation as novel foods at EU level (Italian Law 172/2023; Spoto, 2025; Lanzoni *et al.,* 2024). Comparable prohibitions on the production and sale of cultivated meat have been advanced or adopted in other jurisdictions, including Hungary, through a draft act notified to the European Commission under the TRIS procedure, and U.S. states such as Florida, where SB 1084 (2024) bans the manufacture for sale, sale and distribution of cultivated meat (European Commission, 2024; Florida Senate, 2024; Fortin, 2024). These restrictive measures coexist with more enabling regulatory pathways and are likely to influence where and how CM technologies, including insect-cell platforms, can be translated into commercial products. Having clear guidelines can help to increase consumer confidence and trust. According to research in the literature, the main obstacles to the adoption of cultured meat include contextual issues like price, emotions like fear and disgust, and cognitive problems like lack of familiarity (Grasso *et al.,* 2019; Dupon and Fiebelkorn, 2020; Valente *et al.,* 2019). In response, several tactics have been put out to boost consumer acceptance of cultured meat. These tactics include educating customers about the production method and the advantages of CM, as well as facilitating production scale- up point to present the product at a lower price. This is going to be a major motivator for consumer adoption (Humbird,2021; Arango *et al.,* 2023; Monaco *et al.,* 2024). CM advocacy will be greatly aided by influencer collaborations and educational efforts, and it should

become a common choice for people looking for ethical, sustainable, and healthy food options as the sector develops.

1.12 Conclusion

Cultivated meat has the potential to provide consumers with the nutrition they need while significantly reducing the animal suffering, environmental, and human health issues associated with conventional meat farming. Due to the differences between insect and mammalian cells, *in vitro* CM from insects has considerable advantages for the environment and for large-scale production with a more cost-efficient approach. These include:

- the adaptability of insect cells to both adherent and suspension growth.

- cost reduction of culture media. Mammalian cells require larger amounts of culture medium and components due to the high rates of glucose consumption and accumulation of toxic by-products during cell expansion. Insect cells consume less glucose during growth, accumulate less lactic acid due to slower cell metabolism, and are less sensitive to toxic compounds.

- tolerance of a wide range of environmental variations, including pH (6.0-7.0) and temperature (20-32°C).

- they typically grow in absence of carbon dioxide, and the immortalisation process can occur spontaneously.

CHAPTER 2

1. *Hermetia illucens* as sustainable source of food and feed

This chapter consolidates the technological, regulatory, and adoption landscape for Hermetia illucens and anchors the thesis's system choice for downstream cell- agriculture work. This chapter is based on the following publication: Parisi, G., Piccolo, G., Ouazri, S., Secci, G., Scieuzo, C., & Falabella, P. (2026). Hermetia illucens as sustainable source of food and feed. In F. Bovera & P. Falabella (Eds.), The black soldier fly (Hermetia illucens): Sustainable applications in food, feed, and beyond (pp. 101–124). Academic Press. https://doi.org/10.1016/B978-0-443-29896-7.00006-8 Hermetia illucens

Nearly 2000 insect species have been consumed by humans in entomophagous societies; *Hermetia illucens,* the black soldier fly (BSF), is not as common or well- liked. As ethnographers are not often entomologists, and local people are not always inclined to use the scientific name of an insect when eating it, this prevents precise identification of the species being consumed (Mitsuhashi, 2016; Ramos-Elorduy *et al.,* 1997). Indeed, finding proof of human consumption of *H. illucens* is difficult (Mitsuhashi, 2016). Currently, the only known population consuming *H. illucens* as food is in Sabah Province, Malaysia, on the island of Borneo, where more than 60 species of insects are consumed, mainly by indigenous people. Here, black soldier fly larvae (BSFL) are ingested raw along with a locally produced fermented drink called tapai (Chung *et al.,* 2002). Their high nutritional value, minimal environmental footprint, and potential for large-scale production make them a promising solution for addressing global food security challenges (Kaczor *et al.,* 2022; Nayak *et al.,* 2023; Wang & Shelomi, 2017). Despite these benefits, several barriers hinder the widespread use of *H. illucens* for human consumption due to cultural resistance, safety concerns, and regulatory hurdles. Many societies, especially in Western cultures, view insect consumption (entomophagy) with disgust or skepticism. Several reasons stand beyond this approach; first, countries with limited exposure to entomophagy view it as unconventional or primitive. This cultural unfamiliarity leads to

skepticism and a lack of willingness to try insect-based foods (Wang & Shelomi, 2017). However, even in Western countries, eating insects evoked adventurous, daring, and wild emotions, both in males and females, even if the first seemed favorable to eat insect or insect-based foods (Tucillo *et al.,* 2020). Nevertheless, another study revealed no effect of gender on the probability of eating insects as food, while low levels of phobia for new foods and specifically for insects have been reviewed as one of the main barriers (Moruzzo *et al.,* 2021). To overcome food neophobia, processing and adding edible insects as ingredients to familiar consumer goods have been suggested as useful strategies to improve insects' acceptance, production, and consumption while also concealing their undesirable aspects. As suggested by Higa *et al.* (2021), among American participants in their study, 22% would taste whole roasted BSFL when included in their favorite cereals without any financial incentive, and only 10% asserted that they would not try them. Furthermore, during the same experiment, consumers said that they would be mostly likely to eat BSFL if they were called earth caviar (36% of participants), followed by black butter pods (29%), Malaysian butter grub (18%), but only the 10% would eat BSFL called with its name (Higa *et al.,* 2021). As a matter of fact, the appearance of whole insects or insect larvae can be off-putting to consumers unfamiliar with such foods. The larvae can be processed into flour or protein isolates for human consumption and can be used in innovative foods such as protein bars, snacks, and meat alternatives. Recently, it has been observed that when processed into less recognizable forms (e.g., powders or flours), BSFL seemed to be more acceptable than the whole larvae (Sogari *et al.,* 2018). Furthermore, when a sensory evaluation was performed on three cricket-based foods (dried whole crickets, focaccia bread with bits of dried crickets, cricket flour- based crackers) with 52 Italians, results revealed that a low level of

insect visibility was preferred (Tucillo *et al.,* 2020). The potential of using BSFL fat as an ingredient in bakery goods is examined in the study by Delicato *et al.* (2020). For cakes, cookies, and waffles, a total of 344 respondents' sensory and emotional profiles, willingness to pay, liking, and product preference were investigated. To replace butter, 0%, 25%, or 50% BSFL lipids were used in the formulation of each bakery product. According to the results, BSFL can substitute 25% of the butter in these baked goods without affecting the final flavor or experience. Without affecting consumer acceptance, the substitution in waffles could even reach 50%. Moreover, the aversion for insects stems from cultural norms that categorize insects as pests or contaminants rather than food (Wang and Shelomi, 2017). The idea of consuming insects like BSFL, which can convert waste into larval biomass, provides them with a significant advantage over other insects, adding value and closing nutrient cycles while reducing costs and pollution. However, this all- encompassing benefit is also its main drawback, as it contributes to the existing taboos against consum- ing insects by making it socially unacceptable to consume organisms that feed on waste (Bessa *et al.,* 2021; Wang & Shelomi, 2017). This has been confirmed by Higa *et al.* (2021), who found that Americans felt the least negative about eating BSFL fed on leftovers, followed by BSFL fed on vegetables, but whose parents had been fed on feces, and finally, BSFL fed on feces. Hence, the growing substrates greatly affect people's acceptance and willingness to consume insect-based food. This connection to waste, although environmentally beneficial, can generate concerns about contamination and safety. Despite being a promising and sustainable source of nutrients, there is limited knowledge regarding the food safety of consuming BSF (Costa-Neto, 2013; Mitsuhashi, 2016; Wang & Shelomi, 2017). Overall, insect consumption raises safety concerns related to the

potential presence of chemical and biological contaminants. One of the most significant biological risks associated with edible insects is the potential for pathogenic and poisonous microorganisms to contaminate them throughout their life cycle. These microorganisms can be introduced during breeding, harvesting, or processing and may lead to foodborne illnesses if not properly managed. Additionally, the risk is heightened if insects are not kept in sanitary conditions or if they are not subjected to adequate cooking or processing methods to ensure their safety for consumption. Therefore, rigorous hygiene practices, effective pest management, and thorough processing protocols are crucial to mitigate these risks and ensure the safety of edible insects (European Food Safety Authority, 2021; Garofalo *et al.,* 2019). The Netherlands Food and Consumer Product Safety Authority has issued a pre- liminary report aimed at assisting in the identification of potential microorganisms relevant to edible insects and proposing limits for them. However, there are currently no regulations governing the use of BSFL in food products (Potting, 2014) Numerous studies have assessed the safety of consuming BSFL, considering microbial content, heavy metal levels, and allergen presence (Bessa *et al.,* 2021; Proc *et al.,* 2020; van der Fels-Klerx *et al.,* 2020). For direct human consumption, the safety of BSFL is influenced by many factors, such as the food substrate provided to the larvae, the rearing methods employed, the farming environment, the extent of handling, interactions with other microbial communities, and the origin of the BSFL parent (Liguori *et al.,* 2022). This highlights the importance of implementing sufficient decontamination measures to ensure food safety, such as the blanching method or microwave sanitization (Bessa *et al.,* 2021; Wynants *et al.,* 2019). Food regulation is intricately linked to issues of food supply and safety. In regions without a long history of entomophagy, such as Europe, stringent regulations are

in place for insects categorised as “novel foods” under Regulation (EU) 2015/2283, which came into effect in 2018. These regulations require comprehensive assessments of the insects’ safety and nutritional value before they can be marketed for human consumption. This regulatory framework is designed to prioritize risk avoidance and ensure that all novel foods, including edible insects, meet established safety standards. Since food proteins are often the primary source of allergens, the analysis and identification of potential allergenicity in novel proteins is a critical activity. Evaluation and compliance with these regulations are essential to address potential health risks and facilitate the integration of edible insects into the food supply chain (Knowles *et al.,* 2007; The European Parliament and the Council of the European Union, 2015). This not only allows applicants to meet the requirements for marketing innovative food products but also ensures a high level of food safety for European consumers. The European Food Safety Authority (EFSA) updated its guidelines to reflect advancements in food research and innovation. These updates have applied to all novel food applications submitted to the European Commission starting in February 2025. The revised guidelines provide more detailed information, particularly concerning scientific requirements, aiming to enhance the quality of applications and streamline the risk assessment process (see Navigating Novel Foods: what EFSA’s updated guidance means for safety assessments EFSA visited on 5/22/2025 at https://www.efsa.europa.eu/en/news/navigating-novel- foods-what-efsas-updated- guidance-means-safety-assessments). According to EFSA, the summary of applications submitted within the meaning of Article 10(1) of Regulation (EU) 2015/2283, there is currently one application concerning the use of *H. illucens* as novel food, it is called Hermetia meal, and it was submitted

in 2018 by a company from Flemming, Denmark. On the contrary, BSFL are frequently used in feeding programs for several animal species. In this regard, scientific literature shows a growing interest in the use of insects in general, specifically on BSF, as feed for a varied range of species. Even when larvae are raised on vegetable waste, BSFL meal and oil have a high protein and lipid content, respectively, making them a recognized animal substitute for fishmeal and fish oil in animal diets (European Regulations 2017/893 and 2021/1372) (Wang & Shelomi, 2017) (Figure 1). Several studies have shown that the inclusion of BSFL in animal feed is not only a viable alternative to traditional protein sources such as soybean and fishmeal, but can also improve aspects such as growth, immunity, and meat quality due to the nutritional management of the larvae and their protein- and fat-rich composition (Franco *et al.,* 2021b). The number of research articles has grown exponentially, and the published reviews follow the same trend. Table 1 depicts some of the relevant reviews published from 2015 on this topic. Most of the reviews focus on the aquaculture and poultry sectors, which are the leading drivers of animal production in terms of volume worldwide. The same groups were the first to be authorized in Europe to be fed on processed insects. Despite the advantages connected to the use of BSFL in feed as a valuable substitute for the commercial protein sources, namely soybean meal and fishmeal, its inclusion level has been deeply studied and proposed for the different species. Research highlights that defatted BSFL meal, with digestibility coefficients often exceeding 85%, provides an exceptional protein source for swine, poultry, and aqua- culture species. Its high lysine and methionine content further supports its potential as a viable alternative to conventional protein sources (Henry *et al.,* 2015; Makkar *et al.,* 2014).

1.2 Fishes

H. illucens is used as a substitute protein source for many fish species in aquaculture (Tschirner & Kloas, 2017). Since it contains a lot of crude protein, BSFL meal has been shown to be an excellent substitute for fish meal. Many aquaculture fish species have responded favorably to various BSFL inclusion levels. However, in many cases, when levels exceed 50%, growth characteristics frequently suffer, likely because of the high chitin content, high crude fat content, and incapacity of the fish species and growth stage to use an insect-based diet (Priyadarshana *et al.,* 2021). For example, Belghit *et al.* (2019) demonstrated the effectiveness of BSFL as a fish meal substitute by showing that adding up to 600 g of insect meal per kg of food, together with BSFL fat, had no detrimental effects on feed intake, growth performance, or feed conversion ratio in Atlantic salmon. In addition, BSFL enriched with fish waste showed high levels of omega-3 fatty acids, including ALA, EPA, and DHA, making them a viable alternative to recycle essential fatty acids and improve the nutritional quality of the feed (Barroso *et al.,* 2017). A 2020 study analyzed the effect of BSF inclusion in the diet of juvenile mirror carp (Cyprinus carpio var. specularis). The authors concluded that this fish could be fed BSF at a level below 131 g/kg without having a deleterious impact on their ability to grow or their intestinal health (Xu *et al.,* 2020). Still on C. carpio, which was one of the leading species in global aquaculture production in 2019, another study showed that the BSF oil combined with highly unsaturated fatty acids had substantially increased growth and immunity in the fish (Jahan *et al.,* 2021).

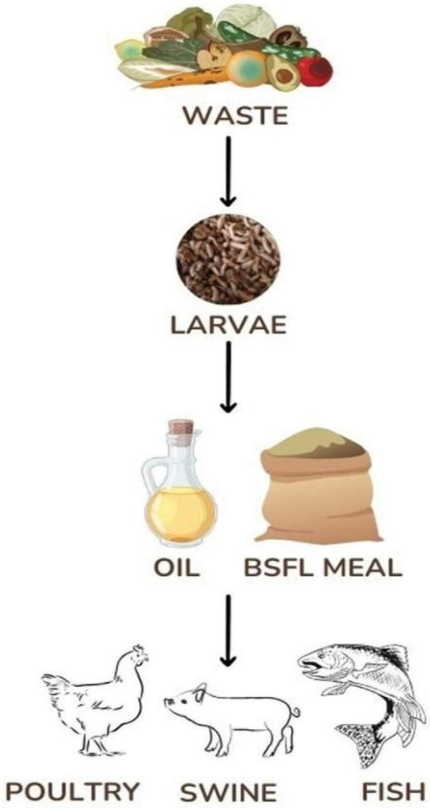


Fig 1. *Organic waste is used to feed the larvae and prepupae of H. illucens, which are subsequently processed into BSFL oil and meal*. These products serve as sustainable and nutritionally valuable ingredients for feeds intended for poultry, swine, and fishes (Image Created with Canva.com).

Tab 1: *Reviews on "Hermetia illucens + feed + review" Extract from Scopus*© (no filters were applied).

TARGET GROUP	SPECIFIC SUB-GROUP (IF ANY)	REFERENCES
FISH		Henry *Et al.* (2015)
		Tomberlin *Et al.* (2015)
		Barragán-Fonseca *Et al.* (2017)
	Salmonids	Papuc *Et al.* (2020)
	Finfish	Priyadarshana *Et al.* (2021)
		Maulu *Et al.* (2022)
		Nairuti *Et al.* (2021)
		Mohan *Et al.* (2022)
		Priyadarshana *Et al.* (2021)
	Salmonids	Weththasinghe *Et al.* (2022)
		Tran *Et al.* (2022)
		Macusi *Et al.* (2023)
FISH, CRUSTACEAN		Calinski *Et al.* (2024)
POULTRY		Tomberlin *Et al.* (2015)
		Barragán-Fonseca *Et al.* (2017)
		Abd El-Hack *Et al.* (2020)
		Dalle Zotte 2021
		Sverguzova *Et al.* (2021)
		Adli (2021)
		Lu *Et al.* (2022)
	Broiler	Ahmed *Et al.* (2023)
	Chicken	Zaid *Et al.* (2023)
	Broiler and layer	Hossain And Bhuiyan (2023)
		Dalmoro *Et al.* (2023)
		Salahuddin *Et al.* (2024)
	Quails	Polubesova *Et al.* (2024)
	Laying hens	Fikri *Et al.* (2024)
	Broiler	Vasilopoulos *Et al.(2024)*

		Silva *Et al.* (2024)
LIVESTOCK		Tomberlin *Et al.* (2015)
PIG		Barragán-Fonseca *Et al.* (2017)
SWINE		Lu *Et al.* (2022)

Despite the different species, even the marine finfish gilthead seabream (*Sparus aurata*), a species of great interest for European aquaculture, can be fed with BSFL included in aquafeed up to 11% (Di Rosa *et al.,* 2023) and 15% (Busti *et al.,* 2024) to partially replace fishmeal without impairing growth performance, blood biochemistry, or stress-related parameters. When inclusions higher than 15% were tested, the insect- based diets were responsible for a reduction of the intestinal mucosa and submucosa integrity (Di Rosa *et al.,* 2023). Considering the marketable traits, such as weight, body length, and fillet yield, it is possible to include up to 27.6% in a diet for gilthead seabream (Pulido *et al.,* 2022); nevertheless, at this inclusion level, a deep effect on lipid composition has been observed by the authors. Indeed, the dietary interventions tested showed no difference in terms of saturated fatty acids when BSFL amount was at 9.2 g/100 g as feed, while a significant increase in this undesirable lipid fraction resulted in fillets from fish fed BSFL at 18 or 27 g/100 g. Interestingly, irrespective of the dietary BSFL content, the overall n-3 PUFA positioned in the sn- 2 of triglycerides was not affected (Pulido *et al.,* 2022), thus resulting in a possible advantage for human nutrition since that position is the one better absorbed by the human body. On the contrary, the fish species considered “similar” to S. aurata, namely European seabass (*Dicentrarchus labrax*), seemed to tolerate a BSFL inclusion level up to 19.5% (Moutinho *et al.,* 2021); in this case, neither fish growth nor fillet nutritional characteristics were negatively affected. Many studies were also conducted on different families of fish other than Cyprinidae,

like Salmonidae, Clariidae, Cichlidae, Latidae, and Acipenseridae, as highlighted in the review by Mohan *et al.* (2022). The cited studies and the inconsistencies that emerge once again highlight the importance of adopting species-specific research while also emphasizing that multiparametric and multidisciplinary approaches can help clarify the effects of including BSFL in fish diets.

1.3 Poultry

Since BSFL naturally establishes itself in and decomposes poultry manure, and be- cause populations are frequently maintained by poultry farms for the sake of waste management and pollution reduction, BSFL has also been utilised in poultry feed as a partial replacement for maize- or soy-based feeds (Bradley & Sheppard, 1984; Bradley *et al.,* 1984). This approach offers a protein-rich alternative while allowing larvae to be reared directly on the manure of the poultry that consume them, thereby optimizing waste recycling and minimizing the environmental impact of poultry farming (Wang & Shelomi, 2017). In broiler feeds throughout their production cycle, meal from BSFL replaced 50% or 100% of soybean oil, while still ensuring satisfactory production performance, carcass characteristics, and overall meat quality; the same level of preference for the basic feed and the diet with 100% BSFL fat was also shown by chickens, suggesting that BSLF could be a promising new component of broiler feeds (Schiavone *et al.,* 2017). Moreover, the inclusion of BSFL-derived fats in poultry feed has been shown to improve the fatty acid profile of tissues and increase the total antioxidant capacity, contributing to enhance meat quality and nutritional sustainability (Kim *et al.,* 2020). Egg production is another sector of interest. In this regard, Fikri *et al.* (2024) have reviewed the role of BSFL in laying hen performances and egg quality, affirming that BSFL meals can be effectively used to optimize feed efficiency, Haugh units, albumen, eggshell quality, liver, renal,

and cellular physiology of laying hens. However, they found no significant impact on body weight gain, egg production, or hematological profiles. This outcome can be attributed to multiple factors; notably, many of the positive effects could be linked to the modulation of gut microbiota, especially by reducing harmful bacteria such as E. coli and Salmonella, due to the presence of lauric acid and chitin that have shown antibiotic properties (Lee *et al.,* 2018). Another frontier in the use of BSFL is its possible inclusion as live insects in poultry nutrition. Concerning this, authors have considered such inclusion not only as a mere nutrient, but also as an environmental enrichment able to increase poultry welfare. For instance, live BSFL reduced frustration in broilers (Biasato *et al.,* 2022) and decreased the duration of tonic immobility (Ipema *et al.,* 2020) by promoting increased activity, particularly foraging behavior. However, fear-related behaviors were not significantly affected (Huang *et al.,* 2024; Tahamtani *et al.,* 2021).

1.4 Swine

BSFL meal is a useful component for pig meals due to its high calcium and amino acid content as well as its pleasant taste (Barragán-Fonseca *et al.,* 2017). Evidence that BSFL is a promising protein source for pigs came from Yu *et al.* (2019), who demonstrated that adding 2% of full-lipid BSFL meal to the diets of weaned pigs had a substantial impact on growth and organ weight. However, due to its high level of fat, BSF meals should be combined with other protein-containing ingredients for pig diets (Newton *et al.,* 1977). Furthermore, methionine and cystine must be added to balanced diets due to its relative lack of those amino acids in BSFL meals (Makkar *et al.,* 2014). According to a study conducted in 2020, adding 0.3% and 0.9% fat from BSFL to pigs' diets had no negative effects on their general health and increased their average daily weight

gain by up to 9%. It also improved the concentration of total protein in tissues, leukocytes, and the population of Bifidobacteria in the large intestine (Nekrasov *et al.,* 2018). Researchers have shown how replacing up to 100% fishmeal with BSFL meal is suitable for the growth and development of pigs (Large White and Landrace crosses). In fact, pigs fed diets with BSFL meal replacing fishmeal at 50%, 75%, or 100% had heavier carcasses than swine fed a control diet, while the crude protein content of pig tissues was high (65%–93% on a dry matter basis) in all groups (Chia *et al.,* 2021).

1.5 BSF and pet nutrition

Pets, particularly dogs and cats, have become increasingly significant in households across the globe. According to a report by Health for Animals (2025), the number of pet owners is rising due to various social and economic factors that vary from one country to another. In high-income countries, there is a trend toward smaller family sizes and delayed parenthood, whereas middle-class populations are expanding in many emerging markets. The United States of America, China, and the European Union host a pet population of 496 million, equally divided between dogs and cats. In Brazil, there are around 54 million dogs and 23 million cats. Globally, the benefits of pet ownership are well-recognized. As reviewed by Hussein *et al.* (2021), "A pet will help to reduce anxiety levels, improve physical activity persistence, and enhance social interaction. Owning a pet contributed to modulating mental illness, reducing cardiovascular problems, improving the outcomes of many mental diseases such as depression, and it seems a helpful therapy for Parkinsonian patients." This flourishing sector has driven significant demand for pet food, a crucial factor influencing pet health and a key concern for owners. Over the years, the pet food market has seen remarkable growth in both volume and value. Production

increased from over 41 billion kg in 2018 to nearly 53 billion kg in 2024, generating a revenue of USD 151 billion in 2024—an increase of USD 62.2 billion compared to 2018 (data sourced from https://www.statista.com/outlook/cmo/food/pet-food/worldwide#analyst-opinion, last accessed May 2025). As the global pet population is projected to grow, further market expansion is forecast. Additionally, the trend of "pet humanization" has spurred demand for natural and organic pet food products, reflecting an emphasis on health and wellness within the pet food industry. To meet these evolving preferences, companies are introducing novel ingredients and formulations. In this context, insects have garnered attention from the pet food industry, with the first insect-based dog food in Europe launched in 2015 (Siddiqui *et al.,* 2023). A recent study has identified 43 brands offering insect-based pet food, primarily focusing on dry and wet food for dogs (Siddiqui *et al.,* 2023). Among the insect species used, the BSF is the most prevalent, owing to its exceptional nutritional properties. Studies on BSF digestibility showed analogous or improved protein and calcium digestibility in comparison with other common protein sources as poultry and venison meal (Abd El-Wahab *et al.,* 2021; Freel *et al.,* 2021; Penazzi, 2021).

Tab 2*: Incidence of food cutaneous adverse food reactions verified in dogs and cats as reviewed by Mueller et al. (2016).*

Dogs		**Cats**	
Source	**Incidence (out of 297 dogs)**	**Source**	**Incidence (out of 78 dogs)**
Beef	34%	Beef	**18%**
Dairy Products	17%	Fish	**17%**
Chicken	15%	Chicken	**5%**
Wheat	13%	Wheat	**4%**
Lamb	14.5%	Corn	**4%**
Soy	6%	Dairy products	**4%**

Corn	4%	Lamb	**3%**
Egg	4%		
Pork	2%		
Fish and rice	2% each		
Other less commonly reported: barley, rabbit, chocolate, kidney bean		**Other less commonly reported:** egg, barley and rabbit	

As concluded by Kepin´skaPacelik & Biel (2022), BSF, both as meal and oil, can be included in dogs' diet without affecting digestibility and overall health. Not surprisingly, claims like "gut health and easy digestibility" have been utilized by 19 brands of pet food out of 43 investigated (Siddiqui *et al.,* 2023). However, its potential as a food attractant remains uncertain. Since dietary preferences vary among dog breeds, further research with a species-specific approach is needed to clarify this aspect. Another aspect deserving attention is the use of insect diets as a potential risk for allergies. Dogs and cats are subjected to food allergies, whose common clinical manifestations are skin diseases, such as erythematous dermatitis, and gastrointestinal signs are typically related to food allergy in dogs (Hensel *et al.,* 2015). These manifestations are generally associated with an immunological reaction to specific proteins contained in foods (IgE-mediated food allergy), but they can also derive from a cross-reaction (the so-called "pan-allergy"). As explained by Premrov Bajuk *et al.* (2021), "Cross- reactivity is an immune- mediated phenomenon in which IgE antibodies recognize and bind similar allergenic molecules and trigger the immune response." In general, the sources mainly associated with allergy are briefly summarized in Table 2. Insects contain a variety of potential allergens for humans. Among these, tropomyosin, alpha-amylase, arginine kinase, and hexamerin have been identified as responsible for primary sensitization (Barre *et al.,* 2019). Additionally, cross- reactivity between allergens from shrimp,

prawns, cockroaches, and house dust mites has been reported. However, a 2021 review by Bosch and Swanson (Bosch & Swanson, 2021) noted that no studies were available on food allergies caused by insect-based pet food. Despite this, the low exposure of dogs and cats to insects as a nutritive source suggests a lower likelihood of sensitization and, consequently, a reduced immune response. This may partially explain why the claim "hypoallergenic" is the most common marketing label for insect-based pet food in Europe (Siddiqui *et al.,* 2023). Emerging evidence supports this hypothesis. For instance, a recent case study demonstrated the effectiveness of BSFL meal in alleviating symptoms of food allergy in a 5-year-old spayed Beagle diagnosed with poultry-induced food allergy (Cesar *et al.,* 2024). The complete substitution of poultry byproduct meal with BSFL meal resulted in the resolution of the dog's clinical manifestations of food allergy within 2 days of administration. These findings are promising; however, caution is warranted due to the limited number of studies and the numerous factors that may influence the outcomes, such as pet species, breeds, pre-existing allergies, insect species, and the distinction between primary and cross-reactions.

1.6 Cell lines for cultured meat

The concept of cultured meat (CM) emerged as a response to the pressing problems associated with conventional meat production. Protein production is a critical issue, affecting food security, sustainability, energy costs, and economic resilience. By 2050, global demand for conventional proteins is projected to rise by 57% for meat and 48% for dairy, driven by economic growth, particularly in Asia. However, climate change is already impacting food production, highlighting the need to explore alternative protein sources (FAO, 2025). Several alternative protein sources have been proposed as part of a multifaceted solution

to the global protein challenge. These include plant-based proteins, derived from legumes and grains; microbial proteins, produced via fermentation processes; edible insects, which offer high nutritional value with a minimal environmental footprint; proteins derived from microorganisms like algae and yeast; mycoproteins, cultivated from fungi; and CM. These protein sources offer sustainable solutions to mitigate the environmental impact of traditional agriculture while addressing the global protein gap (van Huis *et al.,* 2021; Melzener *et al.,* 2021). By 2035, alternative proteins are expected to account for 11% of the global protein market, offering a more sustainable solution to growing global protein demands (Lähteenmäki-Uutela *et al.,* 2021). The global food system poses the most substantial threat to the planet's biodiversity, as it bears sole responsibility for almost 80% of species extinctions and habitat losses worldwide (Benton *et al.,* 2021). These harmful effects manifest in both the short and long term, including the substantial greenhouse gas emissions stemming from the food system, which significantly contribute to climate change. Agriculture stands out as one of the primary industries driving climate change, accounting for 24% of global greenhouse gas emissions. Also, intensive livestock production, within this industry, emerges as a major greenhouse gas emitter, responsible for approximately 14.5% of all emissions, according to estimates from the FAO (2025) and WWF Italia (2025). In addition to issues related to resource consumption and pollution, it is projected that ruminant cattle will account for up to 37% of anthropogenic methane emissions released into the atmosphere. This highlights the widely recognized unsustainability of current industrial meat production practices (Ashizawa *et al.,* 2022). The burgeoning field of CM (also called cultivated meat, clear meat, lab- grown meat, etc.), aimed at producing sustainable meat substitutes without relying on traditional

animal farming methods, represents a significant re- sponse from biotechnology to address the challenges of combating climate change and feeding a growing global population.

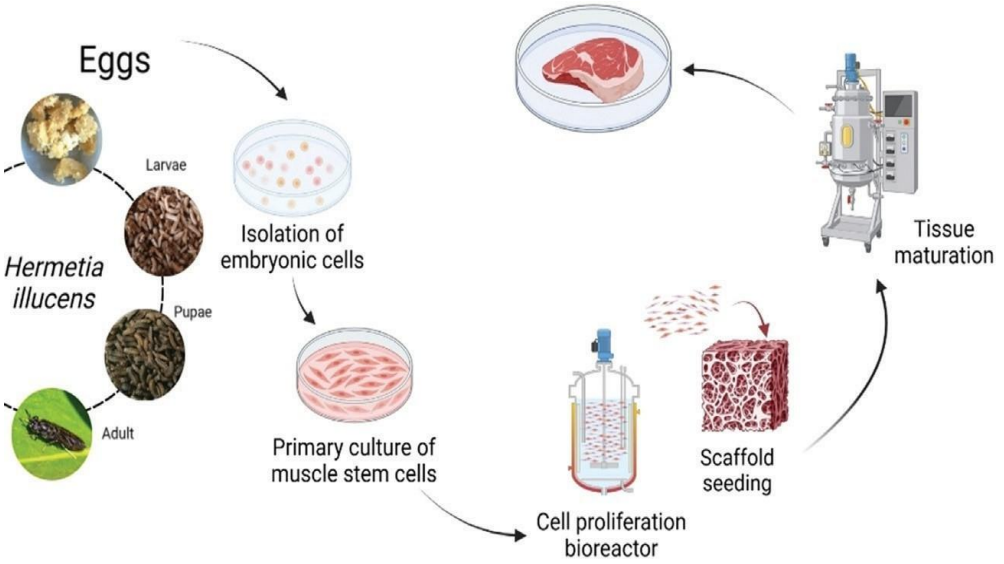


Fig 2. *Overview of the production process for cultivated meat starting from Hermetia illucens cells* (Image Created with BioRender.com).

The process begins with the collection of eggs and progresses through the isolation of embryonic cells, followed by the primary culture of muscle stem cells. These cells are expanded in bioreactors and seeded onto scaffolds to promote tissue maturation. This technology involves applying tissue engineering techniques to cultivate animal muscle and fatty tissues (Ashizawa *et al.,* 2022; Fish *et al.,* 2020). The composition of meat typically comprises approximately 90% muscle fibres, 10% fat and connective tissue, and less than 1% blood (Listrat *et al.,* 2016). To produce CM, several steps must be taken into consideration, including obtaining cells, multiplying them in bioreactors, facilitating their adhesion to specific scaffolds, and promoting their differentiation into muscle and adipose tissues (Figure 2). To make cellular agriculture economically viable and scalable, research should prioritize four key areas: (1) cell sources, (2) culture media, (3) scaffold biomaterials, and (4) bioreactor

design (Fish *et al.,* 2020; Rubio *et al.,* 2019). The environmental impact of large-scale cultivated meat production has been assessed using the life cycle assessment research methodology. Depending on the product being compared, CM consumes 7% to 45% less energy (with only poultry having lower energy consump- tion), emits 78% to 96% fewer greenhouse gases, requires 99% less land, and utilizes 82% to 96% fewer water resources compared to conventionally produced European meat (Tuomisto & de Mattos, 2011). Despite some level of uncertainty, it has been established that the environmental consequences of CM production are considerably less significant than those associated with traditional meat production (Lähteenmäki- Uutela *et al.,* 2021; Sinke *et al.,* 2023; Tuomisto & de Mattos, 2011). However, there are lingering concerns about whether products made from CM can compete economically with traditional meats, a challenge that often arises with any new technology as it advances and scales for commercial use (Ashizawa *et al.,* 2022). The science and technology underpinning CM are still in their early stages of development. Various species' cells, primarily derived from beef, chicken, and pork (Messmer *et al.,* 2023; Zhu *et al.,* 2022), have been utilized in the development of CM. However, exploring cells from less common species could potentially aid in overcoming the current technical impediments that hinder the progress and expansion of cellular agriculture (Rubio *et al.,* 2019). Since insect cells exhibit greater resilience in cell culture when compared to conventional livestock-derived cells, there is a hypothesis that incorporating them into this process could help to reduce certain associated costs, such as advanced bioreactor systems (Franco *et al.,* 2024; Giglio *et al.,* 2024). Along with these benefits, a promising strategy that can improve the sustainability and circularity of the production of CM is the use of chitosan generated from *H. illucens* exoskeletons as a scaffold material. Although

chitosan is already widely used in tissue engineering as a biodegradable and biocompatible scaffold material, obtaining it specifically from *H. illucens* complies with the circular economy and waste valorization principles by incorporating insect farming byproducts into the manufacturing process. Still prospective, this strategy ex- pands on chitosan's proven uses in the food and medicinal industries. Insect cell lines commonly used in bioprocessing (e.g. Sf9, Sf21, *Drosophila* S2) display doubling times on the order of 20–24 h and can reach suspension densities of ≥1–2 × 10^7 cells/mL in serum-free media, with S2 cultures reported up to ~5 × 10^7 cells/mL in bioreactors, while maintaining high viability and productivity (Pamboukian *et al.,* 2008; Moraes *et al.,* 2012; Sigma-Aldrich, 2011); combined with the availability of chemically defined, CO_2-independent serum-free formulations, these properties support more compact and potentially lower-cost high-density culture platforms than many mammalian systems. Additionally, they operate at lower temperatures, reducing energy consumption, and their faster growth cycles decrease the overall need for costly resources like water and labor in large- scale production (Ashizawa *et al.,* 2022). Insect cells possess several characteristics that suggest their suitability for large-scale manufacturing. These include favorable environmental growth conditions, minimal media requirements, and straightforward adaptability to suspension culture systems (Rubio *et al.,* 2019). The culture medium, which typically relies on costly components like fetal bovine serum (FBS) or recombinant protein mixtures, constitutes the primary driver of high production costs. The ability of insect cells to thrive on serum-free media represents a significant advantage for this type of cellular agriculture, also known as entomoculture. This is crucial for CM production as it can reduce both the cost and unpredictability of media while addressing ethical concerns. Biotechnology has already harnessed insect cell

lines such as Sf9 (Spodoptera frugiperda) and S2 (*Drosophila melanogaster*), especially in the synthesis of recombinant proteins (Ashizawa *et al.,* 2022; Chan & Reid, 2016). The most widely used insect cell lines, generated from embryonic or ovarian tissues, include S2, Sf9, Sf21 (Spodoptera frugiperda ovarian), and BTI- TN-5B1-4 (Trichoplusia ni ovarian), commonly referred to as high five (Hi5) cells (Pinillos *et al.,* 2022). Through the development of high-density culture methods, advanced bioreactors, and optimal medium formulations, this field has laid the foundation for utilizing insect cells in food applications. Insect cells are attractive candidates for effective, scalable food production due to many of the same qualities that make them suitable for use in biomanufacturing and soft robotics (Rubio *et al.,* 2019). All the aforementioned factors suggest that utilizing insect cells rather than cells from farmed animals could lead to reduced production costs for lab-grown meat (Ashizawa *et al.,* 2022; Tuomisto & de Mattos, 2011). Harrison, who grew frog embryonic tissues in 1907 and witnessed the differentiation of epidermal, muscular, and nerve cells within droplets of frog lymph, sustained for up to four weeks, is credited with the first description of cell culture (Harrison *et al.,* 1907; Yao and Asayama, 2017). Five years later, Glaser and Chapman published the results of the first study on insect cell cultures, which examined the development of a viral disease in lepidoptera hemocyte cultures (Arunkarthick *et al.,* 2017). The earliest record of an insect cell line, isolated from the silkworm, can be traced back to 1968 in the Chinese Science Bulletin. In this article, the culture medium, known as Grace's Insect Medium, was identified as a key factor contributing to the success of the research. This medium is composed of salts, amino acids, sugars, organic acids, vitamins, antibiotics, and insect plasma obtained from Opodiphthera eucalypti (emperor gum moth) (Grace, 1962). Subsequently, over 800 cell lines have been

established from various insect species, encompassing numerous insect orders, and originating from various tissue sources (Delvendahl *et al.,* 2022). While more than one million insect species inhabit Earth, current data from Expasy-Cellosaurus indicate that approximately 1200 cell lines have been derived from over 170 species (He *et al.,* 2023). In contrast to most vertebrate cells, insect cells exhibit a higher tolerance to variations in the external environment, including temperature and pH fluctuations. They thrive at temperatures to the ambient temperature and show reduced buildup of and sensitivity to toxic substances such as lactate and ammonia. Unlike vertebrate cells, insect cells do not necessitate the addition of recombinant proteins and growth factors during serum-free culture, and they are well-suited for suspension culture, which is advantageous for large-scale production. Additionally, they frequently undergo spontaneous immortalization. As a result, insect progenitor cells can be expanded *in vitro* and induced to differentiate into mature tissues, enabling the creation of meat substitutes (Chan & Reid, 2016; Letcher *et al.,* 2022; Lynn, 2002; Rubio *et al.,* 2020a). The development and physiology of insect muscle cells have been extensively documented, and since the 1970s, various myogenic cell populations from different species and tissue types (e.g., embryonic dorsal, ovarian, leg, vessel) have been successfully cultured *in vitro* (Buckingham & Mayeuf, 2012; Rubio *et al.,* 2020a). However, it is worth noting that insect cells have been cultured for various purposes over the years, but never specifically for food production. Muscle cells in insects can be readily isolated from embryos or stages of meta- morphosis, separated from a heterogeneous cell population, and induced to undergo differentiation through the administration of insect-specific hormones like ecdysone (also known as molting hormone) and methoprene (a juvenile hormone analog). From a food-safety and regulatory perspective, 20-

hydroxyecdysone (the main active ecdysteroid) occurs naturally in edible plants and is already present in some dietary supplements (Dinan *et al.,* 2021), whereas methoprene is currently authorised only as an insect growth regulator with established maximum residue limits (FAO/WHO, 2019). Consequently, the use of these hormones in food-grade cultivated meat processes would require careful assessment and, at present, should be regarded primarily as a research-stage tool rather than as a ready-to-implement technological aid in commercial production (Kraiem *et al.,* 2021). Insect species belonging to the orders Diptera, such as *D. melanogaster* or *H. illucens*, and Lepidoptera, for example, *Antheraea pernyi, Ctenoplusia agnata*, and *Manduca sexta,* can be used as cell sources for studying muscle cells (Rubio *et al.,* 2020a). Research on the dorsal longitudinal muscles, also known as flight muscles, of *D. melanogaster* has provided significant insights into the growth and regeneration of insect muscles; it should also be specified that *D. melanogaster* and vertebrates share many fundamental biological characteristics. *In vitro* categorization of insect muscle cells relies on their spindle-like morphology, differentiation triggered by exposure to ecdysone, and their ability to exhibit spontaneous contractions. Numerous studies have demonstrated that insect muscle cells can function and remain viable *in vitro* for several months without the need for media replenishment (Hegstrom & Truman, 1996; Kurtti & Brooks, 1970; Rubio *et al.,* 2020a; Taylor, 2007). To create myogenic primary cultures, embryos (or tissues) are sterilized with sodium hypochlorite and washed with phosphate-buffered saline, before being homogenized and added to a growth medium (such as *Grace's, Leibovitz's L-15*, or *Schneider's*, or *Shields and Sang M3*) along with FBS supplemented at a rate of 10% to 20%. FBS is typically added at this stage to provide essential growth factors and nutrients necessary for initial cell survival and proliferation.

However, in the process of establishing a continuous insect cell line, FBS is often gradually reduced and ultimately removed as the cells become adapted to grow in serum-free conditions. This step is important to optimize large-scale production and reduce the variability introduced by serum (Rubio *et al.,* 2019, 2020a). Insect muscle cells can survive and contract *in vitro* for longer periods of time without the culture media being changed, thanks to the storage and release of nutrients by fat cells. Important proteins, such as hemolymph proteins, vitellogenin, juvenile hormone-binding protein, and precursors to yolk proteins, are produced by fat body cells (Arrese & Soulages, 2010; Nowock *et al.,* 1975; Rubio *et al.,* 2019; Wyatt, 1988). Indeed, the second vital component contributing to texture and nutrition is fat, specifically referring to adipose tissue, which serves metabolic purposes in insects. In addition to fatty acids, the fat body of insects also contains protein and carbohydrates (Hoshizaki & Chapman, 2012). The lipids that build up in adipose tissue and regulate key organoleptic characteristics of meat result from intracellular anabolism as well as dietary fat intake by the animal. Despite different diets, for example, the fatty acid profile of BSFL consists mainly of lauric acid, saturated fatty acids, followed by palmitic acid, oleic acid, and linoleic acid (Ewald *et al.,* 2020; Franco *et al.,* 2021b). It will be critical to establish cocultures of insect fat tissue and muscle tissue to develop nutrient-rich and appetizing food products. Unlike muscle tissue, adipose tissue does not necessitate a complex structural framework for differentiation. Nevertheless, growing fat cells in a 3D environment could enable forthcoming products to replicate the sensory characteristics of natural fat and potentially improve the traits of cultured fat (Letcher *et al.,* 2022). It is possible for a variety of cell types to differentiate into adipocytes *in vitro*, but it is unclear which will be the best source for creating cell-cultured fat that can be

consumed by humans. Pluripotent stem cells, which are often extracted from embryonic stem cells, mesenchymal stem cells, which are frequently recovered from bone marrow and fat, and dedifferentiated fat cells, which are produced by dedifferentiating mature adipocytes, are all potential substitutes. High proliferative capability, a straightforward and highly effective differentiation process, minimal medium requirements, homogeneity, stability, and resilience to changes in environmental circumstances are traits of the perfect cell source (Rubio *et al.,* 2020a). Insect fat body cells can also synthesize and secrete antimicrobial peptides, which may reduce or eliminate the need for supplementation of external antibiotics during culture (Manniello *et al.,* 2021; Sahoo *et al.,* 2021). Both types of cultures, myogenic and adipocytic, are maintained in a non-humidified incubator without CO_2 exchange, due to the unique metabolic characteristics of insect cells. Unlike mammalian cells, insect cells do not rely on CO_2 to buffer the culture medium, as they can maintain a stable pH without it (Letcher *et al.,* 2022). Finally, it is imperative to ensure the acceptance of cultured insect fat and muscle as an unconventional food source among consumers. While cellular agriculture is still an emerging field, entomophagy is already prevalent in many non-Western regions. Additionally, Western regions have started to explore insect-derived products, such as cricket meal. We anticipate that consumer acceptance can be enhanced through three primary strategies: (1) developing products that closely mimic the flavor, aroma, and texture of familiar meat products, (2) targeting the introduction of insect cell culture products primarily within populations that already include insects in their diets, and (3) potentially creating nutritionally advantageous products, such as those with a higher unsaturated-to-saturated fat ratio compared to currently consumed meat products (Letcher *et al.,* 2022; Tan *et al.,* 2016). While

advancements in cultured meat production highlight the potential for environmental benefits, the regulatory framework for alternative protein sources is evolving to ensure sustainability and consumer safety. These developments not only shape the future of food innovation but also underscore the importance of scientific exploration, including the use of cells from less common species, to overcome current technical impediments and expand the possibilities of cellular agriculture. The regulatory environment is changing to guarantee sustainability and safety as *H. illucens*' use in food and feed applications continues to grow. Future modifications to EU's innovative food regulatory system are intended to address consumer safety concerns and expedite the assessment of products derived from insects. According to these changes, all goods produced from insects that are introduced to the market must undergo thorough safety evaluations, which must include allergenicity and contaminant analyses. These modifications will have two effects: they will increase the standard for scientific innovation, promoting the creation of strong safety evidence and innovative manufacturing techniques, and they will boost consumer confidence in these new products. Adherence to these regulations poses a difficulty as well as an opportunity for *H. illucens*. The industry can position goods produced from *H. illucens* as dependable, sustainable substitutes for traditional protein sources by adhering to these more stringent regulations, which will increase their acceptance by both consumers and regulatory agencies.

1.7 Conclusion

It is well known that BSFL can be used to feed a variety of vertebrates (Tomberlin *et al.,* 2015). This has important implications for sustainable and lower-input agriculture in developing countries (Diener *et al.,* 2011; Nyakeri *et al.,* 2017) and has no effect on how palatable the meats fed to BSFL are to

humans. Although the potential benefits are greatest in the developing countries, BSFL and other insect feeds are expected to play larger roles in advanced economies, like the USA, over time due to food conglomerates' pledges to reduce waste in order to gain approval from regulators and consumers who are becoming more environmentally conscious, as well as the fluctuating prices of fish meal and other feed that are forcing producers to look for alternatives (Klonick, 2017). *H. illucens* holds immense potential as a sustainable and efficient resource for food, feed, pet food and even cellular agriculture. Its use in animal feed—especially in aquaculture, poultry, and swine—has demonstrated benefits in growth, immunity, and nutrient digestibility. In the pet food sector, BSFL-based products are emerging as functional, digestible ingredients, though further studies are needed on palatability and allergenicity. Its high nutritional value, low environmental footprint, and ability to recycle organic waste into valuable biomass position as a key player in addressing global food security and sustainability challenges. Despite these advantages, several barriers hinder its widespread adoption. Cultural resistance, particularly in Western societies, safety concerns regarding contamination and biological risks, and regulatory challenges for its classification as a novel food need to be systematically addressed. Innovative processing methods, such as transforming larvae into flour or isolates, can significantly improve consumer acceptance by reducing the visibility of insects. Creative marketing strategies, including renaming and rebranding insect-derived products, have demonstrated potential in shifting perceptions and increasing willingness to try these unconventional foods. Additionally, advancements in regulatory frameworks that prioritize safety while encouraging innovation are essential to facilitate the integration of BSF derivatives into mainstream markets. In the realm of cellular agriculture, insect

cells, thanks to their resilience, adaptability to serum-free media, and lower energy requirements, offer promising opportunities for scaling CM production. Their application could reduce both costs and environmental impacts while expanding the availability of alternative protein sources. Leveraging these attributes, along with multidisciplinary research into consumer behavior, processing technology, and regulatory alignment, can drive broader acceptance and adoption of *H. illucens*-based solutions. Ultimately, the adoption of BSF products, whether as food, feed, or cultured cell components, will require a collaborative effort across science, policy, and public engagement. By addressing these challenges, *H. illucens* can serve as a cornerstone of a more resilient, sustainable, and inclusive global food system.

CHAPTER 3

1.1 Muscle-oriented *Hermetia illucens* primary cell cultures toward cultivated meat applications

This chapter presents embryo-informed primary cultures of H. illucens and demonstrates quantitative myogenic enrichment consistent with a practical starting point for insect-based cellular agriculture. (Manuscript under consideration for publication; thesis reproduces the submitted version).

The concept of cultivated meat (CM) emerged as a response to the pressing problems associated with conventional meat production. Traditional meat production is a major contributor to environmental pollution, including greenhouse gas emissions, deforestation, water scarcity, and biodiversity loss, while also raising concerns related to animal welfare and disease transmission (zoonosis) (Treich, 2021; FAO,2006; Mancini & Antonioli, 2022; Godfray *et al.,* 2018). In contrast, CM is produced in controlled environments, minimizing the risk of zoonotic disease, antibiotic resistance, and contamination, thereby contributing to a safer and more reliable food supply (Bhat *et al.,* 2019). CM offers a promising solution by generating muscle tissue *in vitro* from animal-derived stem cells, which is then developed into products similar in structure and taste to conventional meat, thereby reducing the environmental footprint, addressing ethical concerns, and improving food safety (Ben-Arye & Levenberg, 2019). Despite the remarkable advances in mammalian cell-based CM, including the successful culture of various animal cells from cattle, pigs, and poultry, and eliminating ethical concerns while ensuring that initial cell lines are sourced ethically and sustainably (Hopkins & Dacey, 2008), the development and commercialization of CM still faces technical, economic, regulatory, and social challenges. The scalability of technology is hindered by stringent growth requirements, expensive media, labor intensive processes and complex bioreactor designs (Post, 2012; Stephens *et al.,* 2018; Danilov *et al.,*2022; Kaplan *et al.,* 2021), and consumer trust and acceptance require thorough assessments of

allergenicity and long-term safety to meet regulatory standards (Bryant & Barnett, 2018; Ong *et al.,* 2023). Mammalian cell cultures require stringent growth conditions, including a controlled temperature of 37°C, high levels of oxygen, CO_2, and complex media (Post, 2012; Specht *et al.,* 2018). The doubling time of mammalian cells typically ranges between 16 and 48 hours, and they are sensitive to environmental fluctuations. Additionally, mammalian cells often require sophisticated and high-cost bioreactors, which are designed to maintain uniform cultivation conditions and efficient nutrient delivery across larger volumes, as well as to manage waste accumulation effectively (Danilov *et al.,* 2022). Considering the challenges summarized above with mammalian cells, researchers have increasingly explored alternative cell sources. Insect cells offer distinct advantages for CM (Giglio *et al.,* 2024), as they generally require lower growth temperatures (20-32°C), simpler media formulations, have no need for CO_2 supplement, and demonstrate shorter doubling times compared to mammalian cells (Smagghe *et al.,* 2009). The composition of culture media is a critical factor that influences both the cost and environmental impact of CM production. For mammalian cells, culture media typically consists of a basal medium, such as DMEM (Dulbecco's Modified Eagle Medium) or RPMI-1640, supplemented with fetal bovine serum (FBS), amino acids, vitamins, inorganic salts, glucose, and growth factors. The use of FBS not only raises ethical concerns, but also significantly increases the costs and environmental footprint of mammalian cell cultures due to the high-water consumption and greenhouse gas emissions associated with its production (Specht *et al.,* 2018; Rodriguez *et al.,*2021; Tuomisto & Teixeira de Mattos, 2011). In contrast, insect cell culture media such as Grace's Insect Medium (Grace, 1962), IPL-41 (Goodwin, 1975), Schneider's Drosophila Medium (Schneider, 1964), and TNM-FH (Trichoplusia

ni Medium - Formulation Hink) (Hink,1970), are generally more affordable and sustainable (Smagghe *et al.,* 2009; Oonincx & de Boer, 2012). Also, many insect cells can be successfully propagated in suspension without the need for complex anchorage systems or microcarriers and can be readily adapted to serum-free media, which simplifies the composition and further reduces costs (Baust *et al.,* 2017; Brunner *et al.,* 2010). In contrast to mammalian cells, insect cells generally do not require exogenous growth factors or hormones (e.g., insulin, transferrin) to proliferate in well-formulated serum-free media (Rubio *et al.,* 2019b). Commercial serum-free insect media are designed as complete formulations; for example, Sf-900™ II and Express Five™ already contain all essential nutrients and surfactants, eliminating the need for additional supplementation. Furthermore, several studies have shown that insect cells can be efficiently maintained in low-cost serum-free formulations based on plant hydrolysates and lipid emulsions (e.g., soy and yeast hydrolysates combined with sterol-enriched lipid emulsions) (Kwon *et al.,* 2003). Thus, the removal of serum does not necessarily entail the use of recombinant hormonal supplements, provided that the medium supplies all key nutrients in a bioavailable form. Compared with mammalian cells, the nutritional requirements of insect cells in culture can be largely met by these basal components, namely an appropriate carbon source (sugars), a complete set of amino acids, vitamins (particularly B-complex), essential lipids/sterols, and inorganic salts (Invitrogen Corp., 2002; B oegel, 2019).The use of insect cells for CM production represents a novel and innovative approach within the broader field of cellular agriculture, aligning well with global sustainability efforts, due to their adaptable growth conditions and eco-friendly profile (Akiyama *et al.,* 2009; Akiyama *et al.,* 2013; Baryshyan *et al.,* 2014). These features enhance their potential to improve the resilience and

scalability of the food supply chain. Unlike mammalian cells, insect cells are smaller (≈ 10–20 μm in diameter versus 20–50 μm for mammalian myoblasts), less multinucleated, which, together with their unique physiological adaptations, supports achieving higher cell densities during cultivation, improved nutrient and oxygen diffusion, and reduced metabolic gradients (Edwards, 1978; Giglio *et al.,*2024; Alberts *et al.,*2022). The growing interest in entomoculture, the use of insect cells for human consumption, reflects these advantages, supported by established infrastructure for large-scale cultivation and the cells adaptability to diverse conditions (Ashizawa *et al.,* 2022). Insect cell lines grown in CM are predicted to have substantially lower media, oxygen consumption, and utility costs than mammalian cells, which translates into reduced production costs per kilogram, according to preliminary technoeconomic study (Ashizawa *et al.,* 2022). Additionally, the ability of insect cells to produce antimicrobial peptides suggests that insect-derived CM could enhance both nutritional value and food safety, and further reduce the need for exogenous additions of antibiotics during CM production (Rubio *et al.,* 2019). Despite the above potential for insect cells for CM, studies exploring insect cells in a food production context remain relatively few. Recent studies on *Drosophila melanogaster* and *Manduca sexta* have demonstrated the feasibility of insect myogenic cell cultures, revealing spontaneous contractility, high protein, iron, and zinc content, and robust growth in serum-free conditions (Schnorrer & Dickson, 2004; Gunage *et al.,* 2017; Rubio *et al.,* 2020a). A non-adherent *M. sexta* cell line has shown promising scalability in animal-free suspension cultures, achieving high cell densities and protein content while maintaining steady growth even in nutrient- limited media, underscoring its potential for cost-effective CM production (Letcher *et al.,* 2024). Furthermore, both *D. melanogaster* and *M. sexta* muscle cells exhibit

spontaneous contraction and sustained function without continuous nutrient supplementation, highlighting their suitability for addressing challenges in CM production, such as the need for cost-effective media and scalable growth systems (Rubio *et al.,* 2020a; Letcher *et al.,* 2022). However, research on non-model species with high biotechnological relevance, such as *Hermetia illucens* (black soldier fly), remains limited. Given the exceptional capacity of this insect species to convert organic waste into high-value biomass and its established role in the circular economy (Diener *et al.,* 2009; Spranghers *et al.,* 2017; Scala *et al.,* 2020; Scieuzo *et al.,* 2023; Franco *et al,* 2022; Franco *et al.,* 2025), *H. illucens* represents a promising platform for sustainable CM production. BSF larvae exhibit a high protein content and a complete amino acid profile. Under standard rearing conditions, harvested larvae contain approximately 41–44% protein and 15–49% lipid on a dry matter basis (Scala *et al.,* 2020). The protein fraction is rich in essential amino acids; leucine and lysine are particularly abundant (e.g. 27.8–78.3 g and 23.0–68.2 g per kg dry matter, respectively), with values exceeding those of plant protein sources such as soybean meal. Other essential amino acids (valine, isoleucine, threonine, phenylalanine) are present at relatively high levels, comparable to those found in fish meal or soybean meal. By contrast, methionine and tryptophan tend to be relatively limiting, similarly to what is observed for soybean meal (Lu *et al.,* 2022). Overall, BSF proteins provide all indispensable amino acids, and their qualitative profile can be partially modulated by altering the composition of the larval diet, as demonstrated by several studies highlighting the substrate-dependent plasticity of both amino acid and fatty acid profiles (Scieuzo *et al.,* 2023; Ianniciello *et al.,* 2024). The lipid fraction of *BSF* larvae is dominated by saturated fatty acids (SFA). In particular, lauric acid (C12:0) and palmitic acid (C16:0) represent the

main components of larval fat (Ewald *et al.,* 2019). Comparative studies have shown that the majority of total larval fat consists of SFA (e.g. 362–783 g/kg dry matter), whereas monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) account for much smaller proportions compared to other edible insect species (Franco *et al.,* 2024a; Ianniciello *et al.,* 2024). Among PUFA, linoleic acid (n-6) and α-linolenic acid (n-3) are present only in modest amounts (on the order of a few grams per kg dry matter) (Lu *et al.,* 2022). Consequently, the n-6/n-3 ratio in the larvae is generally high and the overall lipid profile is relatively unfavourable from a nutritional standpoint when compared with other insect species or marine lipid sources. However, the fatty acid profile of *H. illucens* larvae can be partially modulated by altering the composition of the larval diet. In particular, supplementation with vegetable oils rich in unsaturated fatty acids (e.g. linseed, hemp or rapeseed oil) markedly increases the proportions of UFA and ω-3 fatty acids in the larvae. Experimental studies have shown that inclusion of ω-3-rich oils in the rearing substrate can raise the larval ω-3 PUFA content up to 15–37%, while concomitantly reducing the proportion of SFA (Georgescu *et al.,* 2022). This demonstrates that the lipid profile of BSF larvae (especially linoleic and α-linolenic acid levels) can be partially optimised through dietary manipulation. BSF larvae have been proposed as a food ingredient for both humans and animals. In human nutrition, although direct consumption is not yet widespread, BSF protein meal (produced from dried and ground larvae) and larval oil are envisaged for use in innovative food products (e.g. high-protein snacks, dietary supplements, fat replacers) (Ewald *et al.,* 2019). These products provide high-quality proteins and predominantly saturated fats, and their formulation should take into account this distinctive nutritional profile. To date, the main use of *H. illucens* remains in the animal production sector: dried and

processed larvae as feed ingredients are increasingly being applied in aquaculture, poultry production and livestock farming in general (Scala *et al.,* 2020; Franco *et al.,* 2024a; Ianniciello *et al.,* 2024). To the best of our knowledge, this is first study that outlines protocols for establishing and characterizing primary muscle-enriched cell cultures from *H. illucens*. BSF exhibits a holometabolous life cycle, progressing through embryonic, larval, pupal, and adult stages. Its larvae are a sustainable source of proteins and lipids for animal feed, while pupal exuviae and adults, rich in chitin, which can be converted into chitosan with diverse applications in biomedicine, agriculture, and food preservation (Makkar *et al.,* 2014; Franco *et al.,* 2021; Triunfo *et al.,* 2022; Tafi *et al.,* 2023; Triunfo *et al.,* 2023; Franco *et al.,* 2024; Triunfo *et al.,* 2024; Guarnieri *et al.,* 2024; Vitti *et al.,* 2024; Tedesco *et al.,* 2024; Giani *et al.,* 2025; Ianniciello *et al.,* 2025; Fusco *et al.,* 2025). Here, we describe the isolation, characterization, and myogenic enrichment of primary embryonic cell cultures derived from *H. illucens.* By combining morphological, molecular, and functional analyses, including EGTA-mediated selection and a time-dependent adhesion strategy, we provide a methodology for obtaining muscle-oriented insect cell populations from *H. illucens*.

2. MATERIALS AND METHODS

2.1 H. illucens embryo collection and developmental staging

H. illucens eggs, obtained from Xflies s.r.l. (Potenza, Italy), were used for cell isolation. Eggs were collected within a 3–4 hour oviposition window using dedicated oviposition traps and immediately defined as "freshly laid". These fresh eggs were incubated in sterile 35-mm Petri dishes at 27°C under controlled humidity conditions for additional periods of 15, 19, 22, or 24 hours, resulting in embryos of defined post-collection age. These timepoints aimed to capture

critical transitions in embryonic development associated with myogenic lineage specification. At each stage, embryos were subjected to mechanical dissociation, and the resulting cells were analyzed by both phase-contrast and fluorescence microscopy following immunostaining for muscle-specific markers, enabling the assessment of cell morphology, and myogenic commitment (details follow for each of these steps).

2.2 Embryonic cell isolation

The isolation of cells from embryonated *H. illucens* eggs was conducted following a protocol adapted for *D. melanogaster* from Bernstein *et al.* (1978), with minor modifications to suit the experimental needs of this study. All procedures were carried out within a biosafety cabinet to ensure sterile conditions. All reagents were purchased from Merck (Darmstadt, Germania) unless otherwise indicated, and all solutions were filtered through 0.22 μm sterile filters using an autoclaved glass syringe (Starlab Srl, Milan, Italy). After incubation, eggs were first rinsed with sterile 1X phosphate-buffered saline (PBS) containing 2% Anti/Anti for one minute, followed by a 10-minute bleach wash (50% sodium hypochlorite solution) to ensure surface sterilization. The bleach treatment facilitated the removal of the chorion, the outer protective layer of the egg, a process known as dechorionation (Simcox *et al.,* 2008; Urbán-Duarte *et al.,*2022). Following dechorionation, *H. illucens* eggs were rinsed with sterile 1X PBS containing 2% Anti/Anti to remove residual bleach, and transferred into complete culture medium to facilitate cell adaptation. For homogenization, the eggs were placed in a sterile Dounce homogenizer and mechanically disrupted using 10–15 strokes with the loose pestle. The resulting cell suspension was filtered, and centrifugated to remove excessive yolk

material. Following the initial centrifugation, the pellet, containing large fragments of vitelline membrane, was discarded, and the supernatant was subjected to a second centrifugation at an increased speed, which facilitated the separation of the cells (pellet) from the yolk particles (supernatant). The resulting cell pellet was carefully resuspended in fresh culture medium for seeding.

2.3 Cell culture conditions

Culture medium was prepared with Shields and Sang's M3 insect medium for Diptera cell cultures (Lynn,1996), supplemented with 20% FBS, 2.5 g/L yeast extract, 1g/L bactopeptone, 1% Anti/Anti, 0,2% primocin. Prior to use, the medium was sterile filtered, and the pH adjusted to 6.5. Serial dilutions were then performed to determine the most favorable cell seeding density. All plates were sealed with Parafilm and placed in a humidified incubator at 27°C. Media changes were performed approximately every 7 days, using a 50:50 mix of fresh medium and conditioned medium. Subcultures were initiated at ~80% confluence. If this threshold was reached before the weekly medium change (~7 days), we prioritized passaging over medium replacement; otherwise, only the medium was renewed. Cells were passaged by incubating 0.5% Trypsin-EDTA for 10 minutes at 37°C, followed by centrifugation at 300g for 5 minutes. Cells were then re-seeded at a density of 1,5/2 x 10^5 cells/cm^2. Daily observations of the primary cultures were carried out using an inverted microscope (Nikon Eclipse TE300/TE200, Nikon Corporation, Minato, Tokyo, Japan) to monitor cell morphology, growth characteristics, and the evolving morphological patterns in the cultures. An alternative subculturing method using 0.50% Accutase (Euroclone S.p.A., Cat. No. ECB3056D, Milan, Italy), a non-animal-

derived enzymatic solution, was also evaluated for its ability to preserve cell viability and morphology (Kurtti & Brooks, 1976; Bradl & Jäck, 2001; Genersch *et al.,* 2013; Jayesh *et al.,* 2012) in primary *H. illucens* cultures (*Appendix Figure A1*). However, all subsequent experiments were performed using 0.50% trypsin, which was established as the standard detachment method.

2.4 EGTA- treatment for muscle cell enrichment

Ethylene glycol- *bis*(β-aminoethyl)-N, N, N′, N′-tetraacetic acid (EGTA) was used to chelate free calcium ions, which prevents the attachment of yolk material and contaminating cell types. This selective chelation process allows for the enrichment of myogenic cells by inhibiting the adhesion of unwanted cell populations, thereby promoting a more purified culture of muscle precursor cells (Bernstein *et al.,*1978). To initiate cell cultures, embryos obtained from freshly laid eggs incubated for 19 h were processed following a protocol adapted from previously described methods for the isolation and culture of *H. illucens* myoblasts, with minor modifications. The resulting cell suspension was plated in Shields and Sang's M3 insect medium supplemented with 20% FBS, 2.5 g/L yeast extract, 1g/L bactopeptone, 1% Anti/Anti, 0,2% primocin. and various concentrations of EGTA (0, 1, 5 mM). The cells were allowed to adhere for 2 hours at 27°C. Following this incubation period, non-adherent cells were removed by gently shaking the plates on a rotational shaker at 50 rpm for 10 minutes. The medium containing the nonadherent cells was aspirated and the adherent cells were replenished with fresh EGTA-free medium. Cultures were maintained at 27°C in a humidified incubator, sealed with parafilm to prevent evaporation. Finally, the differentiated cells from the adherent cell culture system were fixed, stained for myosin heavy chain (MHC), and the percentage

of muscle cells was quantified (see section 2.6.2 *Immunofluorescence staining*).

2.5 Time-dependent adherence

To enhance the purity of the muscle cell culture and enrich myoblast populations, a serial pre-plating (PP) protocol based on differential adhesion kinetics was implemented. To enrich for muscle cells, we developed a time-dependent adhesion-based selection method. Cells were isolated from *H. illucens* embryos, 19 h after egg collection - identified as the optimal stage for cell viability and myogenic potential- were seeded and allowed to adhere for 30 min, 90 min, or 150 min. Not-adherent cells were removed at each time point and transferred to fresh wells, generating three sequential populations: PP1 (0–30 min), PP2 (30–90 min), and PP3 (90-150 min). Plates were briefly shaken (100 rpm, 10 min) after each interval to remove weakly attached cells. All populations were fixed and stained for MHC to quantify the myogenic cell content (see section *2.6.2 Immunofluorescence staining*).

2.6 Characterization of isolated cells

2.6.1 Oil Red O staining

Cell staining was performed following the protocol described by Akiduki and Imanishi (2007), with minor modifications. After fixation with 4% (w/v) paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes at room temperature (RT), the cells were washed with distilled water (dH_2O). A pre- incubation step in 60% (v/v) isopropanol (5 min, RT) was performed prior to staining. Cells were then incubated with the working solution of Oil Red O for 20 min at RT. The staining solution was completely removed, and the cells were washed 2-5 times with dH_2O to eliminate any excess stain. After washing, the

cells were covered with 1X PBS for observation. The working solution of Oil Red O was prepared by mixing 6 volumes of the stock solution with 4 volumes of dH_2O. The stock solution consisted of 3.5 mg/mL Oil Red O powder (Sigma-Aldrich, O1391) dissolved in isopropanol. This solution was filtered using a 0.45 µm non- sterile paper filter before use.

2.6.2 Immunofluorescence staining

To accurately distinguish and select desired cell types, immunocytochemistry (ICC) was utilized (Cruz & Bello, 2012; Inoue *et al.,* 1991; Waldherr, 2018; Lynn, 1999). Samples for ICC were prepared following the protocol outlined by Das *et al.,*2007 with minor modifications. The extent of myogenic differentiation was quantified using the fusion index, calculated as the ratio between the number of nuclei within myotubes and the total number of nuclei observed per microscope field. Cells were fixed with 4% (w/v) paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) at RT for 10 minutes, permeabilized for 10 minutes using 0.1% Triton X-100 (Sigma-Aldrich) in 1X PBS and then blocked for 30 min with 1% bovine serum albumin (BSA) in 1X PBS. Subsequently, samples were incubated at RT for 1 hour with a primary monoclonal antibody anti-myosin [MAC 147] (ab51098, Abcam, Cambridge, UK; 1:500). For secondary detection, samples were incubated with AlexaFluor488 goat anti-rat IgG H&L (1:500; Invitrogen, Thermo Fisher Scientific), phalloidin iFluor 488 reagent (ab176753, Abcam; 1:500), and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Thermo Fisher Scientific). Fluorescence images were acquired using a FLoid™ Cell Imaging Station fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence signal intensity was quantified using Fiji software (ImageJ 1.54f, NIH, USA). Negative controls

included omission of the primary antibody (to assess non-specific binding) and omission of both the secondary antibody and phalloidin (to verify absence of autofluorescence).

2.6.3 Live/Dead Cell Viability Assay

Cell viability was assessed using the LIVE/DEAD™ Viability/Cytotoxicity Kit (L3224, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol, with minor modifications. Cells were maintained in culture for a period of two months, and still exhibited significant contractile activity prior to staining. The Calcein-AM reagent (live-cell indicator) was combined with Ethidium homodimer-1 (dead-cell marker) to prepare a 2× working solution, which was added to the cell suspension at a 1:1 ratio. Samples were incubated for 15 min at 20–25°C in the dark to ensure optimal dye penetration and exclusion. Following incubation, fluorescence imaging was performed using a FLoid™ Cell Imaging Station (Thermo Fisher Scientific), enabling the visualization of live (green) and dead (red) cells.

2.6.4 Molecular characterization by gene expression analysis

2.6.4.1 RNA extraction

Total RNA was extracted from *H. illucens* cells cultured in a T25 flask (Passage 0) using the Direct-zol™ RNA Kit (Zymo Research), following the manufacturer's instructions with minor modifications to optimize RNA yield. Specifically, the DNase/RNase-free water used for elution was pre-warmed to 60°C and, after addition to the column, was allowed to incubate for 1–2 min before centrifugation. All materials and solutions were sterilized by autoclaving prior to use. For RNA extraction from fresh eggs, approximately 100 mg of eggs

were placed in 2 mL microcentrifuge tubes, and 1 mL of TRI Reagent (Sigma-Aldrich) was added. Samples were homogenized using a sterile pestle until a uniform suspension was obtained. Total RNA was isolated following the procedure described by Gottshall *et al.* (2008) with minor adaptations.

2.6.4.2 DNase treatment

Residual genomic DNA was removed using Deoxyribonuclease I, Amplification Grade (Invitrogen, Thermo Fisher, Waltham, Massachusetts, Stati Uniti), with modifications to the standard protocol. Following the addition of EDTA, as indicated by the manufacturer, a phenol:chloroform purification step was performed, followed by isopropanol precipitation and ethanol washes to improve RNA purity. RNA integrity was checked on a 0.8% agarose gel stained with ethidium bromide, and RNA concentration and purity were measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). RNA purity was checked by measuring the 260/280 nm absorbance ratio, which was within the range (1.8–1.91) generally considered suitable for downstream applications.

2.6.4.3 cDNA synthesis

First-strand cDNA was synthesized from total RNA using the iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad), following the manufacturer's instructions. All cDNA preparations were adjusted to a final concentration of 300 ng/µL to ensure uniform input for subsequent RT-qPCR analysis. The cDNA was stored at –20°C until use.

2.6.4.4 Quantitative Real-Time PCR (qPCR) and Data analysis

Quantitative real-time PCR (qPCR) was performed to analyze the expression of key developmental and muscle-related genes (MHC, TWIST, and Wingless) in

cultured embryonic cells and whole eggs. These experiments were performed to validate the transcriptional profile of the cell population isolated at 19 h, which was identified as the most promising time point for mesodermal/myogenic commitment based on preliminary morphological and experimental observations. Each reaction mixture contained 1 µL of cDNA (300 ng/µL) in a total volume of 20 µL, using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). Amplification was carried out on an Applied Biosystems 7500 Fast Real-Time PCR System. All samples were run in technical triplicates and included three biological replicates per condition. RPLP8 gene was used as a reference gene for normalization (Lee *et al.,* 2025). Relative expression was calculated using the ΔCt method, and fold changes between samples were determined using the $2^{-\Delta\Delta Ct}$ method. No-template controls (NTC) were included in all runs. The amplification primers were designed using Primer3 4.1.0 software (https://primer3.ut.ee/) (Table 1).

Tab 1. *Primer sequences used for RT-qPCR analysis of H. illucens genes.* Forward and reverse primer sequences (5'→3') designed using Primer3 (https://primer3.ut.ee/) are listed for each target gene. Amplicon sizes (bp) refer to the expected product length.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Amplicon Size (bp)
MHC	AGTAGAACACCAGGAGCGAA	TGCCCTGTGATACGTTCTGTA	170
TWIST	GTGGAAGGACAA CGT AA CGG	CACTGGGCAATGTTGGAATGA	190
WINGLES S	ATAACGAGGCAGGAAGAGCG	ACGTGAAGCTCCATCAATCG	161

2.6.4.4.1 Statistical analysis

Data are presented as mean ± standard deviation (SD) from at least three

independent biological replicates. Statistical analyses of the fusion index across conditions were performed using one-way ANOVA with GraphPad Prism 8.4.2 (GraphPad Software, San Diego, CA, USA). For the qPCR experiments, statistical significance was assessed using unpaired two-tailed t-tests with Welch's correction, performed on $2^{-\Delta Ct}$ values. Variance homogeneity was evaluated using an F test to compare variances. Differences were considered significant at $p < 0.05$.

3. RESULTS

3.1 Establishment and comprehensive characterization of primary *H. illucens* cell cultures

Based on developmental time-point analysis, the 19 h incubation stage was identified as the optimal window for isolating cells with myogenic potential (Figures 1a, b). At this time point, cell cultures were particularly enriched in elongated, contractile cells indicative of myoblasts or early myotubes, providing an optimal balance between cell diversity and differentiation potential for establishing muscle-enriched primary cultures. At 15 h (Figures 1c, d), rounded epithelial-like and adipocyte-like cells predominated, reflecting an early developmental stage with limited differentiation and proliferation. In contrast, 19 h cultures exhibited distinct proliferating myoblast clusters interconnected by fine cytoplasmic extensions and early myotube-like structures, as supported by MHC (red) expression detected via immunofluorescence alongside actin filaments (phalloidin, green) and nuclear staining (DAPI, blue) (Figures 1a, b). At 22 h (Figure 1e–f), a dense surface film with sporadic breaks revealed partially elongated cells, but overall cellular growth appeared reduced, limiting detailed morphological evaluation. By 24 h (Figure 1g, h), the surface was

dominated by a granular, dense coating, with scattered and misaligned actin and myosin signals, suggesting advanced yet disorganized differentiation. At later time points (22–24 h), the dense surface layer observed by phase-contrast microscopy appeared to interfere with actin filament organization, as suggested by the reduced and fragmented phalloidin signal. High cell density and limited nutrient diffusion may have simultaneously triggered differentiation signals and disrupted cytoskeletal alignment. This phenomenon was particularly evident at 24 h, where actin and myosin signals were scattered and poorly aligned, indicating incomplete sarcomeric maturation or stress-induced remodeling of cytoskeletal elements. Collectively, these findings establish 19 h post-oviposition as the most reliable stage for establishing muscle-enriched cultures, and all subsequent experiments were performed using embryos at this time point. Negative controls were performed on cells isolated at the 19 h timepoint, selected as representative since all samples were processed under the same experimental conditions (*Appendix Figure A2).* These controls confirmed the specificity of the staining, showing no non-specific fluorescence when the primary antibody, or both the secondary antibody and phalloidin, were omitted.

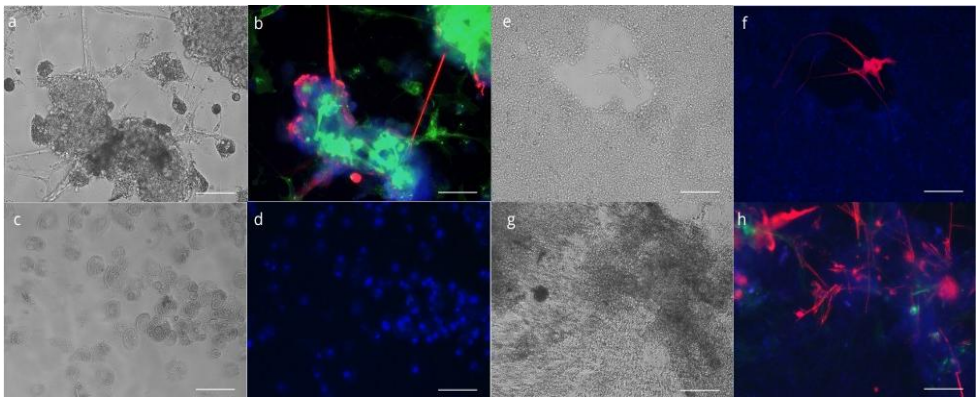


Fig 1. *Representative phase contrast (a, c, e, g) and fluorescence images (b, d, f, h) of H. illucens primary cell cultures established from embryos incubated for 19, 15, 22, and 24 h*

post-oviposition, captured at 1 month of in vitro maintenance. **a,b:** In the 19-hour time point, proliferating myoblast clusters are visible, interconnected by fine extensions, and early muscle fiber formation. **c,d:** At 15 hours, rounded epithelial-like and adipocyte-like cells were observed, indicative of an early developmental stage. **e,f:** By 22 hours, a dense surface coating with occasional breaks reveals partially elongated cells, though cellular growth appears limited. The absence of green signal (phalloidin) may suggest reduced actin polymerization due to high cell density and the presence of a dense layer, which could restrict nutrient access and alter local cell conditions, potentially advancing differentiation and minimizing actin-based structures. **g,h:** At 24 hours, fluorescence staining reveals nuclei (blue, DAPI) and myosin heavy chain expression (red, anti-MHC antibody), confirming muscle differentiation. Although sarcomeric striations were detected, actin and myosin alignment appeared incomplete, suggesting partial maturation of the contractile apparatus. Phase contrast imaging shows only a granular, dense surface coating. Green and red signals appeared scattered rather than interconnected and exhibited some blurring, suggesting incomplete alignment of actin and myosin structures in these later stages of differentiation. Scale bars:100 µm.

Phase-contrast microscopy revealed the heterogeneous composition of primary cell cultures derived from *H. illucens* embryos incubated for 19 h post-oviposition, with distinct morphological features observed under different plating conditions (Figure 2). In cultures seeded with the undiluted pellet (Figure 2a), confluence was reached after approximately 10 days, with diverse cell morphologies observed in early stages, including small spherical cells, elongated myoblast-like cells (M), and irregular giant cells. In contrast, cultures seeded at 1:2 or 1:4 dilutions (Figure 2b, e) formed interconnected networks within 7 days, showing improved organization compared to the undiluted pellet, likely due to more favorable cell density. Over time, the 1:4 dilution cultures (Figure 2d, e) became enriched in elongated muscle- like cells and epithelial-like cells (E), alongside additional cell types such as F-cells (F). Contractile activity, indicative

of muscle cell differentiation, was first observed after 7 days and persisted throughout the culture period (Figure 2c, e). Cell morphology evolved progressively, with elongated cells adhering, spreading, and forming larger aggregates. Extensive cell fusion was noted over time, resulting in multinucleated myotube-like structures and interconnected contractile networks (Figure 2d). Thin cytoplasmic filaments became more prominent as cultures matured (Figure 2b, c). Vesicular structures adhering to cell surfaces were frequently observed (Figure 2b, c), and localized cell degeneration was occasionally detected, characterized by coalescing blebs (B) along cytoplasmic extensions. These observations confirm the intrinsic ability of primary cultures to undergo spontaneous myogenic differentiation, as indicated by progressive cell fusion and multinucleated myotube-like structures, while the presence of neuronal-like projections suggests the coexistence of neurogenic precursors within the heterogeneous cell population.

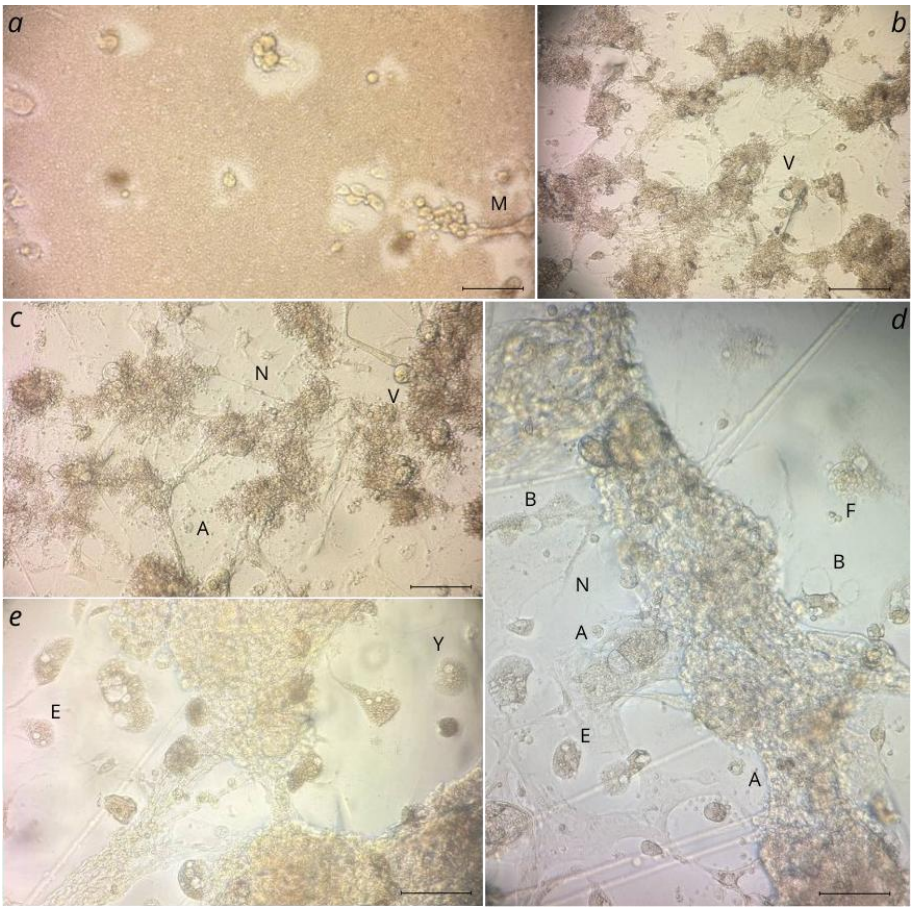


Fig 2. *Phase contrast images of a cell population derived from H. illucens embryos after 19 hours of incubation.* **a.** Culture at 7 days with cell-pellet undiluted. An elongated cell, presumed to be a myoblast (M), is visible alongside a small proliferative cluster. In the other images (b, c, d, e), large cell clumps are also present, accompanied by a well-developed tissue network and visible neuronal extensions. Scale bar: 100 µm; **b.** Culture at 7 days, from a 1:2 pellet dilution. Scale bar: 100 µm; **c.** Culture at 7 days, from a 1:4 pellet dilution. Scale bar: 100 µm; **d.** Culture at 14 days (1:4 dilution). Scale bar: 50 µm; **e.** Culture at 21 days (1:4 dilution). Scale bar: 50 µm. Annotations: **M**: Myoblasts; **E**: Epithelial-like cells: **F**: F-cells; **Y**: Vitellophages (yolk cells); **N**: Neuronal-like cells; **B:** Blebs observed in degenerated areas; **V:** Vesicles.

Phase-contrast microscopy of *H. illucens* primary cultures treated with 0.50% trypsin confirmed cell survival and maintenance of adhesion across passages (Figure 3). At P0 (no passage) (Figure 3a), cell culture showed mixed populations, including rounded clusters and early elongated cells. After P1 (first passage) (Figure 3b) and P2 (second passage) (Figure 3c), the cells remained adherent, forming interconnected networks with visible cytoplasmic extensions. After the P3 (Figure 3d), cell cultures retained viable clusters and adherent cells. No signs of degeneration were detected, and cells-maintained adhesion while forming interconnected, multinucleated aggregates indicative of myogenic differentiation. The persistence of contractile cells and the re-establishment of cytoplasmic networks after passaging confirm that myogenic cells remain functionally active and capable of reconstructing tissue-like structures.

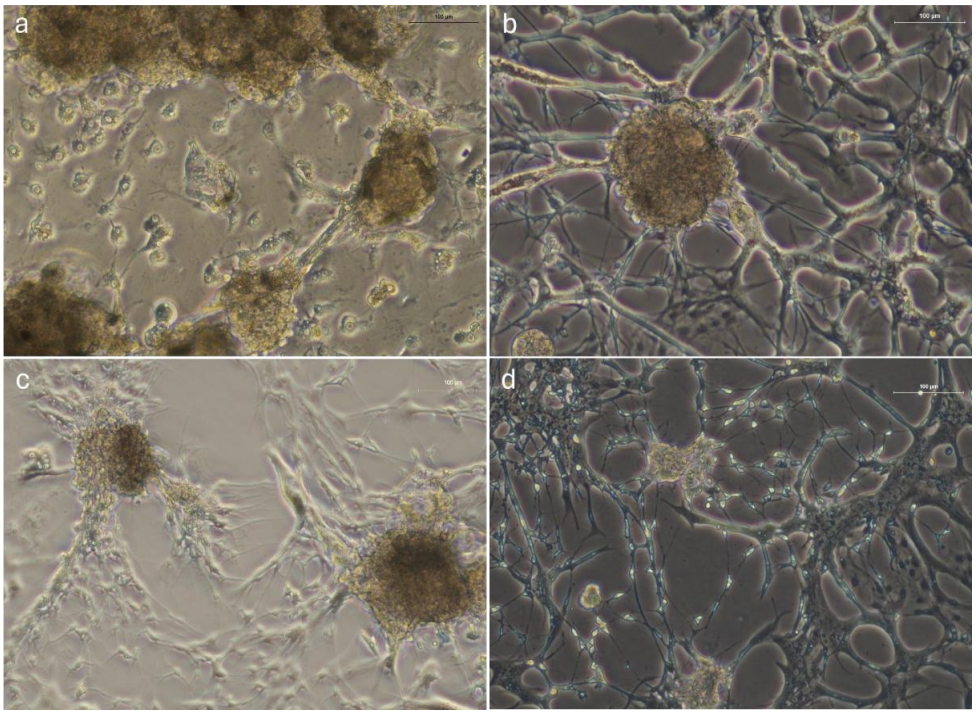


Fig 3. *Phase-contrast images of H. illucens primary cell cultures treated with 0.50% trypsin across passages.* **(a)** Passage 0 (P0); **(b)** Passage 1 (P1); **(c)** Passage 2 (P2); **(d)** Passage 3 (P3). In this series, cells were passaged prior to medium change between P2 and P3. Scale bars: 100 µm.

To evaluate the long-term viability of the primary cultures, a LIVE/DEAD™ viability assay was performed after two months of continuous culture under low-frequency medium changes. The Live/Dead assay confirmed that these cultures maintained high cell viability and contractile functionality, highlighting the metabolic resilience and stability of myogenic cells over extended periods (Figure 4). Notably, viable cells displayed an elongated, interconnected morphology and maintained contractile activity, confirming the persistence of metabolically active myogenic cells within the heterogeneous population, potentially supported by nutrient recycling from yolk-derived cells and trophocytes.

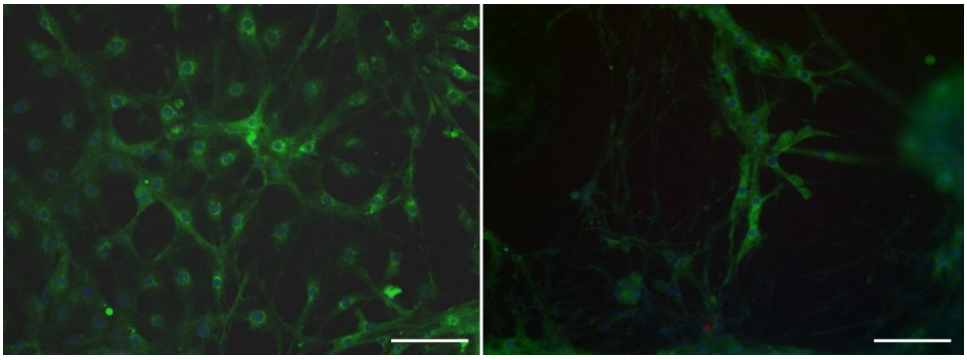


Fig 4. *LIVE/DEAD™ assay of primary H. illucens cell cultures after two months in vitro maintenance.* Representative fluorescence image showing viable cells (green, Calcein-AM) with occasional red signal (Ethidium homodimer-1) possibly indicating dead cells or imaging artefacts. Blue fluorescence corresponds to nuclear staining (DAPI). Scale bar: 100 µm.

Following Oil Red O staining, brightfield images (Figure 5) reveal distinct, red-stained lipid droplets within the cultured cells. The Oil Red O selectively binds to neutral lipids, which were observed as discrete red inclusions interspersed among the elongated muscle-like cells. Lipid inclusions are visible both as isolated droplets and as larger and rounded aggregates formed by multiple lipid droplets. The presence of these lipid-rich areas suggests a notable cellular heterogeneity within the primary culture, with lipid-storing cells coexisting alongside muscle-like cells. This observation highlights the diversity of cell types derived from *H. illucens* embryos.

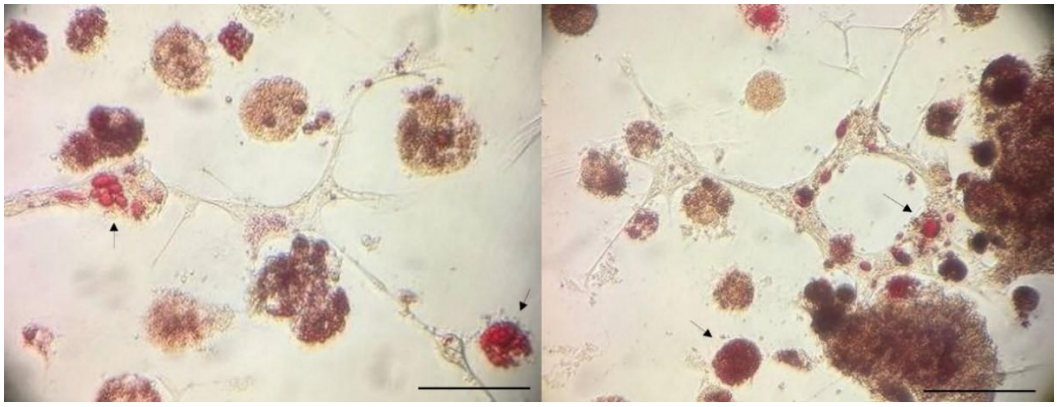


Fig 5. *Brightfield images of primary cells isolated from H. illucens embryos 19 h post-incubation and stained with Oil Red O after 1 month of culture.* Red-stained lipid droplets are observed as discrete inclusions interspersed among muscle-like cells. Lipid deposits appear as both isolated droplets and larger clusters (black arrows), indicating the presence of adipocyte-like cells within the heterogeneous cell population. Scale bars, 50 µm

qPCR analysis revealed that MHC expression, calculated as $2^{-\Delta Ct}$, was on average higher in cultured cells compared to *H. illucens* eggs. The $\log_{10}(2^{-\Delta\Delta Ct})$ plot confirmed a significant upregulation of MHC (Myosin Heavy Chain), a canonical structural and functional marker of muscle fibers, in cultured cells compared to eggs, exceeding the increase observed for TWIST and reflecting the presence of cells undergoing advanced stages of myogenic differentiation (Figure 6). Similarly, TWIST showed a higher mean expression in cultured cells compared to eggs, with $\log_{10}(2^{-\Delta\Delta Ct})$ values indicating a moderate upregulation, although lower than that of MHC (Figure 6). In contrast, Wingless was strongly downregulated in cultured cells relative to eggs, with Welch's t-test confirming a statistically significant difference between the two groups ($p = 0.0129$). Relative expression values ($2^{-\Delta Ct}$) showed that Wingless was expressed in eggs at levels approximately 65–85 times higher than in cultured cells. Consistently, the $\log_{10}(2^{-\Delta\Delta Ct})$ showed a negative value below –2, confirming the pronounced reduction of Wingless expression after cell isolation. Overall, the integrated analysis of MHC, TWIST, and Wingless revealed a transcriptional pattern characterized by increased expression of mesodermal and myogenic genes (MHC and TWIST) and a reduction in early embryonic patterning genes (Wingless). These findings demonstrate that the 19 h time point yields a cell population enriched for mesodermal and early myogenic markers, providing a molecular framework for the establishment of muscle-oriented insect cell cultures.

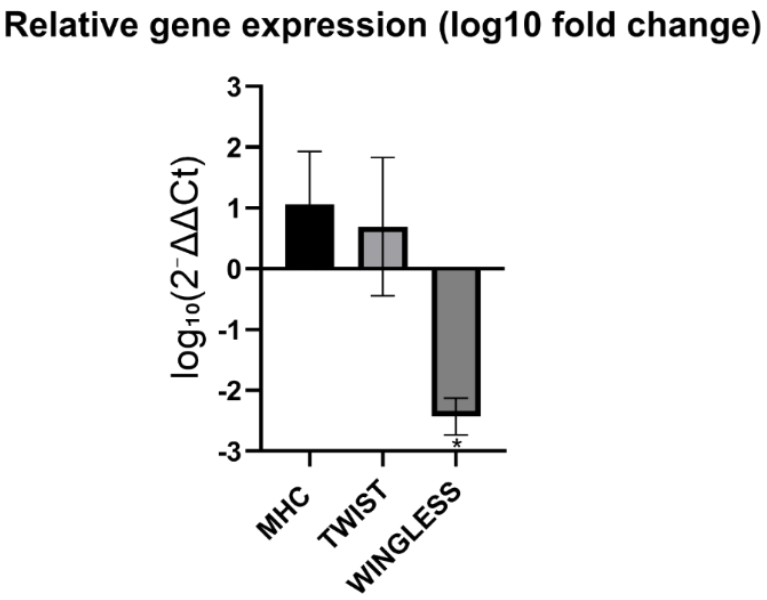


Fig 6. *Relative expressions of MHC, TWIST, and Wingless in cultured cells and eggs of H. illucens*. $Log_{10}(2^{-\Delta\Delta Ct})$ representation of fold changes for all genes, where MHC and TWIST display positive values indicative of increased expression in cultured cells, while Wingless shows a negative value (< –2), confirming strong downregulation. Statistical analysis was performed on ΔCt values (mean ± SD, n = 3) using an unpaired two-tailed t-test with Welch's correction ($p < 0.05$).

3.2 Muscle cell enrichment strategies: EGTA treatment and time-dependent adherence

EGTA treatment modulates muscle cell morphology and myogenic enrichment in *H. illucens* embryonic cultures. Immunostaining for MHC and DAPI revealed EGTA-dependent changes in the spatial organization and morphology of MHC-expressing cells. At 0 mM, MHC-positive cells appeared isolated, displaying short, thickened cytoplasmic extensions and limited alignment (Figure 7a). At 1 mM EGTA, muscle cells exhibited a more elongated, spindle-shaped morphology with pronounced bipolar extensions (Figure 7b). At 5 mM, MHC-positive cells showed increased interconnectivity, with long, thin processes forming overlapping, fiber-like structures suggestive of early bundle formation (Figure 7c).

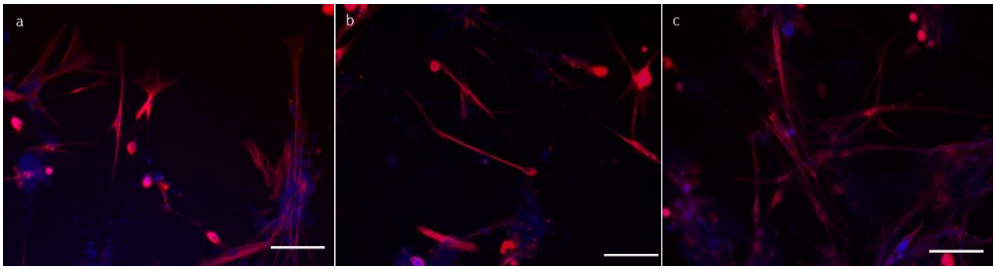


Fig 7. *Immunofluorescence of H. illucens embryonic cells treated with EGTA for 2 hours*. Representative images of cultures treated with (a) 0 mM, (b) 1 mM, and (c) 5 mM EGTA. MHC is shown in red and nuclei in blue (DAPI). Scale bars: 100 µm.

Quantitative analysis showed that a 2-hour exposure to 0–5 mM EGTA followed by gentle agitation increased the myogenic index, with enrichment values of 22.5 ± 6.1 %, 28.8 ± 3.7 %, and 27.0 ± 7.6 % at 0, 1, and 5 mM EGTA, respectively (Figure 8), although without statistically significant differences. The trend indicates that 1 mM EGTA provided the highest relative myogenic enrichment, while 5 mM EGTA resulted in the highest absolute number of myogenic nuclei, despite a slightly lower fusion index. By demonstrating comparable morphological and functional responses to EGTA, the data provides the first evidence that *H. illucens* myogenic cells can be selectively enriched under calcium-depleted conditions, even in the absence of extracellular matrix coatings or genetic techniques. Furthermore, the quantitative analyses revealed that EGTA concentration not only influences myogenic purity but also modulates the balance between adhesion and cell survival, an important parameter for optimizing yield in primary cultures. This outcome reflects a concentration-dependent window of efficacy, highlighting the ability of EGTA-based calcium chelation to modulate myogenic enrichment in *H. illucens* primary cultures and providing a quantitative basis for future optimization in non- model insect systems.

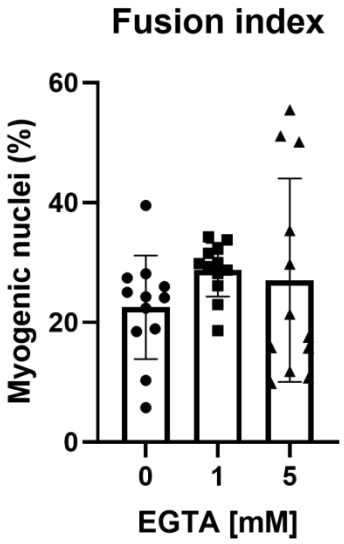


Fig 8. *H. illucens* Fusion index of *H. illucens* embryonic cell cultures treated with 0, 1, and 5 mM EGTA. Bars represent mean ± SD (n = 3 independent biological replicates per condition), and individual data points (12 per condition) correspond to two measurements for each of two technical replicates per biological replicate. Statistical analysis was performed in GraphPad Prism 8.4.2 using one-way ANOVA with Tukey's post hoc test; no significant differences were detected between conditions (all $p > 0.05$; pairwise comparisons: 0 vs 1 mM, $p = 0.1167$; 0 vs 5 mM, $p = 0.6641$; 1 vs 5 mM, $p = 0.9237$).

Also, to investigate an alternative enrichment strategy, the potential of time-dependent differential adhesion to separate myogenic subpopulations was assessed. *H. illucens* primary cells were fractionated based on adhesion time and analyzed after 30 min (PP1, pre-plating), 90 min (PP2), and 150 min (PP3).

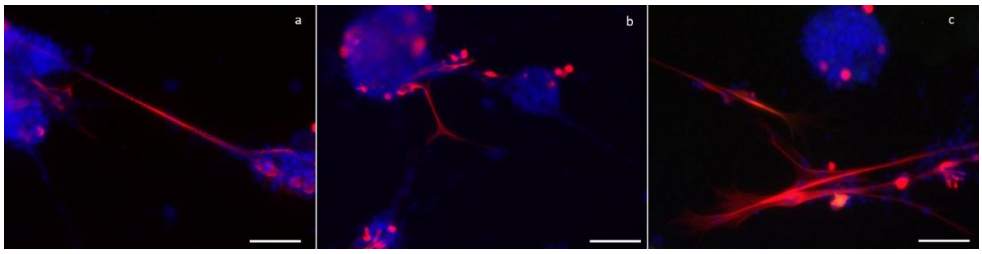


Fig 9. *Cells stained for MHC (red) and nuclei (DAPI, blue).* While PP1(a) and PP2 (b) predominantly displayed mononucleated cells with thin or stellate morphology, PP3 (c) included thick, elongated, multinucleated myotubes with strong MHC signal. Scale bars: 100 µm.

Fusion index quantification was performed by calculating the percentage of nuclei within MHC-positive myotubes. Although PP3 displayed the highest average fusion index and PP1 the lowest, no statistically significant differences were observed among the groups. Nevertheless, morphological assessment revealed distinct qualitative features across the three populations (Figure 9). PP1 cultures predominantly contained elongated, MHC-positive mononucleated cells with thin cytoplasmic extensions. In contrast, PP2 cells exhibited sparse MHC staining with disorganized morphology and reduced cytoplasmic spreading. PP3 cultures showed the most advanced myogenic phenotype, with large, thick, multinucleated myotubes and dense MHC signal, consistent with late-stage differentiation. Given the absence of prior data on adhesion kinetics of insect myogenic cells, these findings provide experimental basis for adapting vertebrate-derived enrichment paradigms to invertebrate systems, thereby expanding the current understanding of insect muscle cell biology. These qualitative differences are in agreement with the higher fusion index values recorded in PP3 and suggest functional divergence between fractions.

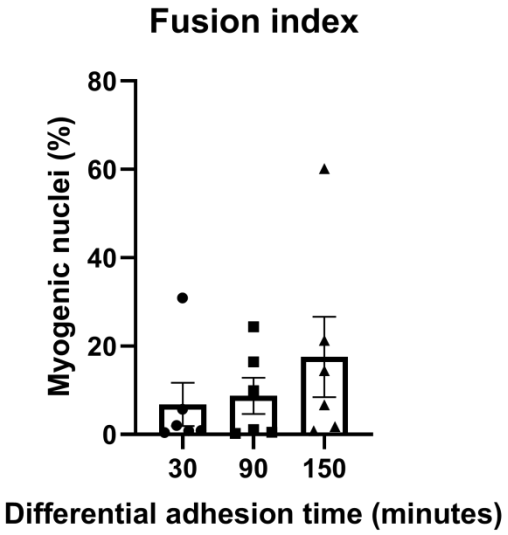


Fig 10. Fusion index calculated as the percentage of nuclei within MHC-positive myotubes in PP1 (0–30 min), PP2 (30–90 min), and PP3 (90–150 min). Bars represent mean ± SEM (n = 3 independent biological replicates per condition), and individual data points (six per condition) correspond to technical duplicates for each biological replicate. Statistical analysis was performed in GraphPad Prism 8.4.2 using one-way ANOVA with Tukey's post hoc test; no significant differences were detected between conditions (PP1 vs PP2, $p = 0.9624$; PP1 vs PP3, $p = 0.1895$; PP2 vs PP3, $p = 0.7207$).

4. DISCUSSION

Primary cell cultures derived from *H. illucens* embryos displayed remarkable morphological heterogeneity, reflecting the coexistence of multiple embryonic tissue lineages, as similarly observed in other holometabolous insects (Rey *et al.,* 2000; Miranda *et al.,* 2008; Ardila *et al.,* 2005). The co-occurrence of epithelial-like, myogenic, and neurogenic cells is a recurrent hallmark of insect embryogenesis and underscores the intrinsic plasticity of these systems. The sustained contractile activity observed in the cultures in the present study is probably attributable to the synergistic interactions with yolk-derived trophocytes, which have been described as critical reservoirs of metabolic energy and growth- supporting factors (Baryshyan *et al.,* 2012). Moreover, the presence of vesicular structures on the cell surface, indicative of active exocytosis and membrane remodeling, mirrors the dynamic vesicle trafficking reported in established insect cell lines (Bello *et al.,* 1999; Suddep *et al.,* 2005; Echeverry *et al.,* 2009; Segura *et al.,* 2012; Goodman *et al.,* 2012). The concomitant appearance of neuronal-like extensions, as previously described in *Lucilia sericata* cultures (Echeverry *et al.,* 2009), further supports the concept of embryonic cellular plasticity. Collectively, these findings position *H. illucens* embryonic cultures as a versatile and underexplored platform for dissecting insect cell differentiation and highlight their untapped potential for cellular agriculture and CM meat. In line with this interpretation, Rubio *et al.* (2019) and Letcher *et al.* (2022) have demonstrated that insect muscle and fat can be cultivated *in vitro*, providing proof-of-concept for the generation of edible tissues with contractile and metabolic activity. Our morphological evidence of contractile myogenic cells parallels these pioneering studies, confirming that insect-derived cells can be directed toward food-oriented tissue engineering

applications. The morphological evidence is reinforced by the transcriptional profiling of TWIST, MHC, and Wingless, which provides molecular validation of myogenic commitment. TWIST, a bHLH transcription factor and pivotal regulator of mesodermal specification and early myogenesis, was significantly upregulated in cultured cells compared with eggs. This observation is consistent with its well-documented role in establishing and maintaining mesodermal identity in *Drosophila melanogaster* and other insects (Leptin, 1991; Baylies & Bate, 1996; Sandmann *et al.,* 2007). TWIST acts as a first-tier transcriptional regulator capable of driving non-myogenic cells toward a somatic muscle fate, while its sustained expression prevents premature differentiation of adult muscle precursors (Cripps & Olson, 1998; Carmena & Baylies, 2006). MHC expression typically coincides with sarcomere assembly and myofiber maturation during insect myogenesis (Bernstein *et al.,* 1978; Weitkunat & Schnorrer, 2014), and its strong induction confirms the presence of terminally differentiating myoblasts in our cultures. Conversely, the sharp downregulation of Wingless—a key regulator of early embryonic patterning—indicates the transition from pluripotent embryonic states to specialized mesodermal lineages. Together, this transcriptional signature delineates a heterogeneous yet muscle-oriented population and confirms that 19 h post-oviposition represents a critical developmental window for isolating cells with high myogenic potential. These insights lay a molecular and functional foundation for the establishment of *H. illucens*-derived muscle cell platforms in CM production. Comparable transcriptional dynamics have been highlighted by Rubio *et al.* (2019), who reported that insect muscle cultures exhibit sarcomeric protein expression and functional contractility reminiscent of vertebrate systems, supporting the robustness of insect myogenesis *in vitro*. Similarly, Giglio *et al.* (2024)

emphasized that the regulatory cascades governing muscle specification are conserved across taxa, further validating our molecular evidence as relevant for cultivated meat applications. A central observation of this study is that EGTA-mediated Ca^{2+} chelation supports a feasibility-level enrichment approach, showing qualitative myogenic reorganization despite non-significant fusion-index differences. Our findings align with seminal studies on *D. melanogaster*, where EGTA pre-plating selectively enriched myoblasts capable of forming multinucleated myotubes (Bernstein *et al.,* 1978). Donady and Fyrberg (1979) further showed that EGTA exposure induces the selective expression of muscle-specific actin isoforms, confirming the molecular fidelity of this selection strategy. Similar approaches have been successfully adapted to *Manduca sexta*, where EGTA treatment combined with 20- hydroxyecdysone yielded long-lived, contractile myotube cultures with robust metabolic resilience (Baryshyan *et al.,* 2012). This suggests that calcium homeostasis is a conserved determinant of muscle-specific cell fate across phylogenetically distant insect taxa (Loeb, 2004). The use of time-dependent adhesion to enrich myogenic cells has been extensively described in vertebrate systems, where fibroblasts generally attach to culture substrates faster than myoblasts, enabling their separation through sequential pre-plating (Yablonka- Reuveni & Nameroff, 1977; Decker *et al.,* 1984; Blau & Webster, 1981). Here, we report the first application of this approach to insect-derived cells, using *H. illucens* primary embryonic cultures. Although no statistically significant differences in fusion index were observed among adhesion-based fractions, PP3 displayed the most advanced myogenic morphology, including thick, multinucleated myotubes with strong MHC signal, consistent with the enrichment of later-adhering myogenic cells described in vertebrate models. Our integrated morphological and transcriptional data

advance a practical starting point for insect-based CM methods, highlighting methodological feasibility rather than statistically significant enrichment. While most entomoculture studies have relied on established cell lines from *D. melanogaster* or *M. sexta*, our work extends muscle cell culture strategies to *H. illucens*, a species of high biotechnological relevance due to its role in bioconversion and circular economy systems (Athanassiou *et al.,* 2024). Techno-economic and life cycle assessments further support the potential of insect entomoculture to achieve cost-effective and environmentally sustainable production of alternative proteins, with lower resource inputs compared to mammalian cell lines (Ashizawa *et al.,* 2022; Sinke *et al.,* 2023; CE Delft, 2021). By integrating morphological, molecular, and functional analyses, we provide a benchmark for deriving muscle-oriented cell populations from *H. illucens*. This aligns with the broader framework proposed by Rubio *et al.* (2020a) and CE Delft (2021), who emphasized that the scalability and sustainability of CM depend on identifying robust, low-cost cellular platforms. Our data suggest that insect-derived cells, due to their resilience and reduced metabolic requirements, could represent a pivotal innovation in the diversification of sustainable protein sources. Such advances expand the entomoculture toolbox and align with recent perspectives emphasizing the nutritional and sustainability benefits of insect cell- derived proteins (Siddiqui *et al.,* 2025; USDA-ERS, 2025).

5. CONCLUSION

A reproducible protocol for the isolation, characterization, and myogenic enrichment of primary embryonic cell cultures from *H. illucens*, a non-model insect species of high biotechnological relevance was demonstrated. By identifying 19 h post-oviposition as the optimal developmental stage and

combining morphological, molecular, and functional analyses, we generated stable cultures enriched in contractile myogenic cells, as demonstrated by the upregulation of MHC and TWIST and the downregulation of Wingless. The implementation of EGTA- mediated calcium chelation and time-dependent adhesion provided complementary enrichment strategies, yielding morphologically distinct muscle cell populations with sustained viability and contractile activity. Given the species' established role in circular economy systems, *H. illucens*-derived myogenic cultures offer a promising route towards cost-effective, scalable, and environmentally responsible muscle tissue biomanufacturing. Future research should focus on serum-free media optimization, long-term maintenance of purity, and integration with edible scaffolds to accelerate the translation of insect cell culture technologies into industrial cultivated meat applications.

CHAPTER 4

1. From edible insect to *in vitro* muscle: a cellular-agriculture platform based on *Tenebrio molitor* myogenic primary cultures

This chapter establishes embryo-derived primary cultures of Tenebrio molitor, implements EGTA-assisted selective adhesion and depending time pre-plating, and validates myosin labeling as evidence of myogenic organization. (Editorial status: original, unpublished work conducted as part of this thesis; not submitted for publication at the time of deposit.)

The transformation of food systems requires a profound rethinking of protein sources considering environmental constraints, supply chain vulnerabilities, and evolving consumer expectations (Galanakis, 2024). In this context, edible insects have consolidated scientific foundations, hygiene and safety standards, and regulatory instruments, with *Tenebrio molitor* (yellow mealworm) occupying a prominent position for its nutritional, environmental, and processing characteristics. From a biological perspective, *T. molitor* is a holometabolous beetle which, when reared under controlled thermo-hygrometric conditions (temperatures around 25–28 °C and relatively high humidity), ensures reliable growth rates and biomass yields. The regular duration of its developmental stages allows for harvest planning and experimental repeatability. Moreover, biomass quality can be modulated through diet and stage management, making this species a model for food production (Wang *et al.,* 2011; Li *et al.,* 2013; Soares Araújo *et al.,* 2019). Larvae of *T. molitor* are an edible insect with a very high protein content, typically around 50–60% of dry matter. They provide a complete amino acid profile, with all essential amino acids present in adequate amounts (Syahrulawal *et al.,* 2023; Franco *et al.,* 2024a; Ianniciello *et al.,* 2024). Among the EAAs, leucine, valine and lysine are particularly abundant (e.g. leucine ~55.5 g/kg, valine ~44.2 g/kg and lysine ~32.4 g/kg on a dry matter basis). Tryptophan and methionine, although present, are the least represented (approximately 8.7 and 9.8 g/kg of dry matter, respectively). In practical terms,

these values correspond to contents of essential amino acids ranging from tens to hundreds of milligrams per 100 g of edible larvae. In addition, compared with bovine meat, *T. molitor*proteins contain higher amounts of isoleucine, leucine, valine, phenylalanine and tyrosine, conferring a high biological value to their protein profile (Feng *et al.,* 2023). Protein digestibility in mealworm is high, especially when the chitin content (the exoskeletal fibre) is reduced through appropriate processing steps (Syahrulawal *et al.,* 2023). As a result, the bioavailability of amino acids is comparable to that of conventional animal proteins.

From a lipid perspective, *T. molitor* larvae exhibit a high proportion of unsaturated fatty acids, which is generally considered beneficial for human health. On average, more than 20–30% of total lipids are mono- or polyunsaturated (Syahrulawal *et al.,* 2023). Oleic acid (ω-9) is the most abundant fatty acid (approximately 37–45% of total larval fat), followed by linoleic acid (ω-6, about 25–35%). The ω-3 fraction (α-linolenic acid) is present at much lower levels (less than 2–3%). Overall, total PUFA may account for approximately 20% up to 60% of larval lipids (depending on the rearing substrate), with a predominance of ω-6 over ω-3 fatty acids (FeedTables by INRAE & AFZ, n.d.). The relatively low proportion of saturated fats (compared with *H. illucens* and many types of meat) together with the high content of mono- and polyunsaturated fatty acids confers a high lipid quality index to *T. molitor* (Syahrulawal *et al.,* 2023). Moreover, *T. molitor* larvae contain substantially less cholesterol than conventional meats, making them an attractive ingredient for diets aiming to reduce the intake of animal sterols. Nutritional analyses confirm that the inclusion of *T. molitor* flour markedly increases the protein content of food preparations, while maintaining a favourable lipid profile

and a good balance of essential amino acids (Franco *et al.,* 2024a; Ianniciello *et al.,* 2024). Chitin contributes to the fiber fraction and requires specific evaluation regarding digestibility and technological suitability (Rumpold & Schlüter, 2013; Zielińska *et al.,* 2018; De Castro *et al.,* 2018). On the technological side, *T. molitor* meal and protein isolates show favorable hydration, emulsification, and gelation properties, useful for the formulation of high-protein or hybrid products. At the same time, processing choices (drying, roasting, grinding) strongly influence lipid oxidation, color, and the development of off-flavors, requiring precise management of water activity, packaging, and storage stability according to integrated safety principles (Barroso *et al.,* 2017; De Castro *et al.,* 2018). From an environmental perspective, Life Cycle Assessment (LCA) is the most appropriate comparative framework. At equal functional units (such as edible fraction or kilogram of protein), studies consistently place mealworms in a favorable position, with lower climate footprint and land use than many conventional livestock chains. The main hotspots are feed production and energy- intensive phases (climate control and drying) (Oonincx & de Boer, 2012; Smetana *et al.,* 2019; Parodi *et al.,* 2018). A crucial but often underestimated aspect is circularity: the use of suitable agro-industrial by-products as substrates can significantly reduce the feed footprint, while frass (a mixture of excreta and rearing residues) represents a co-product with potential agronomic value. Explicit inclusion of these flows within LCA boundaries, and the adoption of transparent allocation rules, is essential to avoid distortions and to capture systemic benefits correctly (van Huis & Oonincx, 2017; Franco *et al.,* 2024). From a regulatory perspective, in the European Union Regulation (EU) 2015/2283 classified edible insects as novel foods. For *T. molitor*, key milestones included the authorization of dried larvae (2021) and, subsequently, the extension to frozen, dried, and

powdered forms (2022), with specifications for use, labeling requirements, and allergen warnings. More recent updates have further expanded the authorized categories. EFSA opinions have confirmed the safety of *T. molitor* under authorized conditions while emphasizing the need for transparent consumer information (EFSA NDA Panel, 2021a; 2021b). Outside the EU, the situation is heterogeneous but converges on key principles of traceability, risk management, and labeling: for example, post-Brexit the United Kingdom adopted a transitional and assessment-based regime, while Switzerland has admitted selected species, including *T. molitor*, under FSVO requirements (Penedo *et al.,* 2022).

The acceptance of insect-based products, particularly in cultural contexts without a tradition of entomophagy, is influenced by psychological barriers (such as disgust), perceptions of risk, and novelty. Incorporating *T. molitor* proteins into familiar food matrices (bread, pasta, bakery products), providing clear labeling (including allergen information), and transparent communication of both benefits and limitations increases consumer willingness to try and purchase, especially after repeated exposure (Mancini *et al.,* 2019; Hartmann & Siegrist, 2018; Elhassan *et al.,* 2019). Supply chain safety requires a systemic approach: microbiological hazards (spores, pathogens from feed or processing), chemical hazards (residues or heavy metals from substrates), and physical hazards, along with allergenicity risks (tropomyosins with potential cross-reactivity to crustaceans and mites), can be mitigated through hygiene prerequisites, Good Hygiene and Manufacturing Practices (GHP/GMP), appropriate thermal treatments, feed controls, management of water activity and oxidation, batch traceability, and proper labeling. Standardization of these barriers and monitoring systems in industrial settings is fundamental to ensuring compliance

and consumer trust (EFSA, 2015; Klunder *et al.,* 2012; Fernandez-Cassi *et al.,* 2020; IPIFF, 2024). Correct accounting of these flows in LCA studies and business models strengthens the competitiveness of mealworms compared to conventional protein sources (Franco *et al.,* 2024; van Huis & Oonincx, 2017). Considering this framework, the present chapter adopts *T. molitor* as a case study to integrate aspects related to edible use and the perspective of cellular agriculture. It aims to lay the conceptual foundations for the use of insect cell cultures within the scope of entomoculture and cultivated meat.

2. MATERIALS AND METHODS

2.1 Embryonic cell isolation

Primary cell isolation was performed using *T. molitor* eggs obtained from Vermont Mealworm Farm (Braintree, VT, USA). Unless otherwise stated, all reagents were procured from Thermo Fisher Scientific (Waltham, MA, USA). Egg processing prior to cell isolation was conducted using two distinct protocols, both initiated under sterile conditions. All protocols were designed to optimize cell viability and enhance myogenic differentiation potential for downstream applications. In the first, eggs were dissected with a sterile scalpel under aseptic conditions; in the second, eggs were homogenized. A detailed description of each protocol is outlined below. Cell isolation cutting embryonated *T. molitor* eggs involved sterilization steps performed in 35 mm Petri dishes. Each egg was sectioned with a sterile scalpel, and 2 mL of culture medium was added to the dish. The wells containing samples were sealed with Parafilm and incubated at 27°C. For the homogenization protocol, *T. molitor* eggs were collected in 1X phosphate buffered saline (PBS) supplemented with 2% antibiotic-antimycotic (A/A) solution and gently rinsed using a Pasteur pipette. Sterilization was

achieved by immersing the eggs in 70% ethanol, followed by treatment with 50% bleach to remove the chorion (Urbán-Duarte *et al.,* 2021). The eggs were subsequently rinsed with 1X PBS and culture medium. Sterilized eggs were transferred to a 1.5 mL Eppendorf tube (Sigma-Aldrich, Saint Louis, USA) containing fresh medium and homogenized using a polypropylene pestle. The homogenate was filtered into a sterile dish and centrifuged at 300 × g for 5 minutes. The supernatant was plated directly, while the pellet was resuspended in fresh medium, centrifuged, and both the resulting supernatant and resuspended pellet were plated at a density of 150,000 cells/cm^2.

2.2 Neonate-larvae cell isolation

T. molitor neonate larvae (referred to as "neo-larvae" and used interchangeably with "neonates") were obtained from eggs sourced from Vermont Mealworm Farm (Braintree, VT, USA). Eggs were maintained under standard rearing conditions until hatching; newly emerged larvae were then used for isolations. For the purposes of this study, neonate larvae were operationally defined as 0–48 h post-hatch. Prior to processing, *T. molitor* neonate larvae were euthanized by brief heat-kill: immersion in ~70 °C water for 30 s followed by rapid cooling on ice for 3 min. The external surface was then sterilized by immersion in 70% ethanol, after which all manipulations were conducted under a biosafety cabinet. Cell isolation proceeded exactly as described for the eggs, employing the same homogenization workflow, solutions, decontamination sequence, centrifugation settings, and plating density, with a minor modification. After the first centrifugation, the initial supernatant (SN1) was plated; the pellet was resuspended in fresh medium, centrifuged again under the same conditions, and the second supernatant (SN2) was plated. Finally, the pellet was resuspended

and plated as the pellet fraction (P).

2.2.1 Enzymatic dissociation of T. molitor neonate larvae

Following heat-kill and surface sterilization, all manipulations were conducted in a biosafety cabinet. For each condition (see Table 1 and Table 2), five neonate larvae were transferred to a sterile 12-well plate and minced with a scalpel into ~2–3 mm fragments. Each well was filled with 2 mL of enzyme solution prepared in 1X PBS containing 1% A/A. Incubations were performed at 27 °C with gentle agitation; samples were mixed every 20 min. Cell suspensions were gently minced with a Pasteur pipette, filtered through a 100 µm sterile cell strainer, and centrifuged at 300 × g for 5 min. Supernatants were aspirated, pellets were resuspended in complete medium, and cells were seeded in tissue-culture plastic (~1.5×10^5 cells cm^{-2}) and maintained at 27 °C. Two exploratory trials were conducted; see Table 1 and Table 2 for the precise enzyme identities, concentrations, and exposure times.

Tab 1: *Trial 1: enzyme conditions.* Note: Collagenase I (Gibco, Thermo Fisher Scientific; Cat. 17100-017) and Trypsin–EDTA (Sigma-Aldrich, Saint Louis, USA; Cat. C41010).

ID	Enzyme(s)	Working concentration	Incubation time
T1-C1	Trypsin–EDTA	0.05%	30 min
T1-C2	Trypsin–EDTA	0.05%	1 h
T1-C3	Trypsin–EDTA	0.25%	30 min
T1-C4	Trypsin–EDTA	0.25%	1 h
T1-C5	Collagenase type I	0.015%	1 h
T1-C6	Collagenase type I	0.015%	4 h

Tab 2*: Trial 2 enzyme conditions (prolonged exposure and higher concentrations).*

ID	Enzyme(s)	Concentration	Incubation time
T2-C1	Collagenase I	0.5%	1 h
T2-C2	Collagenase I	0.5%	2 h
T2-C3	Collagenase I	2.5%	1 h
T2-C4	Collagenase I	2.5%	2 h
T2-C5	Trypsin–EDTA	0.05%	1 h

T2-C6	Trypsin–EDTA	0.05%	17 h (overnight)
T2-C7	Trypsin–EDTA	0.25%	1 h
T2-C8	Trypsin–EDTA	0.25%	17 h (overnight)

2.3 Cell culture conditions

The culture medium for *T. molitor* primary cell culture was prepared with Shields and Sang's M3 insect medium, supplemented with 2.5 g/L Bactopeptone, 1 g/L yeast extract, 20% fetal bovine serum (FBS; Gibco, USA), 1%A/A, and 0.2% Primocin (Invitrogen, USA). Once cell populations reached approximately 80% confluency, subcultures were initiated using 0.25% Trypsin-EDTA. Cell viability and density were assessed using Trypan blue staining and a Countess 3 automated cell counter (Thermo Fisher, Waltham, USA).

2.4 EGTA- treatment for muscle cell enrichment

Ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) was used as a Ca^{2+} chelator to transiently modulate cell substrate adhesion and enrich for myogenic precursors in primary cultures of *T. molitor*. Primary cell cultures were first established from embryonated eggs (P0); EGTA treatments were applied at the first passage (P1, see below), adapting the differential-adhesion strategy of Bernstein *et al.,* (1978). Briefly, cells were detached with 0.25% Trypsin–EDTA, pelleted (300 × g, 5 min), and resuspended in the growth medium containing EGTA at 1, 3, or 5 mM. For each condition, suspensions were plated and incubated at 27 °C for 2 h to permit selective adhesion. Plates were then agitated gently at 100 rpm for 10 min, non-adherent cells were removed by careful rinsing, and adherent cells received fresh medium and were cultured for a further 24 h before fixation (*see section 2.6 Immunofluorescence staining*). Non-adherent (NA) cells were operationally defined as the cell population present in

the culture supernatant after the 2 h adhesion period; this supernatant was transferred to a fresh well and cultured under identical conditions to the adherent fraction. Morphology, adherence, and survival were evaluated across EGTA concentrations. Myogenic differentiation was assessed by immunostaining for myosin heavy chain (MHC), using the MAC 147 antibody (Abcam, ab51098; 1:1000), and with nuclear counterstaining (DAPI), following the procedure detailed in Section 2.6 (*Immunofluorescence staining*). For the ImageJ-based quantification, using Fiji software (ImageJ 1.54f, NIH, USA), total nuclei ($DAPI^+$) and nuclei within MHC^+ myotubes were counted, and the fusion index was calculated as: (number of nuclei contained within multinucleated MHC^+ myotubes / total $DAPI^+$ nuclei) × 100.

2.5 Adhesion-time pre-plating assay

Cells used for this assay were derived from homogenized eggs, and seeded onto tissue-culture 96-well plates at 0.75×10^5 cells cm^{-2} per well. Four adhesion windows were tested—10 min, 1 h, 2 h, and 3 h—after which plates were placed on an orbital shaker at 100 rpm for 10 min; the supernatant was transferred to a new well and designated as the non-adherent fraction (NA). The original well was rinsed once with medium to remove loosely attached cells and replenished with fresh medium, thereby defining the adherent cell population (A), and both A and NA were returned to incubation for a further 24 h under identical conditions. Wells were fixed and immunostained for MHC using the MAC 147 antibody (Abcam, ab51098; 1:1000), following the procedure detailed in Section 2.6 (*Immunofluorescence staining*).

2.6 Immunofluorescence staining

Primary cell cultures of *T. molitor* were fixed with freshly prepared 4% paraformaldehyde (PFA) in 1X PBS for 10 min at room temperature (RT). After fixation, wells were washed three times in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT. Samples were then blocked for 30 min in 1X PBS containing 1% bovine serum albumin (BSA). All subsequent antibody dilutions were prepared in the same blocking solution. All incubations were performed at RT.

For the detection of myogenic structures, rat anti-myosin [MAC 147] (Abcam, UK; 1:1000), was used to detect myosin heavy chain; labeling was revealed with a goat anti-rat IgG (H+L) Alexa Fluor 594 secondary antibody (Thermo Fisher, Invitrogen, USA; 1:500). F-actin was counterstained with Alexa Fluor™ 488–Phalloidin (Thermo Fisher Scientific, USA; 1:1000) and nuclei with DAPI (Thermo Fisher Scientific, USA; 1:1000). Primary antibody incubations were 1 h at RT; after primary incubation, samples were washed in PBS/0.1% Tween-20 (PBST; 3 × 5 min), briefly re-blocked (PBS/1% BSA, 30 min), and incubated with secondary antibody for 1 h at RT. Following secondary incubation, samples were washed in PBST (3 × 5 min), rinsed in PBS, and visualized on a Keyence BZ-X800 fluorescence microscope (KEYENCE Corporation, Osaka, Japan). Primary-only and secondary-only negative controls were not repeated for the MAC147 / Alexa Fluor 594 anti-rat IgG(H+L) staining, because this antibody pair had been previously validated in laboratory on the same biological source. To aid cell-type identification in heterogeneous cultures, additional monoclonal antibodies recognizing conserved developmental/neuronal determinants were screened, in a separate exploratory workflow in which phalloidin was omitted; nuclei were counterstained with DAPI only. The exploratory panel is

summarized in Table 3.

Tab 3: *Exploratory primary antibodies screened in T. molitor cultures.*

Target	Antibody	Supplier	Working dilutions	Rationale/notes
Ecdysone receptor (EcR)	10F1	DSHB, USA	1:1000	Probe ecdysteroid signaling activity in insect cells.
Ecdysone receptor (EcR)	9B9	DSHB, USA	1:50, 1:100, 1:500, 1:1000	Complementary EcR epitope to 10F1; broad use across insect models.
Hox proteins Ubx/Abd-A	FP6.87 (Ubx/ABD-A)	DSHB, USA	1:500, 1:1000	Detects conserved Hox epitopes (patterning/identity markers).
Fasciclin I (neuronal CAM)	3B11 (anti–grasshopper Fas I)	DSHB, USA	1:1, 1:20, 1:40, 1:50	Neuronal cell-adhesion marker, frequently used in insects/Orthoptera.
Myosin heavy chain	3E8-3D3	DSHB, USA	1:100	Alternative anti-MHC for myogenic structures.

For all primary antibodies reported in the Table 3, the secondary antibody was Goat anti-Mouse IgG (H+L), Alexa Fluor™ 488, Recombinant Superclonal (Invitrogen/Thermo Fisher, A28175) at 1:1000. In this case negative control was included omitting the primary antibody ("secondary-only"), to quantify background due to secondary binding and/or sample autofluorescence, which is the limiting factor when screening cross-reactivity of antibodies not yet validated in the target species. Given: (a) the lack of yellow mealworm-validated antibodies for the tested targets, and (b) the need to compare multiple candidate primaries under uniform conditions, we prioritized the secondary-only negative control as the most informative and resource-efficient control for this exploration screen. All reagent catalog numbers, host, and full titration ranges are provided in *Appendix Table A1* and *Table A2.*

3. RESULTS

3.1 Egg cutting and homogenization

Both embryo dissection and homogenization yielded viable *T. molitor* primary cell cultures that remained stable for several weeks, displayed diverse morphologies, and exhibited spontaneous contractile activity throughout the culture period. In the embryo dissection (cutting) preparations, early-stage cultures were evaluated for adhesion and spreading. As illustrated in Figure 1, cells displayed an elongated morphology within the first 24 hours (d1, Figure 1A) post-isolation, consistent with early adhesion and spreading. Over time, cell proliferation increased and elongated structures became more prominent, forming larger multicellular clusters. Throughout the culture period, dynamic morphological variations were observed, suggesting the presence of a heterogeneous cell population. Additionally, spontaneous contractions were frequently detected in multiple regions of the culture, appearing intermittently and persisting over several days.

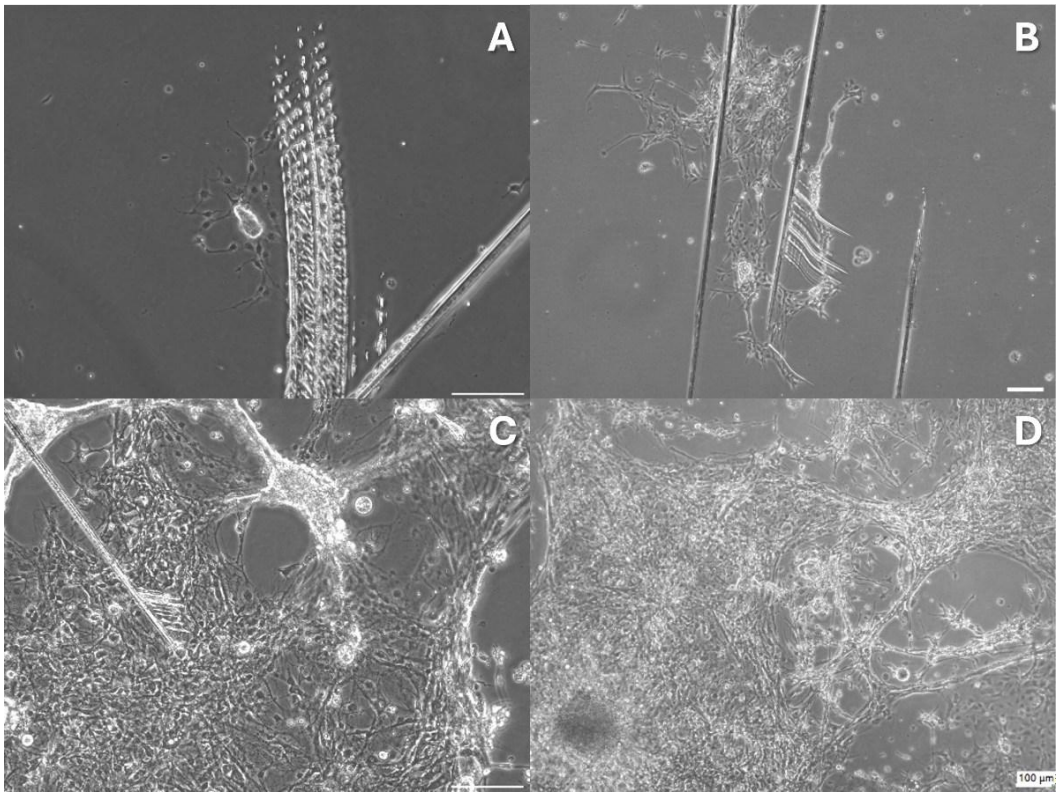


Fig 1. *Brightfield images at different time points post-isolation of primary cells isolated from T. molitor embryos via egg dissection.* Representative images show cellular morphology at day 1 (**A**), where cells exhibit an elongated shape indicative of initial adhesion and spreading. Over time (day 5, **B**; day 19, **C**; day 43, **D**) cells proliferate and form larger multicellular clusters. Spontaneous contractions were detected across multiple regions of the culture, persisting over several days. Scale bar: 100 μm.

Comparable results were obtained when cells were isolated via embryo homogenization. In this method, *T. molitor* eggs were homogenized and the resulting suspension was subjected to centrifugation, yielding two distinct fractions: a pellet and a supernatant. Both fractions were subsequently plated, and Figure 2 and Figure 3 illustrate the cell populations derived from each condition. Figure 2 corresponding to Passage 0, Passage 3, and Passage 5 of pellet-derived cultures depict a progressive increase in cell adhesion, proliferation, and cluster formation over time. In contrast, cells derived from the supernatant (Figure 3) exhibited distinct and highly complex morphological features. These cells appeared significantly larger and more elongated than those in pellet-derived cultures, with prominent branching structures extending in multiple directions. Over an extended incubation period (~1 month), the supernatant-derived cells formed an intricate, interconnected network (Figure 3). Additionally, the arrangement of these cells was not confined to a single plane; instead, some cells overlapped others within the imaging field, producing a stratified appearance. This organization was notably distinct from the compact clusters observed in pellet-derived cultures, indicating potential differences in adhesion properties and cellular interactions between the two fractions.

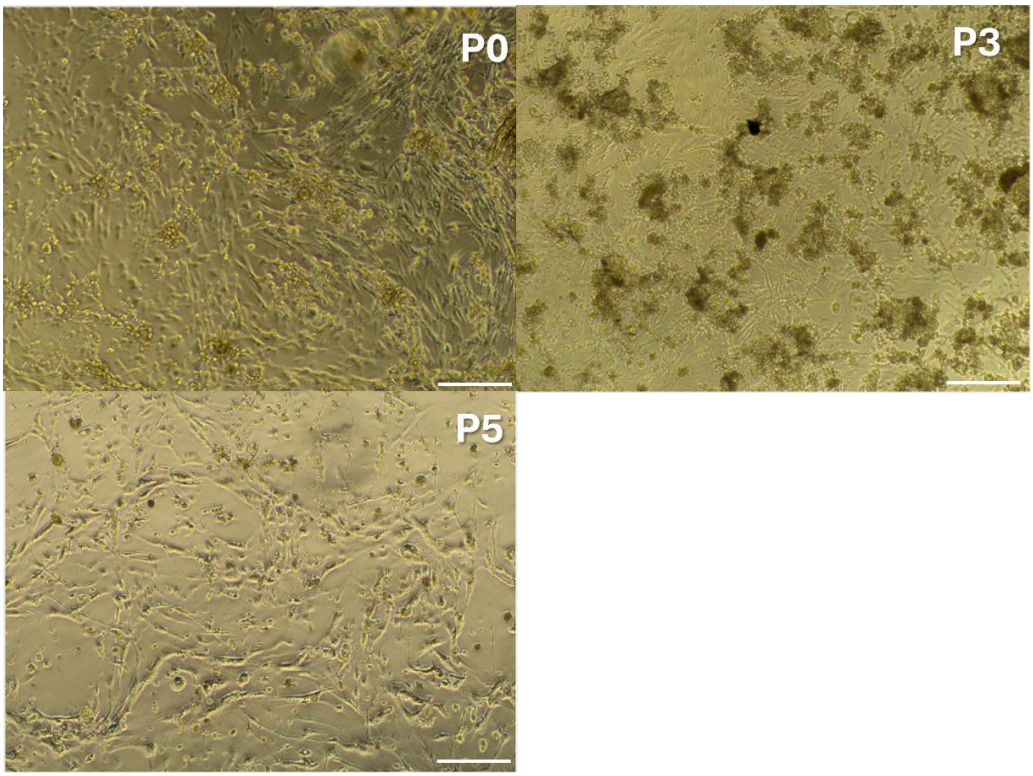


Fig 2. *Representative brightfield images of pellet-derived cells after homogenization at Passage 0 (P0), Passage 3 (P3), and Passage 5 (P5).* Scale bar: 190 µm.

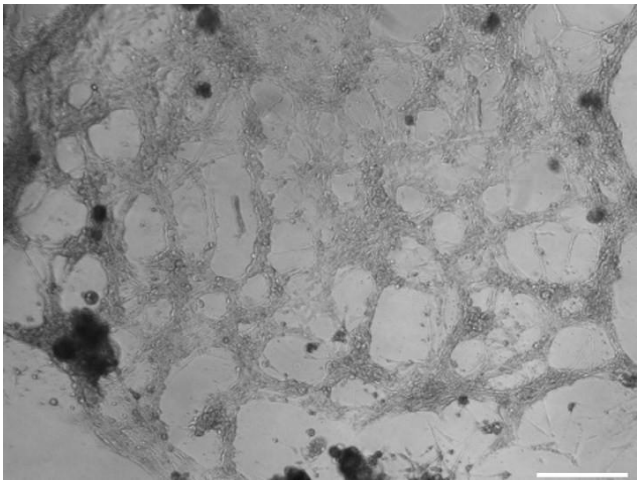


Fig 3. *Supernatant-derived cells after extended culture (~1 month)* form a highly interconnected, network-like structure with extensive cellular elongation and branching. Notably, these cells adopt a multi-layered organization. Scale bar: 190 µm.

3.2 Neonate-larvae cell isolation

Mechanical cutting of neonate larvae followed by direct plating rapidly produced a pronounced darkening of the culture medium within hours (data not shown). This discoloration is consistent with activation of the insect melanization cascade: tissue damage and microbial cues can trigger hemolymph phenoloxidases to oxidize catechols to quinones, which polymerize into melanin, yielding the characteristic brown-black pigments (Binggeli *et al.,* 2014). Under brightfield microscopy, no attached cells were detected in cut-larva preparations, and bacterial contamination became evident (data not shown). Homogenization of larvae produced a comparable medium darkening in the first supernatant (SN1), again in line with hemolymph-driven melanization (Figure 4A). In contrast, the second supernatant (SN2) obtained after re-centrifugation, as well as the pellet (P) fraction showed markedly reduced pigmentation (Figure 4B–C). Across all homogenized fractions, brightfield imaging revealed abundant particulate material and microbial contamination; no attached cells were observed in SN1 or SN2. The pellet (P) was the only condition in which occasional adherent elements were noted, yet these remained sparse and were surrounded by substantial debris and bacteria.

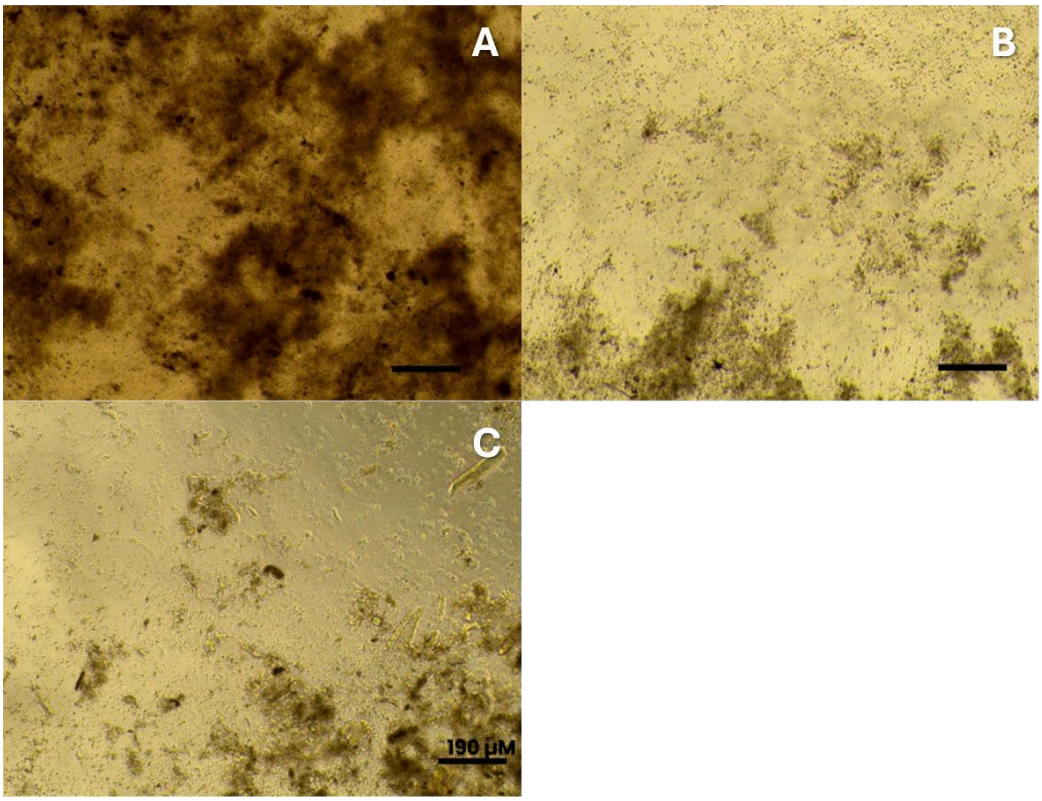


Fig 4. *Brightfield images of fractions derived from homogenized T. molitor neonate larvae.* (A) First supernatant (SN1) showing intense black discoloration compatible with hemolymph melanization. (B) Second supernatant (SN2) after re-centrifugation with visibly reduced pigmentation. (C) Pellet fraction (P) with decreased background darkening and abundant debris; only rare adherent elements were observed. Scale bar: 190 µm.

Phase-contrast imaging of the first enzymatic screen (Figure 5) showed that all conditions yielded suspensions dominated by particulate material, with frequent bacterial contaminants and only rare putative cells. The lowest trypsin concentration (0.05%) for 30 min (Figure 5 T1C1) occasionally revealed isolated, phase-bright cellular profiles (black arrows), but these were infrequent and did not progress to visible attachment after 1 h (Figure 5 T1C2). Trypsin–EDTA at 0.25% for 30–60 min generated fields largely composed of debris with scattered refractile bodies lacking clear signs of spreading (Figure 5 T1-C3, T1-C4). Collagenase I at 0.015% produced sparse, small, rounded elements after 1 h (Figure 5 T1-C5); extending exposure to 4 h increased the load of fragmented

tissue and pigment-like granules without improving the appearance of adherent cells (Figure 5 T1-C6). Across all conditions in Trial 1, no continuous adherent layer was observed and microbial contamination remained evident.

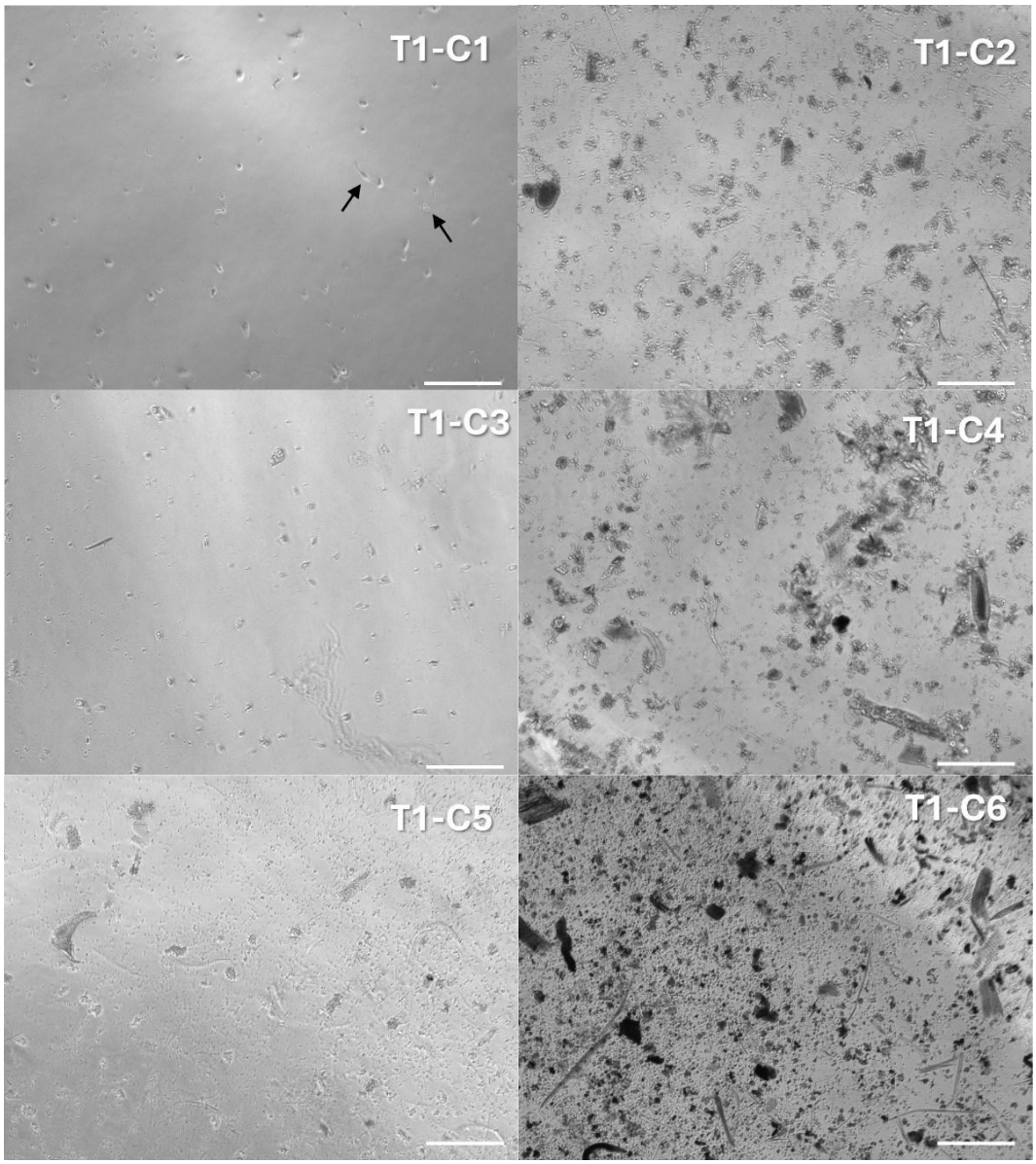


Fig 5. *Brightfield images of primary cells derived from T. molitor newly hatched larvae following enzymatic dissociation (Trial 1).* Images acquired 4 days after plating. Panels are labeled with the Trial–Condition codes corresponding to the enzyme and exposure time used: **T1–C1**, trypsin–EDTA 0.05% for 30 min; **T1–C2**, trypsin–EDTA 0.05% for 1 h; **T1–C3**, trypsin–EDTA 0.25% for 30 min; **T1–C4**, trypsin–EDTA 0.25% for 1 h; **T1–C5**, collagenase type I 0.015% for 1 h; **T1–C6**, collagenase type I 0.015% for 4 h. Black arrows in T1–C1 indicate sporadic adherent cells. Scale bar: 190 µm.

A second screen with prolonged exposures and higher enzyme concentrations (trial 2, Figure 6) similarly failed to produce stable adherent cell cultures. Trypsin–EDTA at 0.05% for 1 h or overnight (≈17 h) (Figure 6 T2-C1 and T2-C2) resulted in dilute suspensions where isolated cell-like elements could be found (black arrow in the figures), particularly after the overnight treatment, but the background remained dominated by debris and bacteria, and no early signs of cell spreading were detected. Trypsin–EDTA at 0.25% (1 h or overnight) did not improve yield or attachment relative to 0.05% (Figure 6 T2-C3, T2-C4). Collagenase I at 0.5% for 1h or 2 h and at 2.5% for 1–2 h increased tissue fragmentation but did not enhance the recovery of viable, adherent cells (Figure 6 T2-C5, T2-C6, T2-C7, T2-C8); fields again showed particulate matter and microbial overgrowth. Taken together, the data indicate that (i) bacterial contamination and (ii) inefficient dissociation/viability under the tested proteolytic conditions were the principal barriers to establishing primary cultures from neonate larvae.

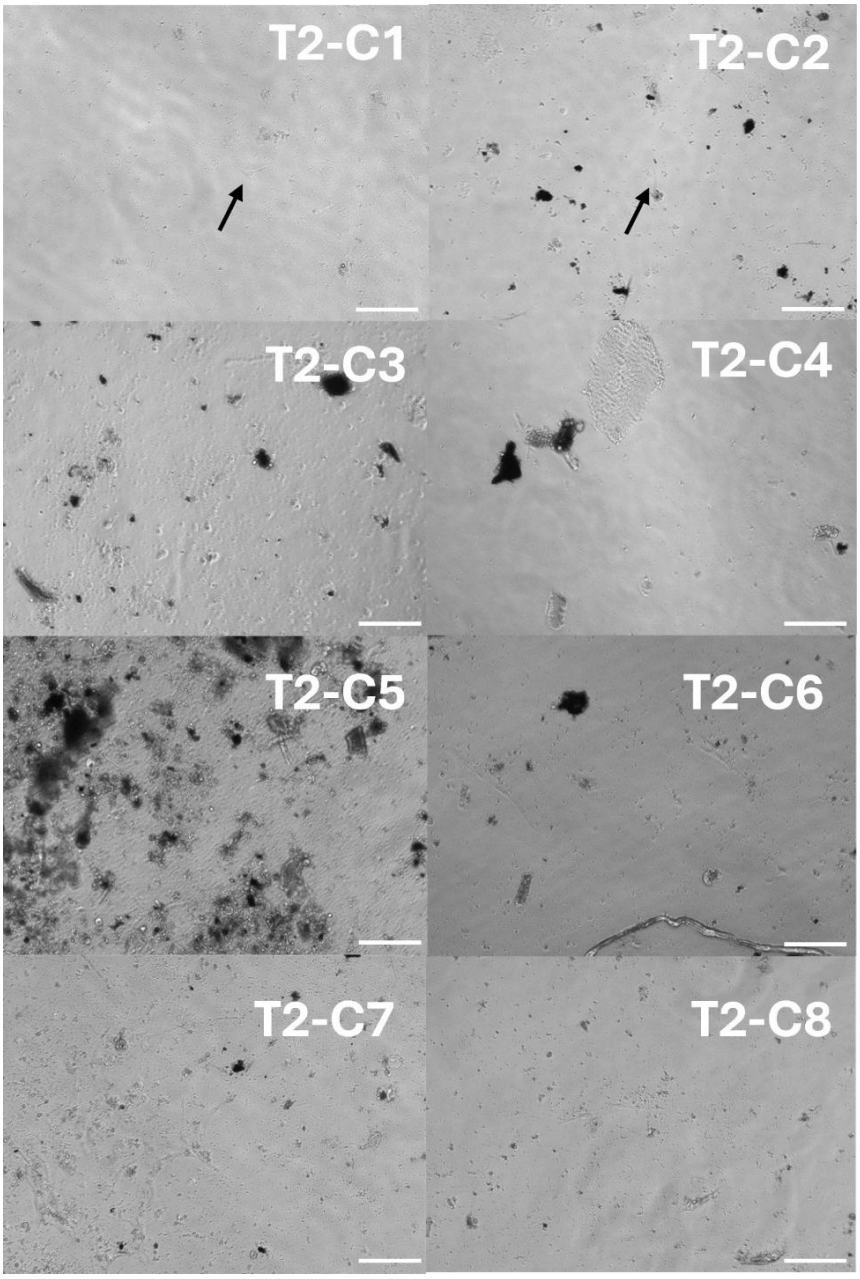


Fig 6. *Brightfield images of primary cells derived from T. molitor newly hatched larvae following enzymatic dissociation (Trial 2).* Images acquired 4 days after plating. Panels are labeled with the Trial–Condition codes corresponding to the enzyme and exposure time used: **T2–C1,** collagenase type I 0.5% for 1 h; **T2–C2**, collagenase type I 0.5% for 2 h; **T2–C3**, collagenase type I 2.5% for 1 h; **T2–C4**, collagenase type I 2.5% for 2 h; **T2–C5**, trypsin–EDTA 0.05% for 1 h; **T2–C6,** trypsin–EDTA 0.05% for ~17 h (overnight); **T2–C7**, trypsin–EDTA 0.25% for 1 h; **T2–C8**, trypsin–EDTA 0.25% for ~17 h (overnight). All fields are dominated by isolation debris and bacterial contaminants across conditions, and no continuous adherent layer is evident. Scale bar: 190 μm.

Across all approaches tested- enzymatic dissociation, mechanical cutting/mincing followed by incubation, and homogenization - cultures initiated from neonate larvae were terminated prematurely due to persistent bacterial contamination and lack of stable attachment. Cutting/mincing rapidly produced medium darkening consistent with hemolymph-driven melanization, and no adherent cells were detected. Homogenization yielded a similar browning in SN1, with reduced pigmentation in SN2 and pellet fractions after centrifugation, yet microbial overgrowth and abundant debris remained; only rare, non-spreading elements were seen in the pellet, insufficient to establish a culture. The failure to sterilize effectively is most plausibly related to the neonates' morphology: unlike later instars, their thin, incompletely sclerotized cuticle is highly susceptible to mechanical damage during rinsing and surface disinfection. To avoid injury, extensive washing could not be performed, likely leaving surface contaminants in place. Holding neonates in 1X PBS supplemented with A/A prior to sterilization did not mitigate contamination and coincided with rapid tissue deterioration, consistent with autolysis and/or osmotic imbalance. Because the gut is a major microbial source, a short fasting period was tested to reduce intestinal load; however, this strategy caused high mortality and structural compromise among survivors. Taken together, these observations indicate that, under the employed conditions, neither enzymatic dissociation nor mechanical cutting nor homogenization produced viable, contamination-free primary cultures from *T. molitor* neonates; bacterial overgrowth and compromised tissue integrity were the principal barriers.

3.3 EGTA- treatment for muscle cell enrichment

Across adherent fractions treated with increasing EGTA (1–5 mM), MHC^{+}

myotubes were detectable in all conditions (representative fields in the Figure 7). The fusion index (FI) displayed a modest, dose-related upward trend but no statistically significant differences among groups. Mean FI (± SEM, n = 3) was 8.17 ± 1.40% at 1 mM, 8.30 ± 2.06% at 3 mM, and 9.02 ± 3.38% at 5 mM (Figure 8).

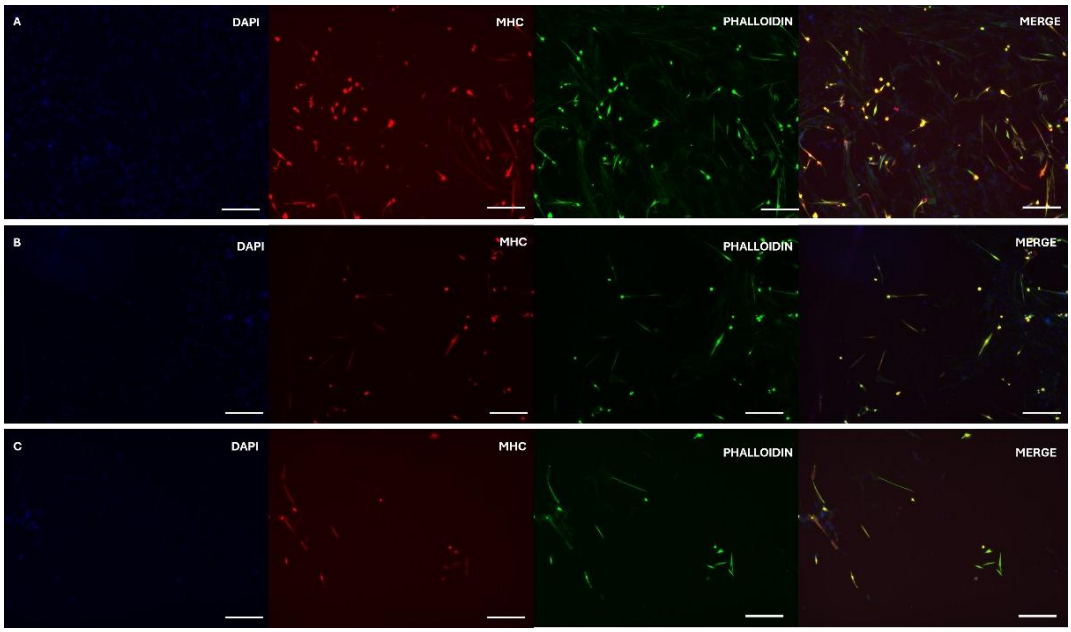


Fig 7. *Adherent cell fractions after EGTA-mediated selective adhesion.* Representative immunofluorescence fields of adherent cultures exposed to 1 mM (*row **A***), 3 mM (*row **B***), and 5 mM (*row **C**)* EGTA. Channels show per columns: DAPI (nuclei, blue), MHC (myosin heavy chain, red), Phalloidin (F-actin, green), and the merged image. Across conditions, MHC^{+} myotubes are evident. Scale bar: 100 µm.

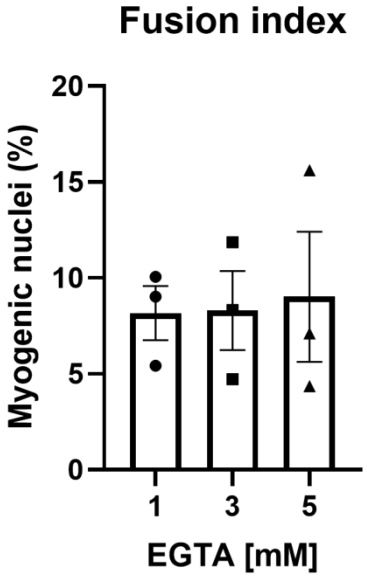


Fig 8. Fusion index of adherent cells after EGTA treatment. Bar graph showing the fusion index (%) of adherent cells exposed to 1, 3, and 5 mM EGTA. Bars represent mean ± SEM (n = 3 biological replicates per condition; individual data points are plotted for each biological replicate. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparisons test (GraphPad Prism 8.4.2); no significant differences were detected between conditions (1 vs 3 mM, p = 0.9855; 1 vs 5 mM, p = 0.9334; 3 vs 5 mM, p = 0.8935).

In parallel, the mean number of total DAPI$^+$ nuclei per field decreased with increasing EGTA - 778.7 at 1 mM, 500.7 at 3 mM (−35.7% vs 1 mM), and 135.7 at 5 mM (−82.6% vs 1 mM) - and the mean number of nuclei within MHC$^+$ myotubes declined from 66.3 to 38.7 to 11.7, respectively. Collectively, these data suggest that while FI tends to rise as EGTA concentration increases, this effect is not statistically significant ($p > 0.05$) and may partly reflect the sharper reduction in overall cell number rather than a clear enhancement of myogenic fusion under the conditions tested.

To contextualize the selective-adhesion step, we examined representative immunostained fields from non-adherent cultures (medium transferred at 2 h and re-plated) alongside their adherent counterparts. Non-adherent cultures displayed

greater morphological heterogeneity, with diffuse and discontinuous MHC labeling distributed in scattered foci and fewer organized multinucleated structures (Figure 9). In contrast, adherent cultures showed more orderly myogenic features, including elongated, aligned fibers with clearer MHC signal and nascent multinucleated myotubes. Overall, these observations are consistent with the EGTA-based adhesion step preferentially retaining cells with higher myogenic potential, while the non-adherent fraction remains comparatively heterogeneous.

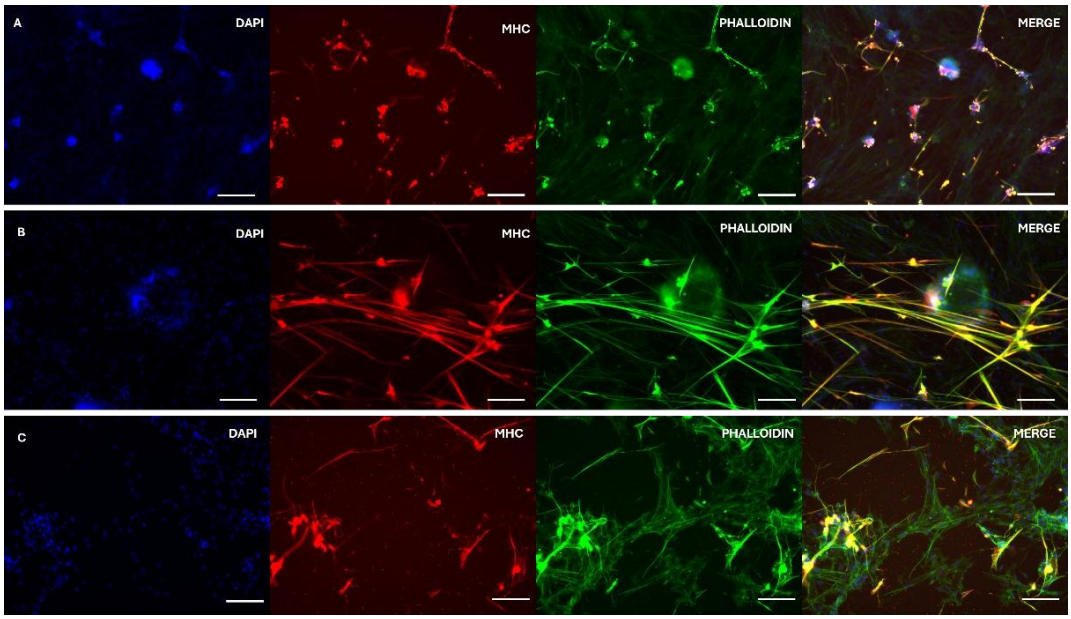


Fig 9. *Non-adherent cell fractions after EGTA selective adhesion.* Representative immunofluorescence fields of non-adherent (replated supernatant collected at 2 h) cultures exposed to 1 mM (*row **A***), 3 mM (*row **B***), and 5 mM (*row **C***) EGTA. Channels shown per column: *DAPI* (nuclei, blue), *MHC* (myosin heavy chain, red), *Phalloidin* (F-actin, green), and merged image. Non-adherent cultures exhibit pronounced morphological heterogeneity with diffuse/discontinuous MHC labeling in scattered foci and prominent phalloidin-positive networks, with limited organized multinucleated MHC^{+} structures. Scale bar: 100 µm.

3.4 Adhesion-time pre-plating assay

Adhesion-time pre-plating produced clear and time-dependent differences between adherent (A) and non-adherent (NA) fractions, with the NA wells consistently showing a visibly higher overall cell yield than their time-matched A counterparts (Figure 10). At 10 min only the NA fraction was captured and already contained numerous elongated MHC-positive profiles (red) with variable phalloidin signal (green), indicating that many putative mesodermal cells had not yet secured firm attachment within this short window. At 1 h, adherent cells contained sparse, elongated MHC^+ cells with occasional red–green overlap, whereas 1 h-NA displayed many long cells dominated by red signal with little or no detectable phalloidin (i.e., MHC-dominant fibers) suggesting assembly or stabilization of myosin-rich contractile structures in cells that remained in suspension. The 2 h condition yielded the most ordered adherent fields: 2 h-A showed long, aligned MHC^+/$phalloidin^+$ fibers and occasional multinuclear profiles, whereas 2 h-NA contained abundant elongated cells and, notably, several elements with clear periodic red banding consistent with nascent sarcomeric striation, underscoring that a sizeable myogenic-like subset still resided in the non-adherent pool at this time. At 3 h, adherent cells appeared comparatively depleted with fewer elongated profiles, while 3 h-NA retained a moderate density of MHC^+ elongated cells. These qualitative readouts indicate that (i) NA fractions are more populated overall across timepoints, (ii) 1–2 h windows capture many elongated, MHC-dominant cells in NA, including striated elements at 2 h, and (iii) among adherent wells, 2 h provides the clearest organization of attached myogenic-like fibers. Given the limited number of analyzable images per condition, no fusion index is reported; these findings are presented as a preliminary qualitative assessment to guide a confirmatory

experiment with quantitative endpoints.

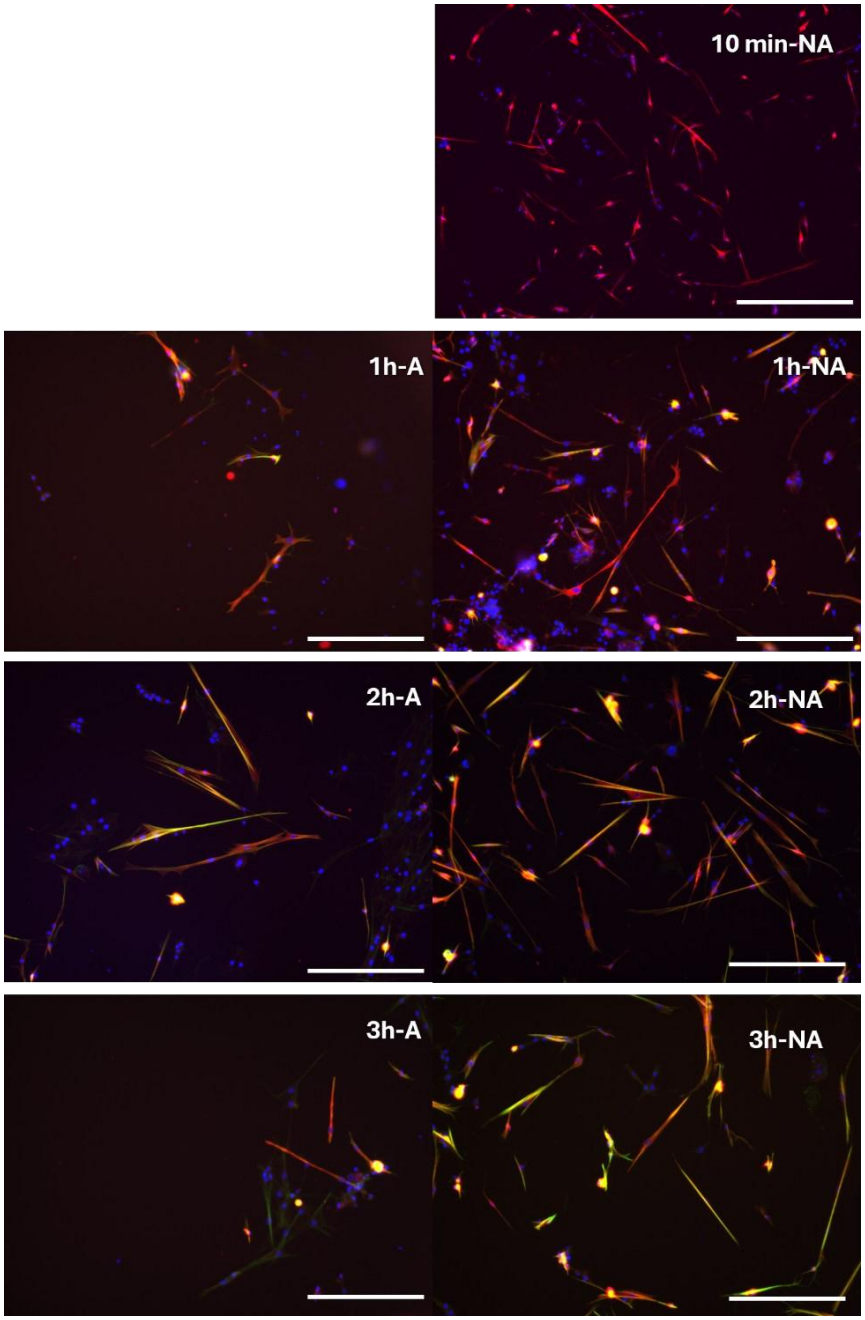


Fig 10 *Adhesion-time pre-plating separates adherent (A) and non-adherent (NA) fractions.* Representative immunofluorescence fields stained for MHC (MAC 147,red), F-actin (Phalloidin, green), and nuclei (Dapi,blue). Scale bar:200 μm.

3.5 Immunostaining

The immunostaining results in primary *T. molitor* cultures highlight both the promise and challenges of applying antibodies from model insects to a new

species. Notably, two antibodies against myosin heavy chain (MHC) identified distinct myogenic cells (MAC 147 and 3E8-3D3) whereas several other developmental markers failed to produce specific labeling. Below we discuss these findings. Immunofluorescence of primary *T. molitor* cultures revealed myogenic features (Figure 11). Phalloidin–Alexa Fluor 488 delineated thick, parallel F-actin bundles in elongated, substrate-adherent cells, and the anti–myosin heavy chain antibody MAC 147 (ab51098) produced a distinct cytoplasmic signal in cells with a filamentous cytoskeleton. In these cells, MHC labeling co-aligned with actin bundles and occasionally appeared banded; where channels overlapped the merge yielded yellow profiles, a pattern compatible with early myofibrillar (sarcomeric- like) organization, although not sufficient to claim definitive sarcomere resolution. DAPI staining showed both isolated nuclei and small multicellular arrays aligned along actin-rich tracts, with MHC^{+} cells occurring as solitary elements or within these organized assemblies.

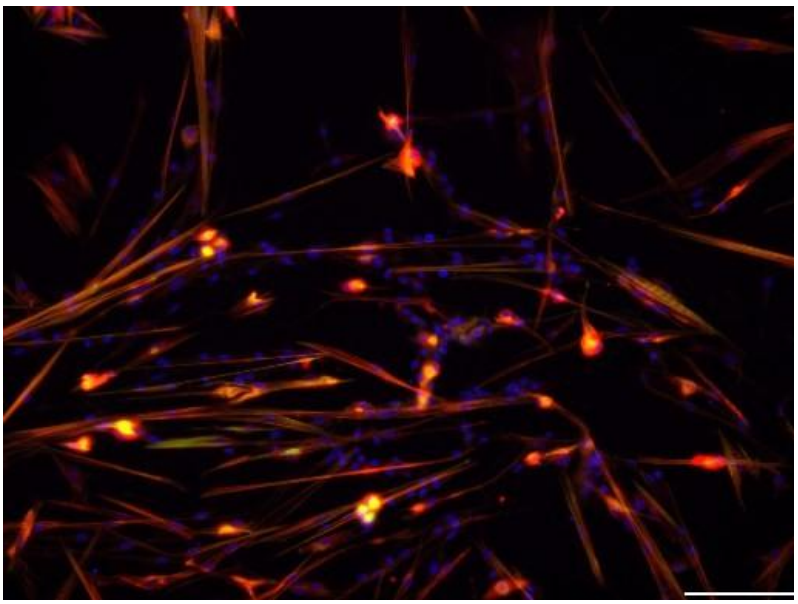


Fig 11. *Immunostaining in primary T. molitor cultures*. Phalloidin–Alexa Fluor 488 (green) labels F-actin; anti-MHC MAC147 (Abcam ab51098) (red); DAPI (blue). Scale bar: 200 μm.

To distinguish different cell types and to identify additional markers suitable for

T. molitor, different antibodies (10F1, 9B9, FP6.87, 3B11, and 3E8-3D3) were screened using DAPI as the only counterstain. The secondary-only control showed no detectable signal, indicating negligible sample autofluorescence and minimal background from the secondary antibody (*Appendix Figure A3*). Under these baseline conditions, EcR (10F1, 9B9), Ubx/Abd-A (FP6.87), and Fas-I (3B11) produced diffuse fluorescence that did not resolve subcellular structures or cell- type–restricted patterns (representative field in Figure 12), consistent with non- specific staining rather than antigen-specific labeling.

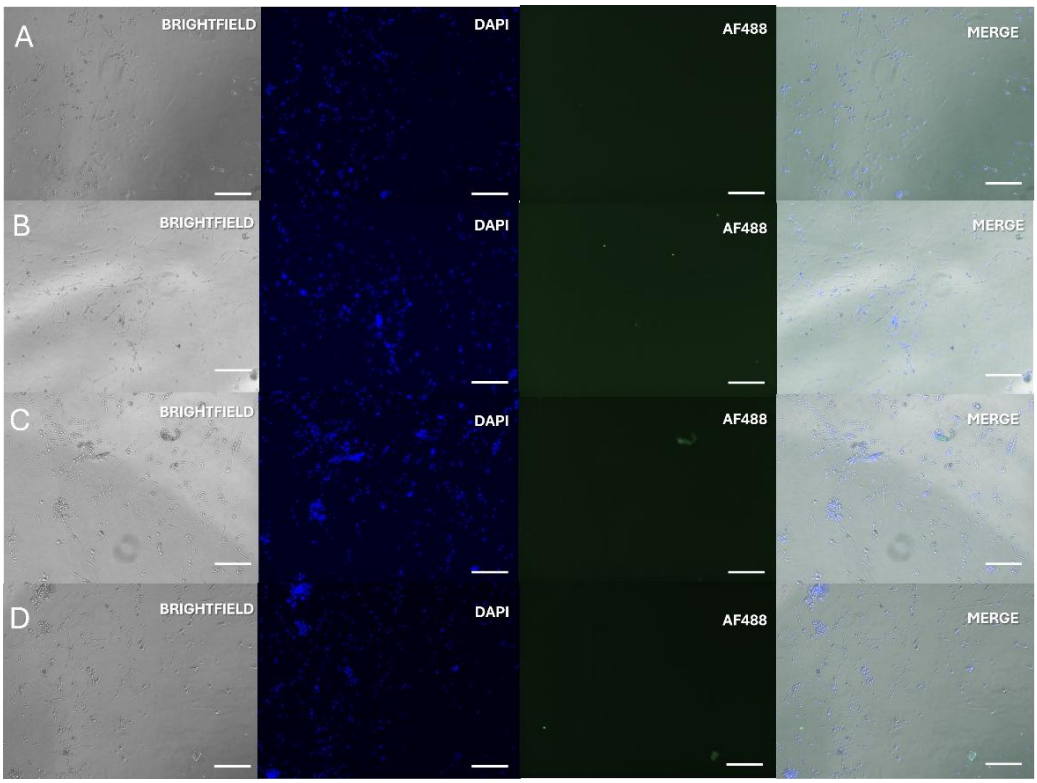


Fig 12. *Exploratory immunostaining in primary T. molitor cultures.* Each row shows one primary antibody*: Row* ***A*****:** 3B11 (1:50). *Row* ***B****:* EcR, 9B9 (1:100). Row **C**: 10F1 (1:1000), *Row* ***D****:* FP6.87 (1:500). Channels show per columns: phase- contrast, *DAPI* (nuclei, blue), *AF488* channel (secondary antibody used A28175, green, 1:1000), and merge. No subcellular or cell-type–restricted AF488 patterns were observed; signals were comparable to the secondary-only baseline (see *Appendix Figure A3*). Scale bars: 100 µm. *Note: During the exploratory screen, no phalloidin was used; only DAPI was included as a nuclear counterstain. Acquisition was restricted to the AF488 channel (secondary) plus DAPI.*

In contrast, the anti–myosin heavy chain antibody 3E8-3D3 yielded discrete labeling in a subset of elongated, adherent cells (Fig. 13). For clarity, only representative images are displayed; full dilution series and control are available in *Appendix Figure A3.* The 3E8-3D3 signal traced fiber-like profiles and was spatially associated with aligned nuclei, a distribution compatible with early myofibrillar organization, although clear sarcomeric banding was not consistently resolved (Figure 13). Taken together, these observations provide preliminary evidence that 3E8-3D3 may serve as a myogenic probe in *T. molitor* under the conditions used. Nevertheless, further validation will be required to confirm specificity and establish its routine utility, whereas the remaining candidates showed no convincing cross- reactivity.

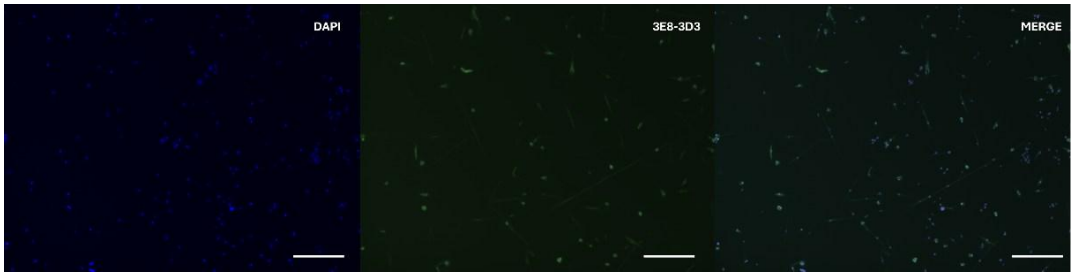


Fig 13. *Myosin heavy chain immunostaining with 3E8-3D3 in primary T. molitor cultures.* Left - right: DAPI (nuclei, blue), AF488 channel detecting mouse anti-MHC 3E8-3D3 (green; 1:100), and merge. A subset of elongated cells shows discrete, fiber-like 3E8-3D3 labeling spatially associated with aligned nuclei, a distribution compatible with early myofibrillar organization. Secondary antibody: goat anti-mouse IgG (H+L)–AF488 (1:1000). Scale bars: 100 µm.

To further evaluate myosin expression, a co-staining with the rat anti–myosin heavy chain MAC147 (red) and the mouse anti–myosin heavy chain 3E8-3D3 (green) was performed, with DAPI counterstain. In merged images (Figure 14), a subset of elongated, substrate-adherent cells showed co-expression of both markers (yellow signal where channels overlapped), whereas additional cells stained predominantly for either MAC147 or 3E8-3D3. Labeling in double-

positive cells followed fiber- like profiles, consistent with a myogenic architecture. Control preparations processed without secondary antibody showed no detectable fluorescence in either channel used for immunodetection, while nuclei (DAPI) and cellular morphology in phase contrast were clearly visible (*Appendix Figure A4*). This confirms that the signals reported for 3E8-3D3 (and the absence thereof for the other primaries) are not attributable to intrinsic sample autofluorescence or optical bleed-through. The partial, non-exclusive overlap between MAC147 and 3E8-3D3 suggests heterogeneity within the myosin-expressing compartment - potentially reflecting distinct differentiation states, myosin isoform composition, or differences in epitope accessibility/fixation. These observations are compatible with the presence of discrete myogenic subpopulations in *T. molitor* cultures.

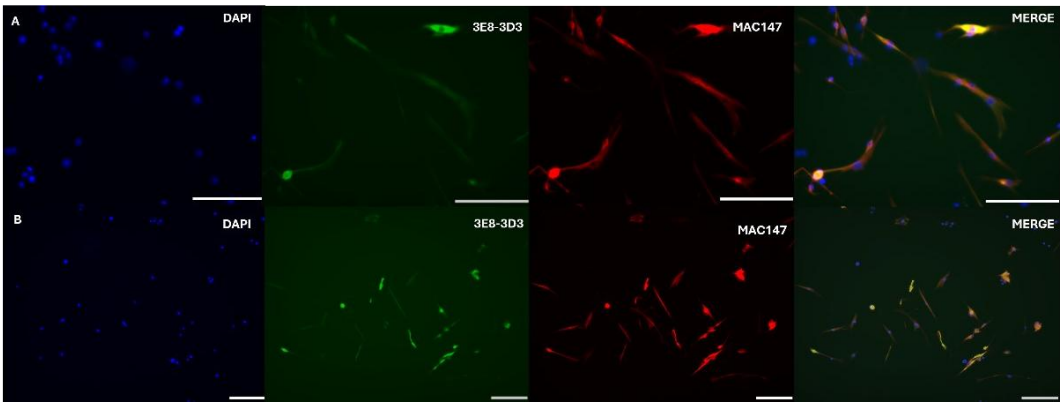


Fig 14 *Co-staining for myosin heavy chain in primary T. molitor cultures*. Left -> right: DAPI (nuclei, blue), 3E8-3D3 (green), MAC147 (red), and merge. *Row **A**:* 3E8-3D3 used at 1:100 (elongated, adherent cells display fiber-like labeling, with partial overlap (yellow) in a subset of cells. Scale bars: 500 µm. *Row **B**:* 3E8-3D3 used at 1:50, showing comparable patterns at the indicated settings. Scale bars: 100 µm. No-secondary controls were negative under identical acquisition parameters (*Appendix Figure A4*)

4. DISCUSSION

Primary cultures from *T. molitor* embryos were reproducibly initiated by both egg dissection and homogenization, yielding heterogeneous populations that

remained viable for weeks and frequently exhibited intermittent spontaneous contractions. Such contractile activity in early mixed insect primary cultures is widely interpreted as consistent with the presence of muscle-like cells, although definitive lineage attribution requires orthogonal validation (e.g., myogenic markers, functional assays) (Sugimoto *et al.,* 2022; Rubio *et al.,* 2019). Notably, spontaneous contractility has been reported across insect primary systems and aligns with the expectation that embryonic isolates can include myocytes among other lineages; dynamic morphological changes observed here are therefore compatible with mixed fates and early myogenic potential (Knežić *et al.,* 2024; Churchill & Caveney, 1993; Lee *et al.,* 1999). A practical distinction emerged between post-homogenization fractions: pellet-derived cultures showed higher apparent yield and faster expansion, whereas supernatant-derived cultures developed larger, elongated, and occasionally ramified morphologies over prolonged culture. This pattern is mechanistically congruent with density-based partitioning, whereby low-speed centrifugation tends to enrich intact cells in the pellet while leaving a greater proportion of fine debris and microbes in the supernatant (Harwood, 1974; Tamir & Gilvarg, 1966). In contrast, none of the neonate-larval approaches (mechanical cutting with direct plating, homogenization, and two enzymatic screens) produced stable adherent cultures under the conditions tested. Two recurring barriers were observed. First, rapid medium darkening - most pronounced in the first supernatant (SN1) - is compatible with activation of the hemolymph prophenoloxidase cascade, which generates quinones/melanin and reactive intermediates known to damage surrounding cells (Lee *et al.,* 1999). The reduced darkening after re-centrifugation (SN2, pellet) suggests partial removal of hemolymph components yet was insufficient to enable robust attachment. Second, persistent microbial

overgrowth is consistent with the documented microbial ecology of yellow mealworms, where high loads reside on the cuticle and in the midgut; fasting and brief heating can reduce, but not abolish bacterial contamination, may impose additional stress if over-applied (Grau *et al.,* 2017; Moruzzo *et al.,* 2021). Enzymatic dissociation parameters likely compounded these issues, both in trial 1 and in trial 2. Comparative work on lepidopteran tissues indicates that collagenase can affect complete dissociation with limited structural damage, whereas trypsin is often incomplete; critically, even "milder" proteolysis can impair post-isolation attachment if concentration or exposure are not precisely bounded (Goodwin, 1975; Murhammer, 2016; Byeun *et al.,* 2022). Methodological parallels from fragile mammalian preparations reinforce the need for tight control of enzyme identity, dose, exposure time, osmolarity, and immediate quenching/debris removal to preserve attachment competence (Yoshioka *et al.,* 2020). Likewise, protocols for delicate larval tissues emphasize very brief, carefully ordered steps to avoid over- digestion and reduce adherent contaminants - principles consistent with the sensitivity observed here (Beňová-Liszeková *et al.,* 2019). Culture media and supplementation were adequate for embryo-derived cells. Shields & Sang's M3 supplemented with bactopeptone, yeast extract, and 20% FBS supported survival and expansion, in line with long-standing use of M3-like formulations across insect taxa for initiating and maintaining primary cultures (Goodwin, 1975; Lynn, 2001; Murhammer, 2016). The difficulty converting to continuous growth is also coherent with the continuous coleopteran lines are comparatively rare and often demand repeated isolations and lengthy empirical optimization; by comparison, successful continuous lines from larval midguts in Lepidoptera have typically required carefully tuned collagenase protocols and medium systems (Sugimoto *et al.,*

2022; Charpentier *et al.,* 2002; Hoshino *et al.,* 2009; Zhang *et al.,* 2015). EGTA-based differential adhesion was explored here as a strategy to enrich myogenic precursors from *T. molitor* embryonic primary cultures. Our qualitative partitioning - more ordered MHC^{+} structures in adherent fractions and greater heterogeneity in non-adherent fractions - is consistent with the original rationale for EGTA pre-plating in insect embryos, where chelating extracellular Ca^{2+} weakens cell–substrate interactions and, preferentially retains myogenic cells (Bernstein *et al.,* 1978; Fyrberg & Donady, 1979). Quantitatively, we observed a modest, dose- related rise of the fusion index (muscle purity) across 1–5 mM EGTA that did not reach significance, together with a monotonic decline in total nuclei in both adherent and non-adherent wells. The drop in yields at higher EGTA is in line with classic *Drosophila melanogaster* data showing that, although EGTA increases myogenic enrichment, too much chelation (and associated shear) weakens even myoblast adhesion and reduces recoverable myoblasts (Bernstein *et al.,* 1978; Fyrberg & Donady, 1979). Mechanistically, an EGTA-dependent fall in cell numbers is expected: extracellular Ca^{2+} supports integrin-mediated adhesion, cytoskeletal organization, and membrane resealing; chelation reduces adhesion strength and renders cells more sensitive to shear or handling (Bernstein *et al.,* 1978). Moreover, work in Lepidopteran systems demonstrates that altering Ca^{2+} availability reprograms proliferation and differentiation of insect cells: decreasing Ca^{2+} with EGTA promotes proliferation and specific lineage choices, whereas elevating Ca^{2+} or facilitating Ca^{2+} influx drives alternative phenotypes (Loeb, 2005). These Ca^{2+}- dependent effects on adhesion and fate offer a plausible basis for the reduced counts at 5 mM EGTA. Comparison to related insect muscle systems reinforces these interpretations. In *Manduca sexta* embryo

cultures, EGTA is explicitly included at initial plating to bias early attachment, after which differentiation is enhanced by endocrine cues such as 20-hydroxyecdysone (Baryshyan *et al.,* 2012). MHC^{+} myotubes formed at all doses, suggesting that selective adhesion worked qualitatively but that maximizing fusion will likely require (i) more refined EGTA concentration within the 2–4 mM range and shorter adhesion windows (30–120 min), and/or (ii) post-enrichment differentiation cues validated for insect myogenesis (Baryshyan *et al.,* 2012; Bernstein *et al.,* 1978). Finally, our non-adherent images align with the literature's expectation that the rinsed fraction retains suspension-prone, non-myogenic populations (e.g., neurons, imaginal cells), whereas adherent fractions concentrate the myogenic lineage after EGTA pre-plating (Bernstein *et al.,* 1978). Taken together, these data indicate that the core mechanism established in Drosophila - EGTA-assisted, time-limited pre- adhesion - translates to *T. molitor,* but species-specific adhesion biology and the absence of pro-differentiation cues likely underlie the modest, non-significant FI gains we observed. Future optimization should therefore balance EGTA dose and adhesion time to preserve yield while adding stage-appropriate differentiation drivers once an enriched adherent fraction is secured. About adhesion-time pre-plating produced consistent, time-dependent partitioning of the culture. Conceptually, this is aligned with the central premise of pre-plating, that distinct cell types differ in adhesion kinetics and substrate preferences, long exploited in vertebrate systems to enrich myogenic cells by controlling attachment time and surface chemistry (e.g., gelatin, collagen, laminin). In those models, early adhering fibroblasts are depleted while more slowly adhering myoblasts are recovered in later fractions (Shahini *et al.,* 2018; Park *et al.,* 2006). Our data suggest that in *T. molitor,* cells displaying a mesodermal, muscle-biased pattern

exhibit delay, accounting for the larger overall cell yield in NA and the more ordered myogenic organization observed in A at intermediate windows, particularly at 2 h. The presence of elongated cells with clearly banded striated signals are consistent with early myofibrillogenesis in which myosin assemblies and Z-disk components can adopt periodic organization before full F-actin polarity sorting and robust filament bundling become evident by phalloidin staining. This implies that assessing NA is not merely a "waste check" but an informative window on differentiation dynamics (Rui *et al.,* 2010; Spletter *et al.,* 2018; Kolley *et al.,* 2024). Immunofluorescence identified a myogenic subpopulation in *T. molitor* primary cultures. The antibody 3E8-3D3 labeled a subset of elongated cells with fibrillar profiles and aligned nuclei, consistent with its use in *D. melanogaster* to visualize myofibrillar organization and actomyosin alterations associated with contractile dysfunction (Dahl-Halvarsson *et al.,* 2020; Birker *et al.,* 2023). In parallel, MAC147 produced a filamentous cytoplasmic signal that was occasionally weakly striated; the clone is reported to react with the subfragment-2 (S2) of myosin II in *Drosophila* and *Lethocerus*, in line with the band-forming patterns we observed (Abcam, n.d.; Szikora *et al.,* 2020). The partial overlap between 3E8-3D3 and MAC147 is compatible with heterogeneity within the MHC-positive compartment (epitope accessibility, maturation state, isoform composition); in *D. melanogaster* a single Mhc gene generates many isoforms via alternative splicing of exon cassettes, with distribution dependent on muscle type and stage, providing a comparative rationale for differences in immunoreactivity (Collier *et al.,* 1990; Hastings & Emerson, 1991; Spletter & Schnorrer, 2014). Conversely, antibodies against EcR (10F1, 9B9), Hox Ubx/Abd-A (FP6.87), and Fasciclin-I (3B11) did not yield specific patterns: FP6.87 and 3B11 are typically effective in embryonic/larval

tissues with strong spatial organization (Bastiani *et al.,* 1987; Kelsh *et al.,* 1994), whereas EcR shows dynamic expression and fixation sensitivity, with variable nuclear/cytoplasmic localization (Vafopoulou, 2012; Vafopoulou *et al.,* 2005). These outcomes, together with known limits of cross-species validation, argue for cautious interpretation and targeted confirmation. In summary, 3E8-3D3 emerges as a promising myogenic marker in *T. molitor* (with partial overlap with MAC147), whereas EcR/Ubx-Abd-A/Fas-I were not informative under the conditions employed. From the perspective of cultivated-meat motivations, the embryo-derived cultures established here meet two early R&D criteria highlighted in the entomoculture literature: reproducible initiation of viable primary cultures and evidence compatible with myogenic potential (i.e., contractility) (Rubio *et al.,* 2019; Ashizawa *et al.,* 2022; Seah *et al.,* 2022). Translation toward scalable *in vitro* insect muscle will likely require (i) contamination-resilient, embryo-focused isolation workflows, (ii) serum-reduced/serum-free media tailored to insect cells, and (iii) strategies for myogenic enrichment and maturation—priorities already emphasized across engineered insect-tissue roadmaps and broader cell-agriculture process design (Ng & Kurisawa, 2021; Stout *et al.,* 2022; O'Neill *et al.,* 2021; Bomkamp *et al.,* 2022).

5. CONCLUSION

Embryo-derived primary cultures of *Tenebrio molitor* are reproducibly established by egg dissection and homogenization, remain viable for weeks, and frequently display intermittent spontaneous contractions consistent with early myogenic potential. Post-homogenization fractions diverge: pellet-derived cultures expand more readily, whereas supernatant-derived cultures form larger,

elongated and occasionally ramified networks over prolonged culture, indicating fraction- dependent adhesion and growth behaviors. In contrast, neonate-larval protocols fail under the tested conditions, primarily due to hemolymph melanization (rapid medium darkening) and persistent microbial overgrowth, which together preclude stable attachment and expansion. As enrichment levers, EGTA-mediated differential adhesion and time-dependent pre-plating partition adherent vs. non- adherent populations and qualitatively bias adherent fractions toward myogenic organization. Immunofluorescence corroborates a myosin-positive subpopulation: 3E8-3D3 and MAC147 label elongated, fiber-like cells (with partial overlap), while “no-secondary” controls are negative, supporting signal specificity. Overall, the chapter delivers an embryo-based, contamination-aware workflow and clarifies practical boundaries for source material and sterilization, providing a tractable foundation for quantitative optimization (e.g., tuned EGTA in the 2–4 mM range and shorter adhesion windows), and maturation cues in future work.

CONCLUSIONS

In this thesis, insect cells are explored as a promising and potentially sustainable platform for next-generation protein biomanufacturing, with a particular focus on cultivated meat. At a conceptual level, the work outlines why insect cells may warrant consideration alongside mammalian systems: they tolerate broader culture conditions, exhibit flexible growth modes, and impose milder demands on media and environmental control, collectively pointing to favorable process economics and operational simplicity at scale. These features, taken together with existing literature, are consistent with the prospect of more favorable process economics and operational simplicity at scale, while also highlighting the boundaries within which insect-based platforms are likely to be most advantageous. At the food-systems interface, the thesis consolidates the role of *Hermetia illucens* (black soldier fly, BSF) across food, feed, pet nutrition, and cellular agriculture. It underscores the alignment between BSF's biological attributes, circular-economy valorization of organic residues, and evolving societal and regulatory expectations, while acknowledging that adoption depends on consumer acceptance, safety management, and fit-for-purpose regulatory pathways. This establishes translational guidelines: technological promise must be matched with risk controls, product design that reduces visual salience, and dossiers that meet novel-food requirements. Methodologically, the thesis advances two complementary primary-culture pipelines for insect myogenesis. In *H. illucens,* it identifies a developmentally informed window for initiating heterogeneous yet myogenesis-prone cultures and demonstrates that calcium chelation and time-dependent adhesion can bias early myogenic organization, with corroboration from morphology and molecular readouts. These findings define a tractable starting point for media minimization, maturation cues, and scaffold integration. In *Tenebrio molitor*, it establishes an embryo-derived

platform that displays sustained viability and intermittent contractility and confirms that EGTA-assisted selective adhesion and timed pre-plating can enrich for myogenic phenotypes; it also delineates constraints of neonate-larvae based isolation due to contamination, thereby mapping practical limits for source material and sterilization regimes. Taken together, the contributions are threefold: (i) a system-level rationale for insect cell platforms grounded in process tolerance and resource efficiency, (ii) species-tailored workflows that shift mixed primary cultures toward muscle-oriented phenotypes without reliance on granular genetic manipulation, and (iii) a translational frame spanning acceptance, safety, and regulation to guide product-ready development. These boundaries delineate a realistic corridor for progress while avoiding overgeneralization beyond demonstrated contexts. In conclusion, this thesis advances a coherent, scientific and translational case indicating the potential of insect cell platforms for sustainable protein production.

REFERENCES

Abd El-Wahab, A., Meyer, L., Kölln, M., Chuppava, B., Wilke, V., Visscher, C., & Kamphues, J. (2021). Insect larvae meal (*Hermetia illucens*) as a sustainable protein source of canine food and its impacts on nutrient digestibility and fecal quality. Animals, 11, 2525. https://doi.org/10.3390/ani11092525

Akiduki, G., & Imanishi, S. (2007). Establishment of a lipid accumulation model in an insect cell line. Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America, 66(3), 109-121.

Akiyama, Y., Iwabuchi, K., Furukawa, Y., & Morishima, K. (2009). Long-term and room temperature operable bioactuator powered by insect dorsal vessel tissue. Lab on a Chip, 9(1), 140–144.

Akiyama, Y., Sakuma, T., Funakoshi, K., Hoshino, T., Iwabuchi, K., *et al.* (2013). Atmospheric- operable bioactuator powered by insect muscle packaged with medium. Lab on a Chip, 13, 4870–4880.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Molecular biology of the cell (4th ed.). New York: Garland Science.

Ardila, A., Escovar, J., & Bello, F. (2005). Características de nuevos cultivos celulares derivados de tejidos embrionarios de *Aedes aegypti* (Diptera: Culicidae). Biomédica (Colombia), 25(1), 65–75.

Arrese, E. L., & Soulages, J. L. (2010). Insect fat body: Energy, metabolism, and regulation. Annual Review of Entomology, 55, 207–225. https://doi.org/10.1146/ annurev-ento-112408- 085356

Arunkarthick, S., Asokan, R., Aravintharaj, R., Niveditha, M., & Kumar, N. K. (2017). A review of insect cell culture: Establishment, maintenance and applications in entomological research. Journal of Entomological Science, 52(3), 261–273. https://doi.org/10.18474/ JES17- 02PT.1

Ashizawa, R. N., Rubio, N. R., Letcher, S. M., Parkinson, A., Dmitruczyk, V., & Kaplan, D. L. (2022). Entomoculture: A preliminary techno-economic assessment. Foods, 11(19), 3037. https://doi.org/10.3390/foods11193037

Athanassiou, C. G., *et al.* (2024). Circular and inclusive utilization of alternative proteins: A European and Mediterranean perspective. Current Opinion in Green and Sustainable Chemistry, 46, 100892. https://doi.org/10.1016/j.cogsc.2024.100892

Barragán-Fonseca, K. B., Dicke, M., & van Loon, J. J. A. (2017). Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – A review. Journal of Insects as Food and Feed, 3(2), 105–120. https://doi.org/10.3920/JIFF2016.0055

Barre, A., Pichereaux, C., Velazquez, E., Maudouit, A., Simplicien, M., Garnier, L., Rougé, P. (2019). Insights into the allergenic potential of the edible Yellow mealworm (*Tenebrio molitor*). Foods, 8, 515. https://doi.org/10.3390/foods8100515

Barroso, F. G., de Haro, C., Sánchez-Muros, M.-J., Venegas, E., Martínez-Sánchez, A., & Pérez- Bañón, C. (2017). Insects as food: Nutritional and functional aspects. Innovative Food Science & Emerging Technologies, 41, 8–18. https://doi.org/10.1016/j.ifset.2017.03.001

Barroso, F. G., Sánchez-Muros, M.-J., Segura, M., Morote, E., Torres, A., Ramos, R., & Guil, J.L. (2017). Insects as food: Enrichment of larvae of *Hermetia illucens* with omega-3 fatty acids by means of dietary modifications. Journal of Food Composition and Analysis, 62, 8–13.

Baryshyan, A. L., Domigan, L. J., Hunt, B., Trimmer, B. A., & Kaplan, D. L. (2014). Self-assembled insect muscle bioactuators with long term function under a range of environmental conditions. RSC Advances, 4(75), 39962–39968. https://doi.org/10.1039/C4RA08438A

Baryshyan, A. L., Woods, W., Trimmer, B. A., & Kaplan, D. L. (2012). Isolation and maintenance- free culture of contractile myotubes from *Manduca sexta* embryos. PLOS ONE, 7(2), e31598. https://doi.org/10.1371/journal.pone.0031598

Bastiani, M. J., Harrelson, A. L., Snow, P. M., & Goodman, C. S. (1987). Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper. Cell, 48(5), 745–755. https://doi.org/10.1016/0092-8674(87)90072-9

Baust, J. M., *et al.* (2017). Best practices in cell culture: An overview. *In vitro* Cellular & Developmental Biology–Animal, 53(8), 669–672. https://doi.org/10.1007/s11626-017-0177-7

Beira, J. V., & Paro, R. (2016). The legacy of *Drosophila* imaginal discs. Chromosoma, 125(4), 573– 592.

Béjar, J., Hong, Y., & Schartl, M. (2003). Mitf expression is sufficient to direct differentiation of medaka blastula derived stem cells to melanocytes. Development, 130(26), 6545–6553. https://doi.org/10.1242/dev.00872

Belghit, I., Waagbø, R., Lock, E. J., & Liland, N. S. (2019). Insect-based diets high in lauric acid reduce liver lipids in freshwater Atlantic salmon. Aquaculture Nutrition, 25, 343–357.

Bello, F., Rodríguez, J., Morales, A., & Olano, V. (1999). Estudio de cultivos celulares primarios de *Psorophora confinnis* (Diptera: Culicidae). Biomédica (Colombia), 19(2), 127–135.

Ben-Arye, T., & Levenberg, S. (2019). Tissue engineering for clean meat production. Frontiers in Sustainable Food Systems, 3, 46. https://doi.org/10.3389/fsufs.2019.00046

Ben-Arye, T., *et al.* (2020). Textured soy protein scaffolds enable the generation of three-dimensional bovine skeletal muscle tissue for cell-based meat. Nature Food, 1(4), 210–220. https://doi.org/10.1038/s43016-020-0046-5

Benjaminson, M. A., Gilchriest, J. A., & Lorenz, M. (2002). *In vitro* edible muscle protein production system (MPPS): Stage 1, fish. Acta Astronautica, 51(12), 879–889. https://doi.org/10.1016/S0094-5765(02)00033-4

Beňová-Liszeková, D., Beňo, M., & Farkaš, R. (2019). A protocol for processing the delicate larval and prepupal salivary glands of *Drosophila* for scanning electron microscopy. Microscopy Research and Technique, 82(7), 1145–1156. https://doi.org/10.1002/jemt.23263

Beňová-Liszeková, D., Beňo, M., & Farkaš, R. (2019). A protocol for processing the delicate larval and prepupal salivary glands of Drosophila for scanning electron microscopy. Microscopy Research and Technique, 82(7), 1145-1156.

Benton, T. G., Bieg, C., Harwatt, H., Pudasaini, R., &Wellesley, L. (2021). Food system impacts on biodiversity loss: Three levers for food system transformation in support of nature. Chatham House, 2–3. https://doi.org/10.13140/RG.2.2.34045.28640

Bentzinger, C. F., Wang, Y. X., & Rudnicki, M. A. (2012). Building muscle: Molecular regulation of myogenesis. Cold Spring Harbor Perspectives in Biology, 4(2),

a008342. https://doi.org/10.1101/cshperspect.a008342

Bentzinger, C., von Maltzahn, J., & Rudnicki, M. A. (2010). Extrinsic regulation of satellite cell specification. Stem Cell Research & Therapy, 1(3), 27.

Bernstein, S. I., *et al.* (1978). Isolation and partial characterization of *Drosophila myoblasts* from primary cultures of embryonic cells. Journal of Cell Biology, 78(3), 856–865. https://doi.org/10.1083/jcb.78.3.856

Bernstein, S. I., Fyrberg, E. A., & Donady, J. J. (1978). Isolation and partial characterization of *Drosophila* myoblasts from primary cultures of embryonic cells. The Journal of Cell Biology, 78(3), 856–865. https://doi.org/10.1083/jcb.78.3.856

Berry, D. C., Stenesen, D., Zeve, D., & Graff, J. M. (2013). The developmental origins of adipose tissue. Development, 140(19), 3939–3949.

Bessa, L. W., Pieterse, E., Marais, J., Dhanani, K., & Hoffman, L. C. (2021). Food safety of consuming black soldier fly (*Hermetia illucens*) larvae: Microbial, heavy metal, and cross- reactive allergen risks. Foods, 10(8), 1934. https://doi.org/10.3390/foods10081934

Bettahalli, N. M. S., Vicente, J., Moroni, L., Higuera, G. A., van Blitterswijk, C. A., Wessling, M., & Stamatialis, D. F. (2011). Integration of hollow fiber membranes improves nutrient supply in three-dimensional tissue constructs. Acta Biomaterialia, 7(9), 3312–3324. https://doi.org/10.1016/j.actbio.2011.06.012

Bhat, Z. F., & Fayaz, H. (2011). Prospectus of cultured meat—Advancing meat alternatives. Journal of Food Science and Technology, 48(2), 125–140. https://doi.org/10.1007/s13197-010- 0198-7

Bhat, Z. F., & Hina Bhat, H. B. (2011). Animal-free meat biofabrication. https://doi.org/10.3923/ajft.2011.441.459

Bhat, Z. F., Kumar, S., & Bhat, H. F. (2017). *In vitro* meat: A future animal-free harvest. Critical Reviews in Food Science and Nutrition, 57(4), 782–789. https://doi.org/10.1080/10408398.2014.924899

Bhat, Z. F., Kumar, S., & Fayaz, H. (2019). *In vitro* meat production: Challenges and benefits over conventional meat production. Journal of Integrative Agriculture, 18(2), 349–363.

Bian, W., & Bursac, N. (2009). Engineered skeletal muscle tissue networks with controllable architecture. Biomaterials, 30(7), 1401–1412. https://doi.org/10.1016/j.biomaterials.2008.11.015

Biasato, I., *et al.* (2022). Welfare implications for broiler chickens reared in an insect larvae-enriched environment: Focus on bird behaviour, plumage status, leg health, and excreta corticosterone. Frontiers in Physiology, 13, 930158. https://doi.org/10.3389/fphys.2022.930158

Bilirgen, A. C., Toker, M., Odabas, S., Yetisen, A. K., Garipcan, B., & Tasoglu, S. (2021). Plant- based scaffolds in tissue engineering. ACS Biomaterials Science & Engineering, 7(3), 926– 938. https://doi.org/10.1021/acsbiomaterials.0c01527

Binggeli, O., Neyen, C., Poidevin, M., & Lemaitre, B. (2014). Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*. PLOS Pathogens, 10(5), e1004067. https://doi.org/10.1371/journal.ppat.1004067

Bio.Tech.Food. (2024). Retrieved April 2024, from https://biotech-foods.com/

Birker, K., Ge, S., Kirkland, N. J., Theis, J. L., Marchant, J., Fogarty, Z. C., … Bodmer, R. (2023). Mitochondrial MICOS complex genes, implicated in hypoplastic left heart syndrome, maintain cardiac contractility and actomyosin integrity. eLife, 12, e83385. https://doi.org/10.7554/eLife.83385

Blau, H. M., & Webster, C. (1981). Isolation and characterization of human muscle cells. Proceedings of the National Academy of Sciences of the USA, 78(9), 5623–5627. https://doi.org/10.1073/pnas.78.9.5623

Blog Kerafast. (2021, July 13). Kerafast's partnership with the Good Food Institute links researchers to cell lines. Retrieved January 2023, from https://news.kerafast.com/2021/07/13/kerafasts- partnership-with-the-good-food-institute-links-researchers-to-cell-lines/

Boegel, S.J.: Towards a chemically defined medium for Sf-9 cell culture: micronutrients reduce dependence on yeast extract; MASc thesis, University of Waterloo, Department of Chemical Engineering, Ontario (Canada). (2019).

Bogliotti, Y. S., *et al.* (2018). Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts. Proceedings of the National Academy of Sciences, 115(9), 2090– 2095. https://doi.org/10.1073/pnas.1716161115

Bomkamp, C., Skaalure, S. C., Fernando, G. F., Ben-Arye, T., Swartz, E. W., & Specht, E. A. (2022). Scaffolding biomaterials for 3D cultivated meat: Prospects and challenges. Advanced Science, 9(3), 2102908. https://doi.org/10.1002/advs.202102908

Bosch, G., & Swanson, K. S. (2021). Effect of using insects as feed on animals: Pet dogs and cats.

Journal of Insects as Food and Feed, 7(5), 795–805.

Bradley, S. W., Booth, D. C., & Sheppard, D. C. (1984). Parasitism of the black soldier fly by *Trichopria* sp. (Hymenoptera: Diapriidae) in poultry houses. Environmental Entomology, 13(2), 451–454. https://doi.org/10.1093/ee/13.2.451.

Bradley, S.W., & Sheppard, D. C. (1984). House fly oviposition inhibition by larvae of *Hermetia illucens*, the black soldier fly. Journal of Chemical Ecology, 10(6), 853–859. https://doi.org/10.1007/BF00987968

Briegel, H. (1990). Metabolic relationship between female body size, reserves, and fecundity of

Aedes aegypti. Journal of Insect Physiology, 36(3), 165–172.

Broucke, K., Van Pamel, E., Van Coillie, E., Herman, L., & Van Royen, G. (2023). Cultured meat and challenges ahead: A review on nutritional, technofunctional and sensorial properties, safety and legislation. Meat Science, 195, 109006. https://doi.org/10.1016/j.meatsci.2022.109006

Brunner, D., Frank, J., Appl, H., Schöffl, H., Pfaller, W., & Gstraunthaler, G. (2010). The serum-free media interactive online database. ALTEX, 27(1), 53–62. https://doi.org/10.14573/altex.2010.1.53

Bryant, C., & Barnett, J. (2018). Consumer acceptance of cultured meat: A systematic review. Meat Science, 143, 8–17. https://doi.org/10.1016/j.meatsci.2018.04.008

Bryant, C., & Barnett, J. (2020). Consumer acceptance of cultured meat: An updated review (2018– 2020). Applied Sciences, 10(15), 5201. https://doi.org/10.3390/app10155201

Buckingham, M., & Mayeuf, A. (2012). Skeletal muscle development. Fundamental Biology and Mechanisms of Disease, 2, 749–762.

Busti, S., *et al.* (2024). *Hermetia illucens* larvae meal as an alternative protein source in practical diets for gilthead sea bream (*Sparus aurata*): A study on growth. Plasma Biochemistry and Gut Microbiota. Aquaculture, 578, 740093. 10.1016/J.AQUACULTURE.2023.740093

Byeun, D. G., Moon, B.-S., Lee, S., & Choi, J. K. (2022). Germ cell isolation and cryopreservation from reproductive organs of brown mealworm. Insects, 13(12), 1108. https://doi.org/10.3390/insects13121108

Caliari, S. R., & Burdick, J. A. (2016). A practical guide to hydrogels for cell culture. Nature Methods, 13(5), 405–414.

Campuzano, S., & Pelling, A. E. (2019). Scaffolds for 3D cell culture and cellular agriculture applications derived from non-animal sources. Frontiers in Sustainable Food Systems, 3, 38. https://doi.org/10.3389/fsufs.2019.00038

Carletti, E., Motta, A., & Migliaresi, C. (2010). Scaffolds for tissue engineering and 3D cell culture. In 3D Cell Culture: Methods and Protocols (pp. 17–39). https://doi.org/10.1007/978-1- 60761-984-0_2

Carmena, A., Speicher, S., & Baylies, M. (2006). The PDZ protein Canoe/AF-6 links Ras-MAPK, Notch and Wingless/Wnt signaling pathways by directly interacting with Ras, Notch and Dishevelled. PLOS ONE, 1(1), e66. https://doi.org/10.1371/journal.pone.0000066

Cesar, C. G. L., *et al.* (2024). An assessment of the impact of insect meal in dry food on a dog with a food allergy: A case report. Animals, 14, 2859. https://doi.org/10.3390/ani14192859

Chal, J., & Pourquié, O. (2017). Making muscle: Skeletal myogenesis *in vivo* and *in vitro*.Development, 144(12), 2104–2122.

Chambers, S. P., & Swalley, S. E. (2009). Designing experiments for high-throughput protein expression. In High-Throughput Protein Expression and Purification: Methods and Protocols (pp. 19–29). https://doi.org/10.1007/978-1-59745-196-3_2

Champlin, D. T., Reiss, S. E., & Truman, J. W. (1999). Hormonal control of ventral diaphragm myogenesis during metamorphosis of the moth, *Manduca sexta*. Development Genes and Evolution, 209(5), 265–274. https://doi.org/10.1007/s004270050252

Chan, L. C., & Reid, S. (2016). Development of serum-free media for lepidopteran insect cell lines. Methods Molecular Biology, 1350, 161–196. https://doi.org/10.1007/978-1-4939-3043- 2_8.

Chapman, R. F., Simpson, S. J., & Douglas, A. E. (2013). The insects: Structure and function. Cambridge University Press.

Charpentier, G., Tian, L., Cossette, J., Léry, X., & Belloncik, S. (2002). Characterization of cell lines developed from the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). *In vitro* Cellular & Developmental Biology–Animal, 38(2), 73–78.

Chen, G., *et al.* (2011). Chemically defined conditions for human iPSC derivation and culture. Nature Methods, 8(5), 424–429. https://doi.org/10.1038/nmeth.1593

Chen, J. (2016). The cell-cycle arrest and apoptotic functions of p53 in tumor initiation and progression. Cold Spring Harbor Perspectives in Medicine, 6(3), a026104. https://doi.org/10.1101/cshperspect.a026104

Chen, Y., Dong, X. J., Zhang, G. R., Shao, J. Z., & Xiang, L. X. (2007). *In vitro* differentiation of mouse bone marrow stromal stem cells into hepatocytes induced by conditioned culture medium of hepatocytes. Journal of Cellular Biochemistry, 102(1), 52–63. https://doi.org/10.1002/jcb.21275

Chen, X., Guo, Z., Tong, X., Wang, X., Liu, X., Nagai, H., Wu, P., Lu, J., Huss, D., Tran, M., Readhead, C., Wu, C., Cao, L., Huang, Y., Zeng, Z., & Ying, Q.-L. (2025). Derivation of embryonic stem cells across avian species. *Nature Biotechnology*. Advance online publication. https://doi.org/10.1038/s41587-025-02833-3

Chia, S. Y., *et al.* (2021). Black soldier fly larval meal in feed enhances growth performance, carcass yield and meat quality of finishing pigs. Journal of Insects as Food and Feed, 7(4), 433–447. https://doi.org/10.3920/JIFF2020.0072

Chen, S.-L., Ye, H.-Q., Sha, Z.-X., & Hong, Y. (2003). Derivation of a pluripotent embryonic cell line from red sea bream blastulas. Journal of Fish Biology, 63(4), 795–805. https://doi.org/10.1046/j.1095-8649.2003.00192.x

Chien, K. B., & Shah, R. N. (2012). Novel soy protein scaffolds for tissue regeneration: Material characterization and interaction with human mesenchymal stem cells. Acta Biomaterialia, 8(2), 694–703. https://doi.org/10.1016/j.actbio.2011.09.036

Chodkowska, K. A., Wódz, K., & Wojciechowski, J. (2022). Sustainable future protein foods: The challenges and the future of cultivated meat. Foods, 11(24), 4008.

Chung, A. Y. C., Khen, C. V., Unchi, S., & Binti, M. (2002). Edible insects and entomophagy in Sabah, Malaysia. Malayan Nature Journal, 56, 131–144.

Churchill, D., & Caveney, S. (1993). Isolation of epidermal cell pairs from an insect, *Tenebrio molitor*, for dual whole-cell recording of large-conductance gap-junctional channels. Journal of Experimental Biology, 178(1), 261–278.

Collier, V. L., Kronert, W. A., O'Donnell, P. T., Edwards, K. A., & Bernstein, S. I. (1990). Alternative myosin hinge regions are utilized in a tissue-specific fashion that correlates with muscle contraction speed. Genes & Development, 4(6), 885–895. https://doi.org/10.1101/gad.4.6.885

Cosenza, Z., & Block, D. E. (2021). A generalizable hybrid search framework for optimizing expensive design problems using surrogate models. Engineering Optimization, 53(10), 1772–1785. https://doi.org/10.1080/0305215X.2020.1826466

Costa-Neto, E. M. (2013). Insects as human food: An overview. Amazônica - Revista de Antropologia, 5(3). https://doi.org/10.18542/amazonica.v5i3.1564

Courtenay, J. C., Johns, M. A., Galembeck, F., Deneke, C., Lanzoni, E. M., Costa, C. A., Scott, J. L., & Sharma, R. I. (2017). Surface modified cellulose scaffolds for tissue engineering. Cellulose, 24(1), 253–267. https://doi.org/10.1007/s10570-016-1111-y

Cruz B., M.; Bello, F.J.: Cultivos celulares primarios *Sarconesiopsis magellanica*; Rev. Cytotechnology. (1996). Cient. 15(2): 313 - 321, 2012 S. https://doi.org/10.1007/BF00350384

Dahl-Halvarsson, M., Olive, M., Pokrzywa, M., Norum, M., Ejeskär, K., & Tajsharghi, H. (2020). Impaired muscle morphology in a *Drosophila* model of myosin storage myopathy was suppressed by overexpression of an E3 ubiquitin ligase. Disease Models & Mechanisms, 13(1), dmm047886. https://doi.org/10.1242/dmm.047886

Danoviz, M. E., & Yablonka-Reuveni, Z. (2012). Skeletal muscle satellite cells:

Background and methods for isolation and analysis in a primary culture system. In Myogenesis: Methods and Protocols (pp. 21–52). Humana Press. https://doi.org/10.1007/978-1-61779-343-1_2

Das, M., Wilson, K., Molnar, P., & Hickman, J. J. (2007). Differentiation of skeletal muscle and integration of myotubes with silicon microstructures using serum-free medium and a synthetic silane substrate. *Nature Protocols, 2*(7), 1795–1801. https://doi.org/10.1038/nprot.2007.229

Datar, I., & Betti, M. (2010). Possibilities for an *in vitro* meat production system. Innovative Food Science & Emerging Technologies, 11(1), 13–22. https://doi.org/10.1016/j.ifset.2009.10.007

Davidenko, N., Campbell, J. J., Thian, E. S., Watson, C. J., & Cameron, R. E. (2010). Collagen– hyaluronic acid scaffolds for adipose tissue engineering. Acta Biomaterialia, 6(10), 3957– 3968. https://doi.org/10.1016/j.actbio.2010.05.005

De Castro, R. J. S., Ohara, A., & Domingues, M. A. C. (2018). Edible insects as a source of high- quality proteins. Food Science and Technology (Campinas), 38(1), 1–5. https://doi.org/10.1590/1678-457X.22916

Decker, E. R., *et al.* (1995). Thermal regimes of the Southern Rocky Mountains and Wyoming Basin in Colorado and Wyoming in the United States. *Tectonophysics, 244*(1–3), 167–184. https://doi.org/10.1016/0040-1951(94)00231-W

Delgado, C., Rosegrant, M., Steinfeld, H., Ehui, S., & Courbois, C. (1999). Livestock to 2020: The next food revolution. Outlook on Agriculture, 30(1), 27–29.

Delicato, C., Schouteten, J. J., Dewettinck, K., Gellynck, X., & Tzompa-Sosa, D. A. (2020). Consumers' perception of bakery products with insect fat as partial butter replacement. Food Quality and Preference, 79, 103755.

Delvendahl, N., Rumpold, B. A., & Langen, N. (2022). Edible insects as food: Insect welfare and ethical aspects from a consumer perspective. Insects, 13(121). https://doi.org/10.3390/ insects13020121

Di Rosa, A. R., Caccamo, L., Pansera, L., Oteri, M., Chiofalo, B., & Maricchiolo, G. (2023). Influence of *Hermetia illucens* larvae meal dietary inclusion on growth performance, gut histological traits and stress parameters in Sparus aurata. Animals, 13(3), 339.

Diener, S., Solano, N. M. S., Gutiérrez, F. R., Zurbrügg, C., & Tockner, K. (2011). Biological treatment of municipal organic waste using black soldier fly larvae. Waste Biomass Valoriz, 2, 357–363.

Diener, S., Zurbrügg, C., & Tockner, K. (2009). Conversion of organic material by black soldier fly larvae: Establishing optimal feeding rates. Waste Management & Research, 27(6), 603– 610. https://doi.org/10.1177/0734242X09103838

Dinan, L., Dioh, W., Veillet, S., & Lafont, R. (2021). 20-Hydroxyecdysone, from plant extracts to clinical use: Therapeutic potential for the treatment of neuromuscular, cardio-metabolic and respiratory diseases. *Biomedicines*, 9(5), 492. https://doi.org/10.3390/biomedicines9050492

Ding, S., *et al.* (2018). Maintaining bovine satellite cells stemness through p38 pathway. Scientific Reports, 8(1), 10808. https://doi.org/10.1038/s41598-018-28746-7

Dohmen, R. G., *et al.* (2022). Muscle-derived fibro-adipogenic progenitor cells for production of cultured bovine adipose tissue. npj Science of Food, 6(1), 6. https://doi.org/10.1038/s41538-021-00122-2

Dulbecco, R., & Freeman, G. (1959). Plaque production by the polyoma virus. Virology, 8(3), 396–
397. https://doi.org/10.1016/0042-6822(59)90043-1

Dupont, J., & Fiebelkorn, F. (2020). Attitudes and acceptance of young people toward the consumption of insects and cultured meat in Germany. Food Quality and Preference, 85, 103983. https://doi.org/10.1016/j.foodqual.2020.103983

Eagle, H. (1959). Amino acid metabolism in mammalian cell cultures. Science, 130(3373), 432–
437. https://doi.org/10.1126/science.130.3373.432

Echeverry, L., Zapata, A., Segura, A., & Bello, F. (2009). Estudio de cultivos celulares primarios derivados de *Lucilia sericata* (Diptera: Calliphoridae). Revista Ciencias de la Salud (Colombia), 7(3), 17–28.

Edwards, J. S. (1978). Insect physiology. Science, 200(4339), 304–305. https://doi.org/10.1126/science.200.4339.304-a

EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA). (2021a). Safety of dried yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal, 19(4), e06443. https://doi.org/10.2903/j.efsa.2021.6443

EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA). (2021b). Safety of frozen and dried formulations from whole yellow mealworm (*Tenebrio molitor* larva) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal, 19(8), e06778. https://doi.org/10.2903/j.efsa.2021.6778

Elhassan, M., Wendin, K., Olsson, V., & Langton, M. (2019). Quality aspects of insects as food— nutritional, sensory, and related concepts. Foods, 8(3), 95. https://doi.org/10.3390/foods8030095

ESA. (2022). ESA investigates cultured meat as novel space food. Retrieved September 2022, from https://www.esa.int/Enabling_Support/Preparing_for_the_Future/Discovery_and_Prepara tion/ESA_investigates_cultured_meat_as_novel_space_food

European Commission. (2001). Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms. Retrieved 2001.

European Food Safety Authority (EFSA). (2015). Risk profile related to production and consumption of insects as food and feed. EFSA Journal, 13(10), 4257. https://doi.org/10.2903/j.efsa.2015.4257

European Food Safety Authority (EFSA) (2021). Edible insects: What science says about novel food evaluations. European Food Safety Authority https://www.efsa.europa.eu/it/news/ edible- insects-science-novel-food-evaluations.

European Parliament, & Council of the European Union. (2015). Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 and repealing Regulation (EC) No 258/97 and Commission Regulation (EC) No 1852/2001. Official Journal of the European Union, L 327, 1–22.

European Parliament, & Council of the European Union. (2003). Regulation (EC) No

1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Official Journal of the European Union, L 268, 1–23.

European Parliament, & Council of the European Union. (2003). Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. Official Journal of the European Union, L 268, 24–28.

European Commission. (2024). TRIS notification 2024/0394/HU – Draft act on the prohibition of the production and placing on the market of food made from cultivated meat. Technical Regulation Information System (TRIS).

Ewald, N., Vidakovic, A., Langeland, M., Kiessling, A., Sampels, S., & Lalander, C. (2020). Fatty acid composition of black soldier fly larvae (*Hermetia illucens*) – Possibilities and limitations for modification through diet. Waste Management, 102, 40–47. https://doi.org/10.1016/j.wasman.2019.10.014Expasy – Cellosaurus. (2023). Retrieved July 2023, from https://www.cellosaurus.org/

Ezashi, T., Telugu, B. P. V., Alexenko, A. P., Sachdev, S., Sinha, S., & Roberts, R. M. (2009). Derivation of induced pluripotent stem cells from pig somatic cells. Proceedings of the National Academy of Sciences, 106(27), 10993–10998. https://doi.org/10.1073/pnas.0905284106

Failla, M., Hopfer, H., & Wee, J. (2023). Evaluation of public submissions to the USDA for labeling of cell-cultured meat in the United States. Frontiers in Nutrition, 10, 1197111. https://doi.org/10.3389/fnut.2023.1197111

FAO/WHO. (2019). Methoprene (147). In *Pesticide residues in food 2019 – Evaluations 2019 Part I: Residues*. Joint FAO/WHO Meeting on Pesticide Residues (JMPR). Food and Agriculture Organization of the United Nations, Rome.

Fernandez-Cassi, X., *et al.* (2020). Microbial communities and food safety aspects of crickets (*Acheta domesticus*) reared under controlled conditions. Journal of Insects as Food and Feed, 6(4), 429–440. https://doi.org/10.3920/JIFF2019.0048

Fernández-Costa, J. M., Fernández-Garibay, X., Velasco-Mallorquí, F., & Ramón-Azcón, J. (2021). Bioengineered *in vitro* skeletal muscles as new tools for muscular dystrophies preclinical studies. Journal of Tissue Engineering, 12, 2041731420981339. https://doi.org/10.1177/2041731420981339

Fikri, F., Purnomo, A., Chhetri, S., Purnama, M. T. E., & Çalışkan, H. (2024). Effects of black soldier fly (*Hermetia illucens*) larvae meal on production performance, egg quality, and physiological properties in laying hens: A meta-analysis. Veterinary World, 17(8), 1904.

Fish, K. D., Rubio, N. R., Stout, A. J., Yuen, J. S., & Kaplan, D. L. (2020). Prospects and challenges for cell-cultured fat as a novel food ingredient. Trends in Food Science & Technology, 98, 53–67. https://doi.org/10.1016/j.tifs.2020.02.005

Fisher, R. A. (1992). The arrangement of field experiments. In Breakthroughs in Statistics: Methodology and Distribution (pp. 82–91). Springer. https://doi.org/10.1007/978-1-4612- 4380-9_8

Flynn, L., & Woodhouse, K. A. (2008). Adipose tissue engineering with cells in engineered matrices. Organogenesis, 4(4), 228–235. https://doi.org/10.4161/org.4.4.7082

Florida Senate. (2024). CS/CS/SB 1084 – Food. 2024 Regular Session, State of Florida Legislature. Retrieved from https://www.flsenate.gov/Session/Bill/2024/1084

Food and Agriculture Organization of the United Nations. (2006). Livestock's long shadow: Environmental issues and options. FAO. https://www.fao.org/3/a0701e/a0701e.pdf

Food and Agriculture Organization of the United Nations (FAO) (2025). FAOSTAT: Food and agriculture data. Retrieved from https://www.fao.org/faostat/en/#data

Fortin, J. (2024, May 2). Florida bans lab-grown meat. *The New York Times*. Retrieved from https://www.nytimes.com

Fox, J. L. (2009). Test tube meat on the menu?. Nature Biotechnology, 27(10), 873–874. https://doi.org/10.1038/nbt1009-873

Franco, A., *et al.* (2021). Lipids from *Hermetia illucens*, an innovative and sustainable source. Sustainability, 13(18), 10198.

Franco, A., *et al.* (2024a). Edible insects: An overview on farming, from processing procedures to environmental impact, with a glimpse to traditional recipes and to future cultured meat. Entomologia Generalis, 44, 813–831. https://doi.org/10.1127/entomologia/2024/2651

Franco, A., Scieuzo, C., Salvia, R., Mancini, I. M., Caniani, D., Masi, S., & Falabella, P. (2024b). Overview of farming process in edible insect industry. Entomologia Generalis, 44, 813– 831. https://doi.org/10.1127/entomologia/2024/2651

Franco, A., *et al.* (2024c). Antimicrobial activity of lipids extracted from *Hermetia illucens* reared on different substrates. Applied Microbiology and Biotechnology, 108, 167.

Franco, A., Pucciarelli, V., Hosseini, S. A., Schmitt, E., Bovera, F., Scieuzo, C., & Falabella, P. (2025). Bioconversion of Meat and Fish-Based Former Foodstuffs by Black Soldier Fly Larvae: A Sustainable Pathway for Reducing Food Waste, Enhancing Nutrient Recovery, with a Circular Economy Approach. Insects, 16(5), 508.

Freel, T. A., McComb, A., & Koutsos, E. A. (2021). Digestibility and safety of dry black soldier fly larvae (BSFL) meal and BSFL oil in dogs. Journal of Animal Science, 99, skab047. https://doi.org/10.1093/jas/skab047

Fusco, A., Guarnieri, A., Scieuzo, C., Triunfo, M., Salvia, R., Donnarumma, G., & Falabella, P. (2025). *Hermetia illucens-derived* chitosan: A promising immunomodulatory agent for applications in biomedical fields. Biomacromolecules, 26(5), 3224–3233. https://doi.org/10.1021/acs.biomac.5c00362

Fyrberg, E. A., & Donady, J. J. (1979). Actin heterogeneity in primary embryonic culture cells from *Drosophila melanogaster*. Developmental Biology, 68(2), 487–502. https://doi.org/10.1016/0012-1606(79)90220-3

Gäde, G., & Auerswald, L. (2003). Mode of action of neuropeptides from the adipokinetic hormone family. General and Comparative Endocrinology, 132(1), 10–20. https://doi.org/10.1016/S0016-6480(03)00159-X

Galanakis, C. M. (2024). The future of food. Foods, 13(4), 506. https://doi.org/10.3390/foods13040506

Gan, L., *et al.* (2016). Cellulose/soy protein composite-based nerve guidance conduits with designed microstructure for peripheral nerve regeneration. Journal of Neural Engineering, 13(5), 056019. https://doi.org/10.1088/1741-2560/13/5/056019

Gardell, A. M., Qin, Q., Rice, R. H., Li, J., & Kültz, D. (2014). Derivation and osmotolerance characterization of three immortalized tilapia (Oreochromis mossambicus) cell lines. PLoS ONE, 9(5), e95919. https://doi.org/10.1371/journal.pone.0095919

Garg, T., & Goyal, A. K. (2014). Biomaterial-based scaffolds—Current status and future directions. Expert Opinion on Drug Delivery, 11(5), 767–789. https://doi.org/10.1517/17425247.2014.891014

Garofalo, C., Milanovi´c, V., Cardinali, F., Aquilanti, L., Clementi, F., & Osimani, A. (2019). Current knowledge on the microbiota of edible insects intended for human consumption: A state- of-the-art review. Food Research International, 125, 108527. https://doi.org/10.1016/ j.foodres.2019.108527

Gazzola, W. H., Benson, R. S., & Carver, W. (2019). Meltblown polylactic acid nanowebs as a tissue engineering scaffold. Annals of Plastic Surgery, 83(6), 716–721. https://doi.org/10.1097/SAP.0000000000002036

Genovese, N. J., Domeier, T. L., Telugu, B. P. V., & Roberts, R. M. (2017). Enhanced development of skeletal myotubes from porcine induced pluripotent stem cells. Scientific Reports, 7(1), 41833. https://doi.org/10.1038/srep41833

Genovese, N. J., Schulze, E. N., & Desmet, D. N. (2023). U.S. Patent No. 11,708,587. U.S. Patent and Trademark Office.

Georgescu, B., Boaru, A. M., Muntean, L., Sima, N., Struți, D. I., Păpuc, T. A., & Georgescu, C. (2022). Modulating the fatty acid profiles of *Hermetia illucens* larvae fats by dietary enrichment with different oilseeds: A sustainable way for future use in feed and food. Insects, 13(9), 801. https://doi.org/10.3390/insects13090801

Gershlak, J. R., *et al.* (2017). Crossing kingdoms: Using decellularized plants as perfusable tissue engineering scaffolds. Biomaterials, 125, 13–22. https://doi.org/10.1016/j.biomaterials.2017.02.011

Giani, M., *et al.,* (2025). *Hermetia illucens*-derived chitosan as a promising sustainable biomaterial for wound-healing applications: Development of sponge-like scaffolds. International Journal of Biological Macromolecules, 304(Pt 2), 140903. https://doi.org/10.1016/j.ijbiomac.2025.140903

Giglio, F., Scieuzo, C., Ouazri, S., Pucciarelli, V., Ianniciello, D., Letcher, S., Salvia, R., Laginestra, A., Kaplan, D. L., & Falabella, P. (2024). A Glance into the near future: Cultivated meat from mammalian and insect cells. Small Science, 2400122. https://doi.org/10.1002/smsc.202400122

Godfray, H. C. J., Aveyard, P., Garnett, T., Hall, J. W., Key, T. J., Lorimer, J., Jebb, S. A. (2018). Meat consumption, health, and the environment. Science, 361(6399), eaam5324. https://doi.org/10.1126/science.aam532

Goodman, C. L., Stanley, D., Ringbauer, J. A., Jr., Beeman, R. W., Silver, K., & Park, Y. (2012). A cell line derived from the red flour beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae). *In vitro* Cellular & Developmental Biology – Animal, 48(7), 426–433.

Goodwin, R. H. (1975). Insect cell culture: Improved media and methods for initiating attached cell lines from the Lepidoptera. *In vitro*, 11(6), 369–378. https://doi.org/10.1007/BF02616373

Gottshall, S. L., Tekin, S., & Hansen, P. J. (2008). Extraction and Purification of Total RNA using Trizol or Tri Reagent. Laboratory Procedures, PJ Hansen Laboratory, University of Florida.

Gourmey. (2024). Retrieved April 2024, from https://www.gourmey.com/

Grace, T. (1962). Establishment of four strains of cells from insect tissues grown *in vitro*. Nature, 195, 788–789. https://doi.org/10.1038/195788a0

Grasso, A. C., Hung, Y., Olthof, M. R., Verbeke, W., & Brouwer, I. A. (2019). Older consumers' readiness to accept alternative, more sustainable protein sources in the European Union. Nutrients, 11(8), 1904. https://doi.org/10.3390/nu11081904

Grau, T., Vilcinskas, A., & Joop, G. (2017). Sustainable farming of the mealworm *Tenebrio molitor* for the production of food and feed. Zeitschrift für Naturforschung C, 72(9–10), 337–349. https://doi.org/10.1515/znc-2017-0033

Grossman, M. R. (2019). USDA and FDA formal agreement on regulation of cultured meat.European Food and Feed Law Review, 14(4), 385–389.

Groux-Muscatelli, B., Bassaglia, Y., Barritault, D., Caruelle, J. P., & Gautron, J. (1990). Proliferating satellite cells express acidic fibroblast growth factor during *in vitro* myogenesis. Developmental Biology, 142(2), 380–385. https://doi.org/10.1016/0012-1606(90)90358-P

Guarnieri, A., *et al.* (2024). Insect-derived chitosan, a biopolymer for the increased shelf life of white and red grapes. International Journal of Biological Macromolecules, 275(Pt 2), 133149. https://doi.org/10.1016/j.ijbiomac.2024.133149

Guerriero, V., & Florini, J. R. (1980). Dexamethasone effects on myoblast proliferation and differentiation. Endocrinology, 106(4), 1198–1202. https://doi.org/10.1210/endo-106-4-1198

Gunage, R. D., Dhanyasi, N., Reichert, H., & VijayRaghavan, K. (2017). *Drosophila adult* muscle development and regeneration. Seminars in Cell & Developmental Biology, 72, 56–66. https://doi.org/10.1016/j.semcdb.2017.11.017

Gupte, M., & Kulkarni, P. (2003). A study of antifungal antibiotic production by Thermomonospora sp. MTCC 3340 using full factorial design. Journal of Chemical Technology & Biotechnology, 78(6), 605–610. https://doi.org/10.1002/jctb.818

Gutierrez, E., Wiggins, D., Fielding, B., & Gould, A. P. (2007). Specialized hepatocyte-like cells regulate *Drosophila* lipid metabolism. Nature, 445(7125), 275–280.

Hahn, T., Roth, A., Ji, R., Schmitt, E., & Zibek, S. (2020). Chitosan production with larval exoskeletons derived from the insect protein production. Journal of Biotechnology, 310, 62–67. https://doi.org/10.1016/j.jbiotec.2019.12.015

Halbleib, M., Skurk, T., de Luca, C., von Heimburg, D., & Hauner, H. (2003). Tissue engineering of white adipose tissue using hyaluronic acid–based scaffolds. I: *In vitro* differentiation of human adipocyte precursor cells on scaffolds. Biomaterials, 24(18), 3125–3132. https://doi.org/10.1016/S0142-9612(03)00156-X

Ham, R. G. (1965). Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proceedings of the National Academy of Sciences, 53(2), 288–293. https://doi.org/10.1073/pnas.53.2.288

Hammond, T. G., & Hammond, J. M. (2001). Optimized suspension culture: The rotating-wall vessel. American Journal of Physiology–Renal Physiology, 281(1),

F12–F25. https://doi.org/10.1152/ajprenal.2001.281.1.F12

Hanga, M. P., Ali, J., Moutsatsou, P., de la Raga, F. A., Hewitt, C. J., Nienow, A., & Wall, I. (2020). Bioprocess development for scalable production of cultivated meat. Biotechnology and Bioengineering, 117(10), 3029–3039. https://doi.org/10.1002/bit.27469

Harada, H., *et al.* (2003). Telomerase induces immortalization of human esophageal keratinocytes without p16INK4a inactivation. Molecular Cancer Research, 1(10), 729–738.

Harris, A. F., Lacombe, J., & Zenhausern, F. (2021). The emerging role of decellularized plant-based scaffolds as a new biomaterial. International Journal of Molecular Sciences, 22(22), 12347. https://doi.org/10.3390/ijms222212347

Harrison, R. G., Greenman, M. J., Mall, F. P., & Jackson, C. M. (1907). Observations of the living developing nerve fiber. Journal of Experimental Zoology, 10, 1–23.

Hartmann, C., & Siegrist, M. (2018). Development and validation of the Food Disgust Scale. Food Quality and Preference, 63, 38–50. https://doi.org/10.1016/j.foodqual.2017.07.013

Harwood, R. (1974). Cell separation by gradient centrifugation. In International Review of Cytology (Vol. 38, pp. 369–403). Elsevier. https://doi.org/10.1016/S0074-7696(08)60930-4

Hastings, G. A., & Emerson, C. P., Jr. (1991). Myosin functional domains encoded by alternative exons are expressed in specific thoracic muscles of *Drosophila*. The Journal of Cell Biology, 114(2), 263–276. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2289080/

He, X., Lu, L., Huang, P., Yu, B., Peng, L., Zou, L., & Ren, Y. (2023). Insect cell-based models: Cell line establishment and application in insecticide screening and toxicology research. Insects, 14(104). https://doi.org/10.3390/insects14020104

Health For Animals. Global state of pet care stats, facts and trends. Retrieved January 22, 2025 HealthforAnimals.org/PetCareReport.

Hegstrom, C. D., & Truman, J. W. (1996). Steroid control of muscle remodeling during metamorphosis in *Manduca sexta*. Journal of Neurobiology, 29, 535–550.

Henry, M., Gasco, L., Piccolo, G., & Fountoulaki, E. (2015). Review on the use of insects in the diet of farmed fish: Past and future. Animal Feed Science and Technology, 203, 1–22.

Hensel, P., Santoro, D., Favrot, C., Hill, P., & Griffin, C. (2015). Canine atopic dermatitis: Detailed guidelines for diagnosis and allergen identification. BMC Veterinary Research, 11, 196. https://doi.org/10.1186/s12917-015-0515-5

Higa, J. E., Ruby, M. B., & Rozin, P. (2021). Americans' acceptance of black soldier fly larvae as food for themselves, their dogs, and farmed animals. Food Quality and Preference, 90, 104119.

Hinds, S., Bian, W., Dennis, R. G., & Bursac, N. (2011). The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. Biomaterials, 32(14), 3575– 3583. https://doi.org/10.1016/j.biomaterials.2011.01.062

Hink, W. F. (1970). Established insect cell line from the cabbage looper, Trichoplusia ni. Nature, 226(5244), 466–467. https://doi.org/10.1038/226466b0

Honka, M. J., Latva-Rasku, A., Bucci, M., Virtanen, K. A., Hannukainen, J. C.,

Kalliokoski, K. K., & Nuutila, P. (2018). Insulin-stimulated glucose uptake in skeletal muscle, adipose tissue and liver: A positron emission tomography study. European Journal of Endocrinology, 178(5), 523–531.

Hopkins, P. D., & Dacey, A. (2008). Vegetarian meat: Could technology save animals and satisfy meat eaters? Journal of Agricultural and Environmental Ethics, 21(6), 579–596. https://doi.org/10.1007/s10806-008-9110-0

Hoshino, K., Hirose, M., & Iwabuchi, K. (2009). A new insect cell line from the longicorn beetle Plagionotus christophi (Coleoptera: Cerambycidae). *In vitro* Cellular & Developmental Biology–Animal, 45(1–2), 19–22. https://doi.org/10.1007/s11626-008-9152-7

Hoshizaki, D. K., & Chapman, R. F. (2012). Fat body. In S. J. Simpson, & A. E. Douglas (Eds.), The insects (pp. 132–146). Cambridge University Press. https://doi.org/10.1017/ CBO9781139035460.009

Hou, D.-R., Jin, Y., Nie, X.-W., Zhang, M.-L., Ta, N., Zhao, L.-H., Yang, N., Chen, Y., Wu, Z.-Q., Jiang, H.-B., Li, Y.-R., Sun, Q.-Y., Dai, Y.-F., & Li, R.-F. (2016). Derivation of porcine embryonic stem-like cells from *in vitro*-produced blastocyst-stage embryos. *Scientific Reports*, 6, 25838. https://doi.org/10.1038/srep25838

Huang, C., Hernandez, C. E., Wall, H., Tahamtani, F. M., Ivarsson, E., & Sun, L. (2024). Live black soldier fly (*Hermetia illucens*) larvae in feed for laying hens: Effects on hen gut microbiota and behavior. Poultry Science, 103(3), 103429.

Hussein, S. M., Soliman, W. S., & Khalifa, A. A. (2021). Benefits of pets' ownership, a review based on health perspectives. Journal of Internal Medicine and Emergency Research, 2(1), 1–9. https://doi.org/10.37191/Mapsci-2582-7367-2(1)-020

Ianniciello, D., Boschi, A., Rinaldi, R., Franco, A., Giglio, F., Scieuzo, C., Salvia, R., & Falabella, P. (2024). A comprehensive review of entomophagy under legal, historical, safety, and nutritional profile. Entomologia Generalis. https://doi.org/10.1127/entomologia/2024/2524

Ianniciello, D., Peláez Montosa, A., Barbosa, R. D. M., García-Villén, F., Salvia, R., Scieuzo, C., & Falabella, P. (2025). Development of chitosan-clay nanocomposite films from *Hermetia illucens*: analysis of chemical, physical, and mechanical properties. https://doi.org/10.1016/j.ijbiomac.2025.143496

Inoue, H., Kobayashi, J., Kawakita, H. *et al.* Insect muscle cell line forms contractile tissue networks*in vitro*. *In vitro* Cell Dev Biol - Animal **27**, 837–840 (1991). https://doi.org/10.1007/BF02630984

International Platform of Insects for Food and Feed (IPIFF). (2024, February). Guide on good hygiene practice. Retrieved September 2025, from https://ipiff.org/good-hygiene-practices/

Invitrogen Corp.: Insect Cell Lines, Version K (cod. 25-0127); Technical manual, Invitrogen, Carlsbad (CA, USA). (2002).

Ipema, A. F., Bokkers, E. A., Gerrits, W. J., Kemp, B., & Bolhuis, J. E. (2020). Long-term access to live black soldier fly larvae (*Hermetia illucens*) stimulates activity and reduces fearfulness of broilers, without affecting health. Scientific Reports, 10(1), 17428.

Italian Republic. (2023). Law No. 172 of 30 November 2023 on provisions concerning the prohibition of the production and placing on the market of synthetic foods and feeds. *Official Gazette of the Italian Republic*, General Series, No. 282, 1–6.

Jahan, R., Tipu, M. M. H., Haque, M. M., & Salam, M. A. (2021). Black soldier fly (*Hermetia illucens*) larvae meal as a fish meal replacement in diets for 1 nursing common carp (Cyprinus carpio) fry. Agricultural Research Archive, 2021. https://doi.org/10.31220/agriRxiv.2021.00030

Kaczor, M., Bulak, P., Proc-Pietrycha, K., Kirichenko-Babko, M., & Bieganowski, A. (2022). The variety of applications of *Hermetia illucens* in industrial and agricultural areas. Biology, 12(1), 25.

Kaplan, D. L., Kim, S. S., Whang, H. G., Yang, Y. P., & Lee, O. J. (2021). Biotechnological advances in cultured meat production. Biotechnology Advances, 49, 107713. https://doi.org/10.1016/j.biotechadv.2021.107713

Kelsh, R., Weinzierl, R. O. J., White, R. A. H., & Akam, M. (1994). Homeotic gene expression in the locust Schistocerca: An antibody that detects conserved epitopes in Ultrabithorax and abdominal-A proteins. Developmental Genetics, 15(1), 19–31. https://doi.org/10.1002/dvg.1020150104

Kepinska-Pacelik, J., & Biel, W. (2022). Insects in pet food industry—Hope or threat? Animals, 12, 1515. https://doi.org/10.3390/ani12121515

Kim, Y. B., Kim, D. H., Jeong, S. B., Lee, J. W., Kim, T. H., Lee, H. G., & Lee, K. W. (2020). Black soldier fly larvae oil as an alternative fat source in broiler nutrition. Poultry Science, 99, 3133–3143.

Klonick, A. (2017). Bug ideas: Assessing the market potential and regulation of insects. Duke University.

Klunder, H. C., Wolkers-Rooijackers, J. C. M., Korpela, J. M., & Nout, M. J. R. (2012). Microbiological aspects of processing and storage of edible insects. Food Control, 26(2), 628–631. https://doi.org/10.1016/j.foodcont.2012.02.013

Knežić, T., Avramov, M., Tatić, V., Petrović, M., Gadjanski, I., & Popović, Ž. D. (2024). Insects as a prospective source of biologically active molecules and pharmaceuticals—biochemical properties and cell toxicity of *Tenebrio molitor* and *Zophobas morio* cell-free larval Hemolymph. International Journal of Molecular Sciences, 25(13), 7491.

Knowles, T., Moody, R., & McEachern, M. (2007). European food scares and their impact on EU food policy. British Food Journal, 109, 110–124. https://doi.org/10.1108/00070700710718507

Kraiem, S., Al-Jaber, M. Y., Al-Mohammed, H., Al-Menhali, A. S., Al-Thani, N., Helaleh, M., Samsam, W., Touil, S., Beotra, A., Georgakopoulas, C., Bouabdallah, S., Mohamed-Ali, V., & Al Maadheed, M. (2021). Analytical strategy for the detection of ecdysterone and its metabolites *in vivo* in uPA(+/+)-SCID mice with humanized liver, human urine samples, and estimation of prevalence of its use in anti-doping samples. *Drug Testing and Analysis*, 13(7), 1341–1353. https://doi.org/10.1002/dta.3032

Kurtti, T. J., & Brooks, M. A. (1970). Growth and differentiation of lepidopteran myoblasts *in vitro*. Experimental Cell Research, 61(2–3), 407–412. https://doi.org/10.1016/0014-4827(70)90465-9

Lähteenmäki-Uutela, A., Rahikainen, M., Lonkila, A., & Yang, B. (2021). Alternative proteins and EU food law. Food Control, 130, 108336. https://doi.org/10.1016/j.foodcont.2021.108336

Lakra, W. S., Swaminathan, T. R., & Joy, K. P. (2011). Development, characterization, conservation and storage of fish cell lines: A review. Fish Physiology and Biochemistry, 37(1), 1–20. https://doi.org/10.1007/s10695-010-9411-x

Lanzoni, D., Rebucci, R., Formici, G., Cheli, F., Ragone, G., Baldi, A., Violini, L., Sundaram, T. S., & Giromini, C. (2024). Cultured meat in the European Union: Legislative context and food safety issues. *Current Research in Food Science*, 8, 100722. https://doi.org/10.1016/j.crfs.2024.100722

Lee, D. E., Lee, S. H., & Kim, J. H. (2025). Validation of reliable reference genes for comparison of gene expression across species in the Anopheles Hyrcanus Group. Scientific Reports, 15(1), 9037.

Lee, H. S., Cho, M. Y., Lee, K. M., Kwon, T. H., Homma, K. I., Natori, S., & Lee, B. L. (1999). The pro-phenoloxidase of coleopteran insect, *Tenebrio molitor*, larvae was activated during cell clump/cell adhesion of insect cellular defense reactions. FEBS letters, 444(2-3), 255-259.

Lee, J., Kim, Y. M., Park, Y. K., Yang, Y. C., Jung, B. G., & Lee, B. J. (2018). Black soldier fly (*Hermetia illucens*) larvae enhances immune activities and increases survivability of broiler chicks against experimental infection of *Salmonella Gallinarum*. Journal of Veterinary Medical Science, 80(5), 736–740

Leiria, L. O., & Tseng, Y. H. (2020). Lipidomics of brown and white adipose tissue: Implications for energy metabolism. Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids, 1865(10), 158788. https://doi.org/10.1016/j.bbalip.2020.158788

Leitner, B. P., *et al.* (2017). Mapping of human brown adipose tissue in lean and obese young men. Proceedings of the National Academy of Sciences, 114(32), 8649–8654. https://doi.org/10.1073/pnas.1705287114

Leptin, M. (1991). twist and snail as positive and negative regulators during *Drosophila mesoderm* development. Genes & Development, 5(9), 1568–1576. https://doi.org/10.1101/gad.5.9.1568

Letcher, S. M., Rubio, N. R., Ashizawa, R. N., Saad, M. K., Rittenberg, M. L., McCreary, A., Ali, A., Calkins, O. P., Trimmer, B. A., & Kaplan, D. L. (2022). *In vitro* insect fat cultivation for cellular agriculture applications. ACS Biomaterials Science & Engineering, 8(9), 3785– 3796. https://doi.org/10.1021/acsbiomaterials.2c00093

Letcher, S. M., Calkins, O. P., Clausi, H. J., McCreary, A., Trimmer, B. A., & Kaplan, D. L. (2025). Establishment and characterization of a non-adherent insect cell line for cultivated meat. *Scientific Reports, 15*(1), 7850. https://doi.org/10.1038/s41598-025-86921-z

Li, L., Zhao, Z., & Liu, H. (2013). Feasibility of feeding yellow mealworm (*Tenebrio molitor* L.) in bioregenerative life support systems as a source of animal protein for humans. Acta Astronautica, 92(1), 103–109. https://doi.org/10.1016/j.actaastro.2012.03.012

Li, S., Yu, X., & Feng, Q. (2019). Fat body biology in the last decade. Annual Review of Entomology, 64(1), 315–333. https://doi.org/10.1146/annurev-ento-011118-112007

Liguori, B., Sancho, A. I., Poulsen, M., & Bøgh, K. L. (2022). Novel foods: Allergenicity assessment of insect proteins. EFSA Journal, 20, e200910. https://doi.org/10.2903/j.efsa. 2022.e200910

Listrat, A., Lebret, B., Louveau, I., Astruc, T., Bonnet, M., Lefaucheur, L., Picard, B., & Bugeon, J. (2016). How muscle structure and composition influence meat and flesh quality. The Scientific World Journal. https://doi.org/10.1155/2016/3182746

Liu, J., Li, X., Huang, J., Yang, G., & Ma, J. (2024). Additive-guided solvation-regulated flame- retardant electrolyte enables high-voltage lithium metal batteries with robust electrode electrolyte interphases. Advanced Functional Materials, 34(16), 2312762. https://doi.org/10.1002/adfm.202312762

Liu, M., Wang, S., Wei, Z., Song, Y., & Jiang, L. (2009). Bioinspired design of a superoleophobic and low-adhesive water/solid interface. Advanced Materials, 21(6), 665–669. https://doi.org/10.1002/adma.200801782

Liu, S. (2020). Bioprocess engineering: Kinetics, sustainability, and reactor design. Elsevier.

Liu, X., Sun, Q., Wang, H., Zhang, L., & Wang, J. Y. (2005). Microspheres of corn protein, zein, for an ivermectin drug delivery system. Biomaterials, 26(1), 109–115. https://doi.org/10.1016/j.biomaterials.2004.02.013

Liu, Y., Li, Y., Liu, T., Xie, C., & Zeng, Q. (2017). Taurine biosynthesis in a fish liver cell line (ZFL) adapted to a serum-free medium. Marine Drugs, 15(6), 147. https://doi.org/10.3390/md15060147

Loeb, M. J. (2005). Altering the fate of stem cells from midgut of *Heliothis virescens*: The effect of calcium ions. Archives of Insect Biochemistry and Physiology, 59(4), 202–210. https://doi.org/10.1002/arch.20060

Lu, S., Taethaisong, N., Meethip, W., Surakhunthod, J., Sinpru, B., Sroichak, T., Archa, P., Thongpea, S., Paengkoum, S., Purba, R. A. P., & Paengkoum, P. (2022). Nutritional composition of black soldier fly larvae (*Hermetia illucens* L.) and its potential uses as alternative protein sources in animal diets: A review. Insects, 13(9), 831. https://doi.org/10.3390/insects13090831

Lynn, D. E. (1996). Development and characterization of insect cell lines. Cytotechnology, 20(1–3), 3–11. https://doi.org/10.1007/BF00350384

Lynn, D. E. (1999). Development of insect cell lines: Virus susceptibility and applicability to prawn cell culture. Methods in Cell Science, 21(3), 173–181.

Lynn, D. E. (2002). Methods for maintaining insect cell cultures. Journal of Insect Science, 2(1), 9. https://doi.org/10.1093/jis/2.1.9.

Makkar, H. P. S., Tran, G., Heuzé, V., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed. Animal Feed Science and Technology, 197, 1–33. https://doi.org/10.1016/j. anifeedsci.2014.07.008

Mancini, M. C., & Antonioli, F. (2022). Italian consumers standing at the crossroads of alternative protein sources: Cultivated meat, insect-based and novel plant-based foods. *Meat Science, 193*, 108942. https://doi.org/10.1016/j.meatsci.2022.108942

Mancini, S., Moruzzo, R., Riccioli, F., & Paci, G. (2019). European consumers' readiness to adopt insects as food: A review. Food Research International, 122, 661–678. https://doi.org/10.1016/j.foodres.2019.01.041

Manniello, M. D., *et al.* (2021). Insect antimicrobial peptides: Potential weapons to counteract the antibiotic resistance. Cellular and Molecular Life Sciences, 78, 4259–4282. https://doi.org/10.1007/s00018-021-03784-z

Melzener, L., Verzijden, K. E., Buijs, A. J., Post, M. J., & Flack, J. E. (2021). Cultured beef: from small biopsy to substantial quantity. Journal of the Science of Food and Agriculture, 101(1), 7-14.

Messmer, T., *et al.* (2023). Single-cell analysis of bovine muscle-derived cell types for cultured meat production. Frontiers in Nutrition, 10, 1212196. https://doi.org/10.3389/ fnut.2023.1212196

Miranda, A., Sarmiento, L., Caldas, M., Zapata, C., & Bello, F. (2008). Morfología y citoquímica de cultivos celulares de *Aedes aegypti* (Diptera: Culicidae) y susceptibilidad a Leishmania panamensis (Kinetoplastida: Trypanosomatidae). Revista de Biología Tropical (Costa Rica), 56(2), 447–458.

Mitsuhashi, J. (2016). Edible insects of the world. CRC Press https://doi.org/10.1201/ 9781315367927

Mohan, K., Rajan, D. K., Muralisankar, T., Ganesan, A. R., Sathishkumar, P., & Re-vathi, N. (2022). Use of black soldier fly (*Hermetia illucens* L.) larvae meal in aquafeeds for a sustainable aquaculture industry: A review of past and future needs. Aquaculture, 553, 738095. https://doi.org/10.1016/j.aquaculture.2022.738095

Moraes, A. M., Jorge, S. A. C., Astray, R. M., Suazo, C. A. T., Calderón Riquelme, C. E., Augusto, E. F. P., Tonso, A., Pamboukian, M. M., & Piccoli, R. A. M. (2012). *Drosophila melanogaster* S2 cells for expression of heterologous genes: From gene cloning to bioprocess development. Biotechnology Advances, 30(3), 613–628. https://doi.org/10.1016/j.biotechadv.2011.10.009

Moruzzo, R., Mancini, S., Boncinelli, F., & Riccioli, F. (2021). Exploring the acceptance of entomophagy: A survey of italian consumers. Insects, 12, 123. https://doi.org/10.3390/ insects12020123

Moruzzo, R., Riccioli, F., Espinosa Diaz, S., Secci, C., Poli, G., & Mancini, S. (2021). Mealworm (*Tenebrio molitor*): Potential and challenges to promote circular economy. Animals, 11(9), 2568. https://doi.org/10.3390/ani11092568

Moutinho, S., Pedrosa, R., Magalhães, R., Oliva-Teles, A., Parisi, G., & Peres, H. (2021). Black soldier fly (*Hermetia illucens*) pre-pupae larvae meal in diets for european seabass (Dicentrarchus labrax) juveniles: Effects on liver oxidative status and fillet quality traits during shelf-life. Aquaculture, 533, 736080. 10.1016/j.aquaculture.2020.736080

Mueller, R. S., Olivry, T., & Prélaud, P. (2016). Critically appraised topic on adverse food reactions of companion animals (2): Common food allergen sources in dogs and cats. BMC Veterinary Research, 12, 1–9. 10.1186/s12917-016-0633-8

Murhammer, D. W. (Ed.). (2016). Baculovirus and insect cell expression protocols (Vol. 1350).

Springer. https://doi.org/10.1007/978-1-4939-3043-2

Nairuti, R.N., Musyoka, S.N., Yegon, M.J., Opiyo, M.A. (2021). Utilization of Black Soldier Fly (*Hermetia illucens* Linnaeus) Larvae as a Protein Source for Fish Feed – a Review. Aquaculture Studies, 22(2), AQUAST697. https://doi.org/10.4194/AQUAST697

Nayak, A., Rühl, M., & Klüber, P. (2023). *Hermetia illucens* (diptera: Stratiomyidae): Need, potentiality, and performance measures. Agriculture, 14(1), 8.

Nekrasov, R., Zelenchenkova, A., Chabaev, M., Ivanov, G., Antonov, A., & Pastukhova (2018). Dried black soldier fly larvae as a dietary supplement to the diet of growing pigs. Journal of Animal Science, 96, 314.

Newton, G. L., Booram, C. V., Barker, R. W., & Hale, O. M. (1977). Dried *Hermetia illucens* larvae meal as a supplement for swine. Journal of Animal Science, 44(3), 395–400. https://doi.org/10.2527/jas1977.443395x

Ng, S., & Kurisawa, M. (2021). Integrating biomaterials and food biopolymers for cultured meat production. Acta Biomaterialia, 124, 108–129. https://doi.org/10.1016/j.actbio.2021.01.017

Nowock, J., Goodman, W., Bollenbacher, W. E., & Gilbert, L. I. (1975). Synthesis of juvenile hormone binding proteins by the fat body of *Manduca sexta*. General and Comparative Endocrinology, 27, 230–239. https://doi.org/10.1016/0016-6480(75)90238-5

Nyakeri, E. M., Ogola, H. J., Ayieko, M. A., &Amimo, F. A. (2017). An open system for farming black soldier fly larvae as a source of proteins for small scale poultry and fish production. Journal of Insects as Food and Feed, 3, 51–56.

O'Neill, E. N., Cosenza, Z. A., Baar, K., & Block, D. E. (2021). Considerations for the development of cost-effective cell culture media for cultivated meat production. Comprehensive Reviews in Food Science and Food Safety, 20(1), 686–709. https://doi.org/10.1111/1541- 4337.12678

Ong, K. J., Tejeda-Saldana, Y., Duffy, B., Holmes, D., Kukk, K., & Shatkin, J. A. (2023). Cultured meat safety research priorities: Regulatory and governmental perspectives. Foods, 12(14), 2645. https://doi.org/10.3390/foods12142645

Oonincx, D. G. & de Boer, I. J. M. (2012). Environmental impact of the production of mealworms as a protein source for humans: A life cycle assessment. PLOS ONE, 7(12), e51145. https://doi.org/10.1371/journal.pone.0051145

Oonincx, D. G., Laurent, S., Veenenbos, M. E., & van Loon, J. J. (2020). Dietary enrichment of edible insects with omega-3 fatty acids. Insect Science, 27(3), 500–509. https://doi.org/10.1111/1744-7917.12669

Orkusz, A. (2021). Edible insects versus meat—Nutritional comparison: Knowledge of their composition is the key to good health. Nutrients, 13(4), 1207. https://doi.org/10.3390/nu13041207

Pajčin, I., Knežić, T., Savić-Azoulay, I., Vlajkov, V., Djisalov, M., Janjušević, L., Grahovac, J., & Gadjanski, I. (2022). Bioengineering outlook on cultivated meat production. Micromachines, 13(3), 402. https://doi.org/10.3390/mi13030402

Pamboukian, M. M., Jorge, S. A. C., Santos, M. G., Yokomizo, A. Y., Pereira, C. A., & Tonso, A. (2008). Insect cells respiratory activity in bioreactor. Cytotechnology, 57(1), 37–44. https://doi.org/10.1007/s10616-007-9118-8

Parodi, A., Leip, A., de Boer, I. J. M., Slegers, P. M., Ziegler, F., & Erb, K.-H. (2018). The potential of future foods for sustainable and healthy diets. Nature Sustainability, 1(12), 782–789. https://doi.org/10.1038/s41893-018-0189-7

Pasitka, L., Cohen, M., Ehrlich, A., Gildor, B., Reuveni, E., Ayyash, M., Wissotsky, G., Herscovici, A., Niv, A., Bitcover, R., Dadia, O., Rudik, A., Voloschin, A., Shimoni, M., Cinnamon, Y., & Nahmias, Y. (2023). Spontaneous immortalization of chicken fibroblasts generates stable, high-yield cell lines for serum-free production of cultured meat. Nature Food, 4, 391–403. https://doi.org/10.1038/s43016-022-00658-w

Pasitka, L., Cohen, M., Regenbaum, S., Ehrlich, A., Gildor, B., Gold, A., & Nahmias, Y. (2025). Spontaneous immortalization of bovine fibroblasts following long-term expansion offers a non-transformed cell source for cultivated beef. Nature Food, 6(11), 1079–1094. https://doi.org/10.1038/s43016-025-01255-3

Penazzi, L. (2021). *In vivo* and *in vitro* digestibility of an extruded complete dog food containing black soldier fly (*Hermetia illucens*) larvae meal as protein source. Frontiers in Veterinary Science, 8, 542–550.

Penedo, A. O., *et al.* (2022). The consumption of insects in Switzerland: Legal framework and consumer perspectives. International Journal of Environmental Research and Public Health, 19(16), 10120. https://doi.org/10.3390/ijerph191610120

Percival, N. J. (2002). Classification of wounds and their management. Surgery (Oxford), 20(5), 114–117. https://doi.org/10.1383/surg.20.5.114.14626

Pinillos, I., Pérez, C., Torres, O., Patarroyo, M. A., & Bello, F. J. (2022). Establishing and characterising a new cell line from *Calliphora vicina* (Diptera: Calliphoridae) fly embryonic tissues. Heliyon, 8(9), e10674. https://doi.org/10.1016/j.heliyon.2022.e10674

Poovathumkadavil, P., & Jagla, K. (2020). Genetic control of muscle diversification and homeostasis: Insights from *Drosophila*. Cells, 9(6), 1543. https://doi.org/10.3390/cells9061543

Post, M. J. (2012). Cultured meat from stem cells: Challenges and prospects. Meat Science, 92(3), 297–301. https://doi.org/10.1016/j.meatsci.2012.04.008

Post, M. J. (2014). Cultured beef: Medical technology to produce food. Journal of the Science of Food and Agriculture, 94(6), 1039–1041. https://doi.org/10.1002/jsfa.6474

Post, M. J., *et al.* (2020). Scientific, sustainability and regulatory challenges of cultured meat. Nature Food, 1(7), 403–415. https://doi.org/10.1038/s43016-020-0112-z

Potting, R. (2014). Advisory report on the risks associated with the consumption of mass-reared insects. https://www.researchgate.net/publication/277716517. May 22, 2025.

Premrov Bajuk, B., Zrimšek, P., Kotnik, T., Leonardi, A., Križaj, I., & Strajn, B. J. (2021). Insect protein-based diet as potential risk of allergy in dogs. Animals, 11, 1942. https://doi.org/10. 3390/ani11071942

Priyadarshana, M. K. C., Walpita, C. N., Naveenan, M., Magamage, M. P. S., & Ruwan-deepika, H.

A. D. (2021). Substitution of fishmeal with black soldier fly *Hermetia illucens* Linnaeus, 1758 larvae in finfish aquaculture – A review. Asian Fisheries Science, 34(2). https://doi.org/10.33997/j.afs.2021.34.2.001

Proc, K., Bulak, P., Wi¸acek, D., & Bieganowski, A. (2020). *Hermetia illucens* exhibits bioaccumulative potential for 15 different elements: Implications for feed and food production. Science of the Total Environment, 723, 138125.
https://doi.org/10.1016/j.scitotenv.2020. 138125

Pulido, L., Secci, G., Maricchiolo, G., Gasco, L., Gai, F., Serra, A., & Parisi, G. (2022). Effect of dietary black soldier fly larvae meal on fatty acid composition of lipids and sn-2 position of triglycerides of marketable size gilthead sea bream fillets. Aquaculture, 546, 737351

Qu, Z. H., Wang, H. J., Tang, T. T., Zhang, X. L., Wang, J. Y., & Dai, K. R. (2008). Evaluation of the zein/inorganics composite on biocompatibility and osteoblastic differentiation. Acta Biomaterialia, 4(5), 1360–1368. https://doi.org/10.1016/j.actbio.2008.03.006

Raikhel, A. S., Deitsch, K. W., & Sappington, T. W. (1997). Culture and analysis of the insect fat body. In The Molecular Biology of Insect Disease Vectors: A Methods Manual (pp. 507– 522). Springer. https://doi.org/10.1007/978-94-009-1535-0_41

Ramos-Elorduy, J., Moreno, J. M. P., Prado, E. E., Perez, M. A., Otero, J. L., & de Guevara, O. L. (1997). Nutritional value of edible insects from the state of

Oaxaca, Mexico. Journal of Food Composition and Analysis, 10(2), 142–157. https://doi.org/10.1006/jfca. 1997.0530

Rao, P. P., Birthal, P. S., & Ndjeunga, J. (2005). Crop-livestock economies in the semi-arid tropics. (Report).

Reddy, N., & Yang, Y. (2011). Potential of plant proteins for medical applications. Trends in Biotechnology, 29(10), 490–498. https://doi.org/10.1016/j.tibtech.2011.05.003

Redshaw, Z., & Loughna, P. T. (2012). Oxygen concentration modulates the differentiation of muscle stem cells toward myogenic and adipogenic fates. Differentiation, 84(2), 193–202. https://doi.org/10.1016/j.diff.2012.06.001

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. (2003). European Commission.

Reiss, J., Robertson, S., & Suzuki, M. (2021). Cell sources for cultivated meat: Applications and considerations throughout the production workflow. International Journal of Molecular Sciences, 22(14), 7513. https://doi.org/10.3390/ijms22147513

Rey, G., Ferro, C., & Bello, F. (2000). Establishment and characterization of a new continuous cell line from *Lutzomyia longipalpis* (Diptera: Psychodidae) and its susceptibility to infections with arboviruses and *Leishmania chagasi*. Memórias do Instituto Oswaldo Cruz (Brasil), 95(1), 103–110.

Rodríguez Escobar, M. I., Cadena, E., Nhu, T. T., Cooreman-Algoed, M., De Smet, S., & Dewulf, J. (2021). Analysis of the cultured meat production system in function of its environmental footprint: Current status, gaps and recommendations. Foods, 10(12), 2941. https://doi.org/10.3390/foods10122941

Rodríguez, K., Renneckar, S., & Gatenholm, P. (2011). Biomimetic calcium phosphate crystal mineralization on electrospun cellulose-based scaffolds. ACS Applied Materials & Interfaces, 3(3), 681–689. https://doi.org/10.1021/am100972r

Roma, G. C., Bueno, O. C., & Camargo-Mathias, M. I. (2010). Morpho-physiological analysis of the insect fat body: A review. Micron, 41(5), 395–401.

Rout, P., Preußner, M., & Önel, S. F. (2022). *Drosophila melanogaster*: A model system to study distinct genetic programs in myoblast fusion. Cells, 11(3), 321.

Rubio, N. R., Fish, K. D., Trimmer, B. A., & Kaplan, D. L. (2019a). *In vitro* insect muscle for tissue engineering applications. ACS Biomaterials Science & Engineering, 5(2), 1071–1082. https://doi.org/10.1021/acsbiomaterials.8b01261

Rubio, N. R., Fish, K. D., Trimmer, B. A., & Kaplan, D. L. (2019b). Possibilities for engineered insect tissue as a food source. Frontiers in Sustainable Food Systems, 3, 24. https://doi.org/10.3389/fsufs.2019.00024

Rubio, N. R., McCartney, N. E., Trimmer, B. A., & Kaplan, D. L. (2020a). Biofabrication with insect cells. Trends in Entomology, 16, 1–17. https://doi.org/10.5281/zenodo.7245722

Rubio, N. R., Xiang, N., & Kaplan, D. L. (2020b). Plant-based and cell-based approaches to meat production. Nature Communications, 11(1), 6276. https://doi.org/10.1038/s41467-020-20061-y

Rumpold, B. A., & Schlüter, O. K. (2013). Nutritional composition and safety aspects of edible insects. Molecular Nutrition & Food Research, 57(5), 802–823. https://doi.org/10.1002/mnfr.201200735

Ryu, W. S. (2016). Molecular virology of human pathogenic viruses. Academic Press.

Sahoo, A., Swain, S. S., Behera, A., Sahoo, G., Mahapatra, P. K., & Panda, S. K. (2021). Antimicrobial peptides derived from insects offer a novel therapeutic option to combat biofilm: A review. Frontiers in Microbiology, 12, 661195. https://doi.org/10.3389/fmicb.2021.661195

Salehi, S., *et al.* (2023). A 3D printed polylactic acid-Baghdadite nanocomposite scaffold coated with microporous chitosan-VEGF for bone regeneration applications. https://doi.org/10.1016/j.carbpol.2023.120787

Sandmann, T., Girardot, C., Brehme, M., Tongprasit, W., Stolc, V., & Furlong, E. E. M. (2007). A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. Genes & Development, 21(4), 436–449.

Saxton, A., Tariq, M. A., & Bordoni, B. (2022). Cardiac muscle. In StatPearls. StatPearls Publishing.

Scala, A., *et al.* (2020). Rearing substrate impacts growth and macronutrient composition of *Hermetia illucens* (L.) (Diptera: Stratiomyidae) larvae produced at an industrial scale. Scientific reports, 10(1), 19448.

Scalia, C. R., *et al.* (2017). Antigen masking during fixation and embedding, dissected. Journal of Histochemistry & Cytochemistry, 65(1), 5–20. https://doi.org/10.1369/0022155416673995

Schätzlein, E., & Blaeser, A. (2022). Recent trends in bioartificial muscle engineering and their applications in cultured meat, biorobotic systems and biohybrid implants. Communications Biology, 5(1), 737. https://doi.org/10.1038/s42003-022-03593-5

Schiavone, A., *et al.* (2017). Partial or total replacement of soybean oil by black soldier fly larvae (*Hermetia illucens* L.) fat in broiler diets: Effect on growth performances, feed-choice, blood traits, carcass characteristics and meat quality. Italian Journal of Animal Science, 16(1), 93–100. https://doi.org/10.1080/1828051X.20161249968

Schmidt, M., Schüler, S. C., Hüttner, S. S., von Eyss, B., & von Maltzahn, J. (2019). Adult stem cells at work: Regenerating skeletal muscle. Cellular and Molecular Life Sciences, 76(13), 2559–2570. https://doi.org/10.1007/s00018-019-03093-6

Schmidt-Nielsen, K. (1997). Animal physiology: Adaptation and environment. Cambridge University Press.

Schneider, I. (1964). Differentiation of larval *Drosophila eye-antennal* discs *in vitro*. Journal of Experimental Zoology, 156(1), 91–103. https://doi.org/10.1002/jez.1401560107

Schnorrer, F., & Dickson, B. J. (2004). Muscle building: Mechanisms of myotube guidance and attachment site selection. Developmental Cell, 7(1), 9–20. https://doi.org/10.1016/j.devcel.2004.06.010

Scieuzo, C., *et al.* (2023). *In vitro* evaluation of the antibacterial activity of the peptide fractions extracted from the hemolymph of *Hermetia illucens* (Diptera: Stratiomyidae). Insects, 14(5), 464. https://doi.org/10.3390/insects14050464

Seah, J. S. H., Singh, S., Tan, L. P., & Choudhury, D. (2022). Scaffolds for the

manufacture of cultured meat. Critical Reviews in Biotechnology, 42(2), 311–323. https://doi.org/10.1080/07388551.2021.1931803

Segura, N., Santamaría, E., Cabrera, O., & Bello, F. (2012). Establishment and characterisation of a new cell line derived from *Culex quinquefasciatus* (Diptera: Culicidae). Memórias do Instituto Oswaldo Cruz, 107(1), 89–95.

Seo, J. W., Jung, W. K., Park, Y. H., & Bae, H. (2023). Development of cultivable alginate fibers for an ideal cell-cultivated meat scaffold and production of hybrid cultured meat. Carbohydrate Polymers, 321, 121287. https://doi.org/10.1016/j.carbpol.2023.121287

Shefer, G., & Yablonka-Reuveni, Z. (2005). Isolation and culture of skeletal muscle myofibers as a means to analyze satellite cells. Methods in Molecular Biology, 290, 281–304. https://doi.org/10.1385/1-59259-838-2:281

Siddiqui, S. A., Brunner, T. A., Tamm, I., van der Raad, P., Patekar, G., Bahmid, N. A., Paul, A. (2023). Insect-based dog and cat food: A short investigative review on market, claims and consumer perception. Journal of Asia-Pacific Entomology, 26(1), 102020. https://doi.org/10.1016/j.aspen.2022.102020

Siddiqui, S. A., Ngah, N., Wu, Y. S., Kalita, T., Yudhistira, B., & Ibrahim, S. A. (2024). Lab-grown insect meat–Chemical and biological insights–A comprehensive review. Journal of Insects as Food and Feed, 11(2), 401-428.

Sigma-Aldrich. (2011). EX-CELL™ TiterHigh™ Sf Insect Medium (I5408) – Product information sheet. Sigma-Aldrich, St. Louis, MO, USA.

Silva, B. C. R., Lehnen, C. R., & Marcato, S. M. (2024). Black soldier fly (*Hermetia illucens*) as a protein ingredient in poultry feed.World's Poultry Science Journal, 80(4), 1123–1154. https://doi.org/10.1080/00439339.2024.2384880

Simcox, A., *et al.* (2008). Efficient genetic method for establishing *Drosophila* cell lines unlocks the potential to create lines of specific genotypes. PLOS Genetics, 4(8), e1000142. https://doi.org/10.1371/journal.pgen.1000142

Sinke, P., Swartz, E., Sanctorum, H., Van Der Giesen, C., & Odegard, I. (2023). Ex-ante life cycle assessment of commercial-scale cultivated meat production in 2030. The International Journal of Life Cycle Assessment, 28(3), 234-254. https://doi.org/10.1007/s11367-022- 02128-8

Smagghe, G., Goodman, C. L., & Stanley, D. (2009). Insect cell culture and applications to research and pest management. *In vitro* Cellular & Developmental Biology – Animal, 45(3–4), 93– 105.

Smetana, S., Schmitt, E., & Mathys, A. (2019). Sustainable use of *Hermetia illucens* insect biomass for feed and food: Attributional and consequential life cycle assessment. Resources, Conservation & Recycling, 144, 285–296. https://doi.org/10.1016/j.resconrec.2019.01.042

Soares Araújo, R. R., dos Santos Benfica, T. A. R., Ferraz, V., & Santos, E. M. (2019). Nutritional composition of insects Gryllus assimilis and Zophobas morio: Potential foods harvested in Brazil. Journal of Food Composition and Analysis, 76, 22–26. https://doi.org/10.1016/j.jfca.2018.11.005

Sogari, G., Menozzi, D., & Mora, C. (2018). Sensory-liking expectations and perceptions of processed and unprocessed insect products. International Journal on Food System Dynamics, 9(4), 314–320. https://doi.org/10.18461/ijfsd.v9i4.942

Song, W.-J., Liu, P.-P., Zheng, Y.-Y., Meng, Z.-Q., Zhu, H.-Z., Tang, C.-B., Li, H.-X., Ding, S.-J., & Zhou, G.-H. (2022). Production of cultured fat with peanut wire-drawing protein scaffold and quality evaluation based on texture and volatile compounds analysis. Food Research International, 160, 111636.

https://doi.org/10.1016/j.foodres.2022.111636

Specht, E. A., Welch, D. R., Clayton, E. M., & Lagally, C. D. (2018). Opportunities for applying biomedical production and manufacturing methods to the development of the clean meat industry. Biochemical Engineering Journal, 132, 161–168. https://doi.org/10.1016/j.bej.2018.01.015

Spletter, M. L., & Schnorrer, F. (2014). Transcriptional regulation and alternative splicing cooperate in muscle fiber-type specification in *Drosophila*. Experimental Cell Research, 321(1), 90– 98. https://doi.org/10.1016/j.yexcr.2013.10.007

Spoto, G. (2024). Lab-grown meat and the proposed Italian ban. Manuscript/preprint available at https://www.researchgate.net/publication/376872537_Lab-grown_Meat_and_the_Proposed_Italian_Ban

Spranghers, T., *et al.* (2017). Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. Journal of the Science of Food and Agriculture, 97(8), 2594–2600. https://doi.org/10.1002/jsfa.8081

Stephens, N., Di Silvio, L., Dunsford, I., Ellis, M., Glencross, A., & Sexton, A. (2018). Bringing cultured meat to market: Technical, socio-political, and regulatory challenges in cellular agriculture. Trends in Food Science & Technology, 78, 155–166. https://doi.org/10.1016/j.tifs.2018.04.010

Stout, A. J., Mirliani, A. B., Soule-Albridge, E., Cohen, J. M., Beltran, A. M., Uiterweer, A., Kaplan,D. L. (2022). Simple and effective serum-free medium for sustained expansion of bovine satellite cells for cell cultured meat. Communications Biology, 5, 466. https://doi.org/10.1038/s42003-022-03423-8

Strachecka, A., *et al.* (2017). Insights into the biochemical defence and methylation of the solitary bee *Osmia rufa* L.: A foundation for examining eusociality development. PLOS ONE, 12(4), e0176539. https://doi.org/10.1371/journal.pone.0176539

Suddep, A., Khushiramani, R., Athawale, S., Mishra, A., & Mourya, D. (2005). Characterization of a newly established potato tuber moth (*Phthorimaea operculella Zeller*) cell line. Indian Journal of Medical Research, 121(3), 159–163.

Sugimoto, T. N., Watanabe, K., Akiduki, G., Imanishi, S., & Mitsuhashi, W. (2022). A new continuous cell line from the pest insect, Anomala cuprea (Coleoptera; Scarabaeidae): Emergence of contractile cells. *In vitro* Cellular & Developmental Biology–Animal, 58(7), 610–618. https://doi.org/10.1007/s11626-022-00707-5

Sun, C., & Zhang, S. (2015). Immune-relevant and antioxidant activities of vitellogenin and yolk proteins in fish. Nutrients, 7(10), 8818–8829. https://doi.org/10.3390/nu7105432

Sverguzova, S. V., Shaikhiev, I. H., Sapronova, Z. A., Fomina, E. V., & Makridina, Y. L. (2021). Use of fly larvae *Hermetia illucens* in poultry feeding: A review paper. Journal of Water and Land Development, 49 (IV–VI), 95–103. https://doi.org/10.24425/jwld.2021.137101

Syverud, B. C., VanDusen, K. W., & Larkin, L. M. (2016). Growth factors for skeletal muscle tissue engineering. Cells Tissues Organs, 202(3–4), 169–179. https://doi.org/10.1159/000444671

Szikora, S., *et al.* (2020). Super-resolution microscopy of *Drosophila* indirect flight muscle reveals a regular pattern of myosin-binding protein distribution. Journal of Cell Science, 133(2), jcs234148.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7842721/

Tafi, E., *et al.* Preliminary investigation on the effect of insect-based chitosan on preservation of coated fresh cherry tomatoes. Sci Rep 13, 7030 (2023). https://doi.org/10.1038/s41598- 023-33587-0

Tahamtani, F. M., Ivarsson, E., Wiklicky, V., Lalander, C., Wall, H., Rodenburg, T. B., & Hernandez, C. E. (2021). Feeding live black soldier fly larvae (*Hermetia illucens*) to laying hens: Effects on feed consumption, hen health, hen behavior, and egg quality. Poultry Science, 100(10), 101400

Takahashi, A., Ohtani, N., & Hara, E. (2007). Irreversibility of cellular senescence: Dual roles of p16INK4a/Rb-pathway in cell cycle control. Cell Division, 2, 10. https://doi.org/10.1186/1747-1028-2-10

Tamir, H., & Gilvarg, C. (1966). Density gradient centrifugation for the separation of sporulating forms of bacteria. Journal of Biological Chemistry, 241(5), 1085–1090. https://doi.org/10.1016/S0021-9258(18)96806-7

Tan, H. S. G., van den Berg, E., & Stieger, M. (2016). The influence of product preparation, familiarity and individual traits on the consumer acceptance of insects as food. Food Quality and Preference, 52, 222–231. https://doi.org/10.1016/j.foodqual.2016.05.003

Taylor, M. V. (2007). Comparison of muscle development in *Drosophila* and vertebrates.

Developmental Biology, 1, 10–25. https://doi.org/10.1007/0-387-32963-3_14.

Tedesco, F., Pietrafesa, R., Siesto, G., Scieuzo, C., Salvia, R., Falabella, P., & Capece, A. (2024). Antimicrobial Activity of Chitosan from Different Sources Against Non-Saccharomyces Wine Yeasts as a Tool for Producing Low-Sulphite Wine. Beverages, 10(4), 105. https://doi.org/10.3390/beverages10040105

The European Parliament and the Council of the European Union (2015). Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods. Official Journal of the European Union.

Thorley, M., *et al.* (2016). Skeletal muscle characteristics are preserved in hTERT/cdk4 human myogenic cell lines. Skeletal Muscle, 6, 43.

Thyden, R., *et al.* (2022). An edible, decellularized plant-derived cell carrier for lab-grown meat.Applied Sciences, 12(10), 5155. https://doi.org/10.3390/app12105155

Tomberlin, J. K., Van Huis, A., Benbow, M. E., Jordan, H., Astuti, D. A., Azzollini, D., & Zheng, L. (2015). Protecting the environment through insect farming as a means to produce protein for use as livestock, poultry, and aquaculture feed. Journal of Insects as Food and Feed, 1(4), 307–309.

Toong, D. W. Y., *et al.* (2020). Bioresorbable polymeric scaffold in cardiovascular applications. International Journal of Molecular Sciences, 21(10), 3444. https://doi.org/10.3390/ijms21103444

Toprak, U. (2020). The role of peptide hormones in insect lipid metabolism. Frontiers in Physiology, 11, 434. https://doi.org/10.3389/fphys.2020.00434

Tran, D. B., Weber, C., & Lopez, R. A. (2019). Anatomy, thorax, heart muscles.

Tran, G., Heuzé, V., Makkar, H. P. S., & Ankers, P. (2014). State-of-the-art on use of insects as animal feed.

Treich, N. (2021). Cultured meat: Promises and challenges. Environmental and Resource Economics, 79, 33–61. https://doi.org/10.1007/s10640-021-00551-3

Triunfo, M., *et al.* (2022). Characterization of chitin and chitosan derived from *Hermetia illucens*, a further step in a circular economy process. Scientific Reports, 12, 6613. https://doi.org/10.1038/s41598-022-10423-5

Triunfo, M., *et al.* (2023). *Hermetia illucens*, an innovative and sustainable source of chitosan-based coating for postharvest preservation of strawberries. iScience, 26(12), 108576. https://doi.org/10.1016/j.isci.2023.108576

Triunfo, M., *et al.* (2024). A comprehensive characterization of *Hermetia illucens* derived chitosan produced through homogeneous deacetylation. International Journal of Biological Macromolecules, 271(2), 132669. https://doi.org/10.1016/j.ijbiomac.2024.132669

Tschirner, M., & Kloas, W. (2017). Increasing the sustainability of aquaculture systems: Insects as alternative protein source for fish diets. GAIA - Ecological Perspectives for Science and Society, 26(4), 332–340. https://doi.org/10.14512/gaia.26.4.10

Tucillo, F., Marino, M. G., & Torri, L. (2020). Italian consumers' attitudes towards entomophagy: Influence of human factors and properties of insects and insect-based food. Food Research International, 137, 109619.

Tuomisto, H. L., & de Mattos, M. J. (2011). Environmental impacts of cultured meat production. Environmental Science & Technology, 45(14), 6117–6123. https://doi.org/10.1021/es200130u

U.S. Food and Drug Administration. (2024). Human food made with cultured animal cells. Retrieved April 2024, from https://www.fda.gov/food/food-ingredients-packaging/human-food- made-cultured-animal-cells

Urbán-Duarte, D., *et al.* (2022). Effect of chemical dechorionation on silkworm embryo viability. Journal of Insect Physiology, 137, 104327. https://doi.org/10.1016/j.jinsphys.2021.104327

Urbani, L., Piccoli, M., Franzin, C., Pozzobon, M., & De Coppi, P. (2012). Hypoxia increases mouse satellite cell clone proliferation maintaining both *in vitro* and *in vivo* heterogeneity and myogenic potential. PLOS ONE, 7(11), e49860. https://doi.org/10.1371/journal.pone.0049860

US Food and Drug Administration. (2022). Cell Culture Consultation 000002. Scientific Memo.Center for Food Safety and Applied Nutrition, College Park, MD.

US Food and Drug Administration. (2023). Cell Culture Consultation 000001. Scientific Memo.Center for Food Safety and Applied Nutrition, College Park, MD.

USDA–ERS. (2025). The economics of cellular agriculture (ERR-342). U.S. Department of Agriculture, Economic Research Service. https://ers.usda.gov/publications/pub- details?pubid=110622

Vafopoulou, X., Steel, C. G. H., & Terry, K. L. (2005). Ecdysteroid receptor (EcR) shows marked differences in temporal patterns between tissues during larval–adult development in *Rhodnius prolixus*: Correlations with haemolymph ecdysteroid titres. Journal of Insect Physiology, 51(1), 27–38. https://doi.org/10.1016/j.jinsphys.2004.11.001

Vafopoulou, X. (2012). Cytoplasmic travels of the ecdysteroid receptor in target cells: Pathways for both genomic and non-genomic actions. Frontiers in Endocrinology, 3, 43. https://doi.org/10.3389/fendo.2012.00043

van der Fels-Klerx, H. J., Meijer, N., Nijkamp, M. M., Schmitt, E., & van Loon, J. J. A. (2020). Chemical food safety of using former foodstuffs for rearing black soldier fly larvae (*Hermetia illucens*) for feed and food use. Journal of Insects as Food and Feed, 6(5), 475–488. https://doi.org/10.3920/JIFF2020.0024

van Huis, A., van Itterbeeck, J., Klunder, H., Mertens, E., Halloran, A., Muir, G., & Vantomme, P. (2013). Edible insects: Future prospects for food and feed security (FAO Forestry Paper No. 171). Food and Agriculture Organization of the United Nations. Retrieved September 2025, from http://www.fao.org/3/i3253e/i3253e.pdf

van Huis, A., & Oonincx, D. G. (2017). The environmental sustainability of insects as food and feed: A review. Agronomy for Sustainable Development, 37(5), 43. https://doi.org/10.1007/s13593-017-0452-8

Van Huis, A., Rumpold, B., Maya, C., & Roos, N. (2021). Nutritional qualities and enhancement of edible insects. Annual review of nutrition, 41(1), 551-576.

Vitti, A., *et al.* (2024). *In vitro* antifungal activity and *in vivo* edible coating efficacy of insect-derived chitosan against *Botrytis cinerea* in strawberry. International Journal of Biological Macromolecules, 279, 135158. https://doi.org/10.1016/j.ijbiomac.2024.135158

Waldherr, S. (2018). Estimation methods for heterogeneous cell population models in systems biology. Journal of the Royal Society Interface, 15(147), 20180530. https://doi.org/10.1098/rsif.2018.0530

Wang, H. C., Liao, H. Y., & Chen, H. L. (2011). *Tenebrio* small-scale ecological farming feasibility study. Advanced Materials Research, 356–360, 267–270. https://doi.org/10.4028/www.scientific.net/AMR.356-360.267

Wang, Y. S., & Shelomi, M. (2017). Review of black soldier fly (*Hermetia illucens*) as animal feed and human food. Foods, 6(10), 91. https://doi.org/10.3390/foods6100091

Weitkunat, M., & Schnorrer, F. (2014). A guide to study *Drosophila muscle* biology. Methods, 68(1), 2–14. https://doi.org/10.1016/j.ymeth.2014.02.037

WWF Italia (2025). Allevamenti nemici del clima. World Wildlife Fund Italia. https://www.wwf.it/pandanews/clima/allevamenti-nemici-del-clima. May 22, 2025.

Wyatt, G. R. (1988). Vitellogenin synthesis and the analysis of juvenile hormone action in locust fat body. Canadian Journal of Zoology, 66, 2600–2610. https://doi.org/10.1139/z88-384

Wynants, E., Frooninckx, L., Crauwels, S., Verreth, C., De Smet, J., Sandrock, C., Wohlfahrt, J., Van Schelt, J., Depraetere, S., Lievens, B., Van Miert, S., Claes, J., & Van Campen-hout, L. (2019). Assessing the microbiota of black soldier fly larvae (*Hermetia illucens*) reared on organic waste streams on four different locations at laboratory and large scale. Microbial Ecology, 77(4), 913–930. https://doi.org/10.1007/s00248-018-1286-x

Xu, C., Jiang, J., Sottile, V., McWhir, J., Lebkowski, J., & Carpenter, M. K. (2004). Immortalized fibroblast-like cells derived from human embryonic stem cells support undifferentiated cell growth. Stem Cells, 22(6), 972–972.

Xu, X., Ji, H., Yu, H., & Zhou, J. (2020). Influence of dietary black soldier fly (*Hermetia illucens* Linnaeus) pulp on growth performance, antioxidant capacity and intestinal health of juvenile mirror carp (*Cyprinus carpio* var. *specularis*).

Aquaculture Nutrition, 26(2), 432–443. https://doi.org/10.1111/anu.13005

Yamamoto, Y., Ito, A., Jitsunobu, H., Yamaguchi, K., Kawabe, Y., Mizumoto, H., & Kamihira, M. (2012). Hollow fiber bioreactor perfusion culture system for magnetic force-based skeletal muscle tissue engineering. Journal of Chemical Engineering of Japan, 45(5), 348–354. https://doi.org/10.1252/jcej.11we237

Yao, T., & Asayama, Y. (2017). Animal-cell culture media: History, characteristics, and current issues. Reproductive medicine and biology, 16(2), 99-117.

Yin, H., Price, F., & Rudnicki, M. A. (2013). Satellite cells and the muscle stem cell niche. Physiological Reviews, 93(1), 23–67. https://doi.org/10.1152/physrev.00043.2011

Yoshioka, K., Kitajima, Y., Okazaki, N., Chiba, K., Yonekura, A., & Ono, Y. (2020). A modified pre- plating method for high-yield and high-purity muscle stem cell isolation from human/mouse skeletal muscle tissues. Frontiers in Cell and Developmental Biology, 8, 793. https://doi.org/10.3389/fcell.2020.00793

Yu, G., Wu, X., Kilroy, G., Halvorsen, Y. D. C., Gimble, J. M., & Floyd, Z. E. (2011). Isolation of murine adipose-derived stem cells. In Adipose-Derived Stem Cells: Methods and Protocols (pp. 29–36). Humana Press. https://doi.org/10.1007/978-1-61737-960-4_3

Yu, M., Li, Z., Chen, W., Rong, T., Wang, G., Li, J., & Ma, X. (2019). Use of *Hermetia illucens* larvae as a dietary protein source: Effects on growth performance, carcass traits, and meat quality in finishing pigs. Meat Science, 158, 107837.

Yuan, Y., & Hong, Y. (2017). Medaka insulin-like growth factor-2 supports self-renewal of the embryonic stem cell line and blastomeres *in vitro*. Scientific Reports, 7, 78. https://doi.org/10.1038/s41598-017-00094-y

Yuen, J. S., *et al.* (2022). Perspectives on scaling production of adipose tissue for food applications.Biomaterials, 280, 121273. https://doi.org/10.1016/j.biomaterials.2021.121273

Yun, S. H., *et al.* (2023). Improved culture procedure for bovine muscle satellite cells for cultured meat. Food Research International, 174, 113660. https://doi.org/10.1016/j.foodres.2023.113660

Zagury, Y., Ianovici, I., Landau, S., Lavon, N., & Levenberg, S. (2022). Engineered marble-like bovine fat tissue for cultured meat. Communications Biology, 5(1), 927. https://doi.org/10.1038/s42003-022-03852-5

Zarrintaj, P., Manouchehri, S., Ahmadi, Z., Saeb, M. R., Urbanska, A. M., Kaplan, D. L., & Mozafari, M. (2018). Agarose-based biomaterials for tissue engineering. Carbohydrate Polymers, 187, 66–84. https://doi.org/10.1016/j.carbpol.2018.01.060

Zhang, G., & Block, D. E. (2009). Using highly efficient nonlinear experimental design methods for optimization of *Lactococcus lactis* fermentation in chemically defined media. Biotechnology Progress, 25(6), 1587–1597. https://doi.org/10.1002/btpr.277

Zhang, G., Mills, D. A., & Block, D. E. (2009). Development of chemically defined media supporting high-cell-density growth of lactococci, enterococci, and streptococci. Applied and Environmental Microbiology, 75(4), 1080–1087. https://doi.org/10.1128/AEM.01416-08

Zhang, G., Olsen, M. M., & Block, D. E. (2007). New experimental design method for highly nonlinear and dimensional processes. AIChE Journal, 53(8), 2013–2025. https://doi.org/10.1002/aic.11226

Zhang, X., Feng, Y., Ding, W.-F., Li, X., & Wang, C.-Y. (2015). A new continuous cell line from Blaps rhynchoptera Fairmaire (Coleoptera: Tenebrionidae). *In vitro* Cellular & Developmental Biology–Animal, 51(2), 151–156. https://doi.org/10.1007/s11626-014-9815-5

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., & Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature, 372(6505), 425–432. https://doi.org/10.1038/372425a0

Zhao, A., *et al.* (2017). Use of real-time cellular analysis and Plackett–Burman design to develop the serum-free media for PC-3 prostate cancer cells. PLOS ONE, 12(9), e0185470. https://doi.org/10.1371/journal.pone.0185470

Zhu, H., *et al.* (2022). Production of cultured meat from pig muscle stem cells. Biomaterials, 287, 121650. https://doi.org/10.1016/j.biomaterials.2022.121650.

Zielińska, E., Baraniak, B., Karaś, M., Rybczynska, K., & Jakubczyk, A. (2018). Selected species of edible insects as a source of nutrient composition. Food Research International, 105, 377–383. https://doi.org/10.1016/j.foodres.2017.11.008

APPENDIX

Figure A1. *Effect of enzymatic dissociation method on Hermetia illucens primary cell viability.*

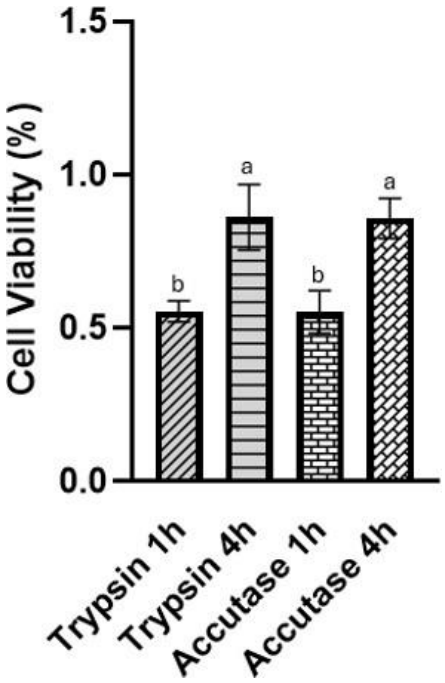


Fig A1. Primary embryonic cells were detached using either 0.50% Trypsin-EDTA or 0.50% Accutase and assessed for metabolic activity using the Alamar Blue assay after 1 h or 4 h of reagent incubation. Mean cell viability (%) ± SD is shown (n = 3 biological replicates, each with two technical replicates). One-way ANOVA with Tukey's *post hoc* test revealed significantly higher viability after 4 h compared to 1 h for both Trypsin-EDTA ($p = 0.0041$) and Accutase ($p = 0.0042$), with no significant difference between enzymatic treatments at either incubation time. Letters above the bars denote statistically homogeneous groups ($p < 0.01$). These findings indicate that Accutase, a non-animal-derived dissociation reagent, can preserve short-term viability of *H. illucens* embryonic cells to a comparable extent as Trypsin-EDTA, supporting its potential as an animal-free alternative for cultivated meat applications.

Figure A2. *Negative controls for immunostaining in 19-h primary cultures of Hermetia illucens: phase-contrast and DAPI/phalloidin fluorescence*

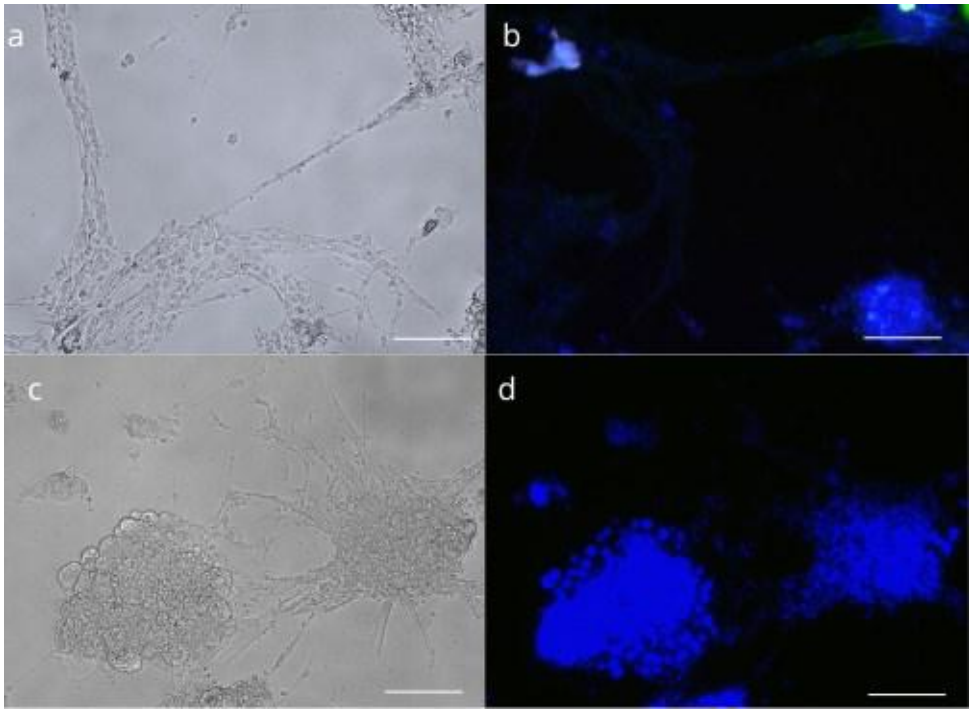


Fig A2. a,b: Negative control 1: phase-contrast and fluorescence images showing only DAPI and actin (phalloidin, green), confirming specificity when the primary antibody is omitted. **c,d:** Negative control 2: no red or green signals are detected when both secondary antibody and phalloidin are omitted. Negative controls were performed on cells from the 19 h timepoint, chosen as representative since all samples followed identical fixation and staining procedures. Scale bars:100 µm.

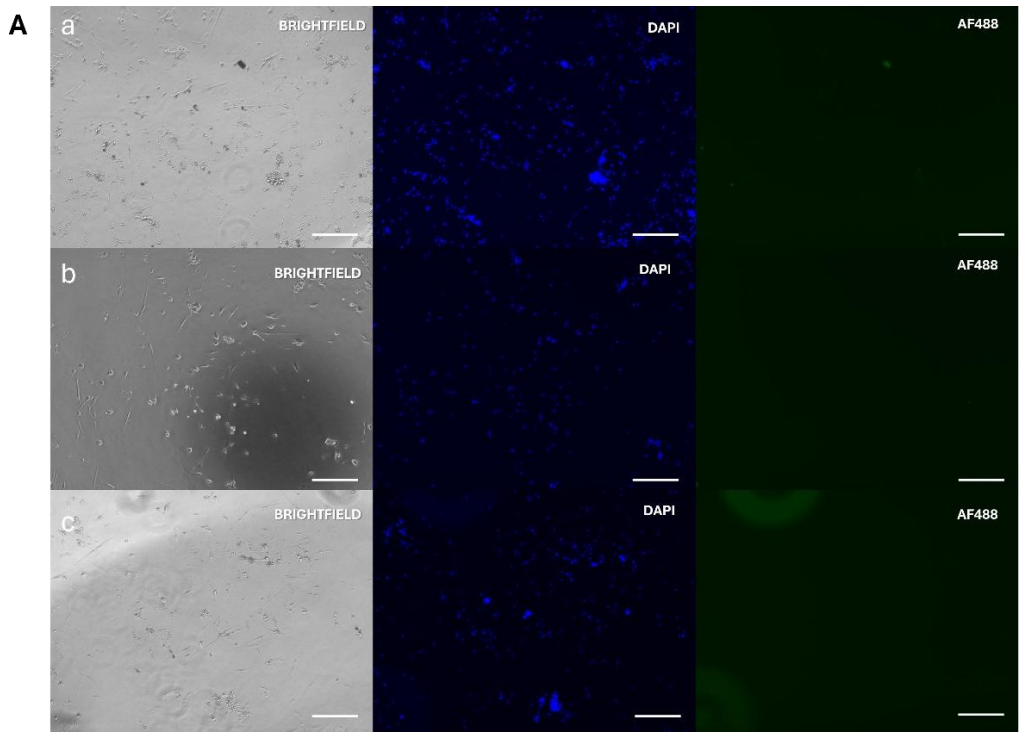
Tab A1: *Antibodies used in this study.*

Target	Antibody	Supplier	Antibody ID	Host	Confirmed Species Reactivity
Myosin (MHC)	*MAC 147*	*Abcam, UK*	*ab51098*	Rat	*Drosophila melanogaster, Waterbug*
Ecdysone receptor (EcR)	*10F1*	*DSHB, USA*	*AB_528210*	Mouse	*Bombyx mori, Drosophila, Locusta migratoria, Manduca, Rhodnius prolixus*
Ecdysone receptor (EcR)	*9B9*	*DSHB, USA*	*AB_528213*	Mouse	*Bombyx mori, Cricket, Manduca*
Hox Ubx/Abd-A	*FP6.87 (Ubx/ABD-A)*	*DSHB, USA*	*AB_10660834*	Mouse	*Crustacean, Drosophila, Drosophila virilis, Gerridae, Grasshopper, Limnoporus, Manduca, Metrobates, Onychophora, Rhagovelia, Spider*
Fasciclin I (neuronal CAM)	*3B11 (anti–grasshopper Fas I)*	*DSHB, USA*	*AB_528314*	Mouse	*Grasshopper*
Myosin heavy chain (alternative)	*3E8-3D3*	*DSHB, USA*	*AB_2721944*	Mouse	*Drosophila melanogaster*
Actin	*Alexa FluorTM 488 Phalloidin*	*Thermo Fisher Scientific, USA*	-	-	-
Nuclei	*DAPI*	*Thermo Fisher Scientific, USA*	-	-	-

Tab A2. *Secondary antibodies for immunofluorescence and working dilutions.*

	Supplier	ID	Host /Anti	Dilutions tested
Goat anti-Mouse IgG (H+L) Alexa Fluor 488	*Thermo Fisher (Invitrogen)*	*A28175*	*Goat anti-mouse*	*1:1000 (for all mouse primaries in the exploratory panel)*
Goat anti-Rat IgG (H+L) Alexa Fluor 488	*Thermo Fisher (Invitrogen)*	*A-11006*	*Goat anti-rat*	*1:500 (for MAC 147)*
Goat anti-Rat IgG (H+L) Alexa Fluor 594	*Thermo Fisher (Invitrogen)*	*A-11007*	*Goat anti-rat*	*1:500*

Fig A3. *Exploratory antibody dilution series and secondary-only control in primary Tenebrio molitor cultures*



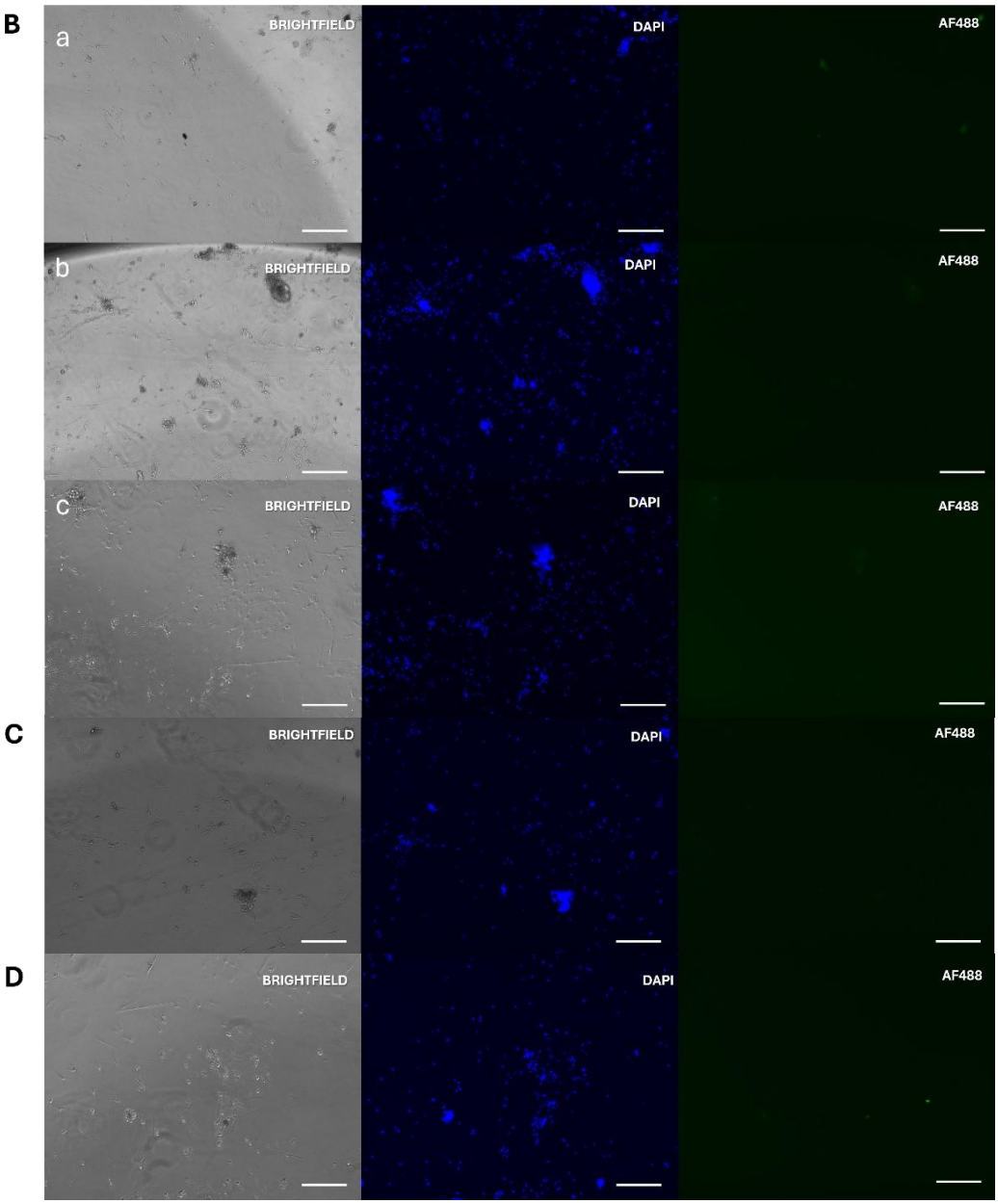


Fig A3. (A–D) Channels show per columns: Phase-contrast, DAPI (nuclei, blue), and AF488 channel (goat anti-mouse IgG [H+L]–Alexa Fluor 488, 1:1000) for the exploratory primaries. **A**: *9B9; Row **a**:* 1:50, *Row* **b:** 1:500, *Row* **c**: 1:1000. **B**:3B11; *Row **a**:* 1:40, *Row* **b:** 1:20, *Row* **c**: 1:10. **C:** *FP6.87* (1:500) **D,** secondary-only control (no primary). Across tested dilutions, AF488 fluorescence did not resolve subcellular or cell-type–restricted patterns and remained comparable to the secondary-only baseline. Scale bars: 200 µm

Figure A4: *No-secondary control for dual myosin co-staining*

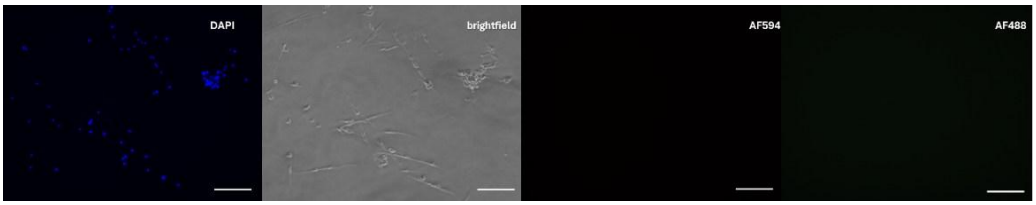


Fig A4. Left -> Right: DAPI (nuclei, blue), brightfield, AF594 channel (MAC147/red), and AF488 channel (3E8-3D3/green). No fluorescence was detected in either AF594 or AF488 under identical acquisition settings; DAPI and brightfield confirm cell presence and morphology. Scale bars: 200 μm