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新水缸潜水模型在斑马鱼焦虑及应激行为研究中的进展

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【摘要】 新水缸潜水实验(novel tank diving test)是专门针对模式动物斑马鱼或其他小型鱼类的焦虑行为研究设计的评价范式,是介于活体和细胞测定之间的药物筛选模型。该模型从小鼠迷宫实验模型发展而来,在行为测试的便捷性上具有一定优势。斑马鱼与人类具有较高的基因同源性,药物介导的斑马鱼焦虑和攻击行为与其内分泌系统中下丘脑-垂体-肾间腺(HPI)轴密切相关,类似于人体下丘脑-垂体-肾上腺(HPA)轴的作用。因此该模式对人类焦虑行为的研究具有重要的指导意义。总的来说,新水缸潜水实验是一种高效、可靠的高通量行为筛选模型,主要应用于动物社会性、成瘾性、睡眠、学习与记忆等研究领域。本文综述了过去15年间,新水缸潜水模型从形成雏形到不断完善,最后广泛应用于各种场景,从模型起源、测定参数、用途、存在问题和未来发展趋势等5个方面进行综述,并与明/暗水缸实验进行比较分析,以期扩宽斑马鱼焦虑及应激行为评价实验的研究范畴和应用范围。

【关键词】 新型水缸;焦虑;斑马鱼;运动参数;药物筛选
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Advances in novel tank diving test for the study of anxiety and stress behaviour in zebrafish

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【**Abstract**】 The novel tank diving test is a drug screening paradigm between *in vivo* and cellular assays, which is specially designed for the anxiety behavioral response of zebrafish or other small fish. This paradigm was derived from the maze model in rodents and has advantages in terms of the convenience of behavior assays. Zebrafish shares high gene homology with humans. Chemically triggered anxiety and aggression in zebrafish are closely related to the hypothalamic-pituitary-renal gland axis in the endocrine system, which is parallel to the effect of the hypothalamic-pituitary-adrenal axis in humans. The novel tank diving test is an efficient and reliable high-throughput behavioral screening model mainly used in studying animal sociality, addiction, sleeping, learning, and memory. The novel tank diving test has been widely used in various scenarios from initiative to continuous improvement in the previous 15 years. This article reviews the origin, measurement parameters, usage, existing problems, and perspectives of this paradigm compared with the light/dark test to broaden the anxiety behavior evaluation paradigm and application scope.

【**Keywords**】 Novel tank diving test; anxiety; zebrafish; behavioral parameters; drug screening
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近年来,斑马鱼逐渐替代啮齿动物成为神经行为研究的模式动物,多用于研究焦虑、恐惧等精神疾病[1-7]。与传统的大小鼠模型相比,斑马鱼模型具有用药量少、实验周期短、成本低等优势[8]。斑马鱼具有经典的脊椎动物神经递质,并且其神经内分泌系统对压力具有强大的生理响应[9-10]。斑马鱼的报警反应与人类焦虑症的发病症状极为相似,且斑马鱼在基因上与人类具有高度同源性,是应激和焦虑研究中的模型生物。通过研究斑马鱼报警反应的神经传导,可以探索与人类神经性疾病的发病和临床治疗相关的途径和机制[11]。

新水缸潜水模型在 2007 年由 Levin 等[5]开始使用,到 2021 年的 15 年间取得了巨大的研究进展。论文发表数逐年上升(图 1),实验条件及参数不断优化,应用范围逐渐扩大。本文将对新水缸潜水模型 15 年来的发展及应用进行综述。

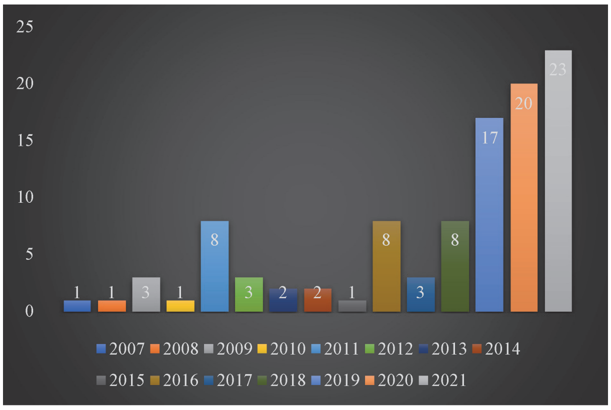

图 1　2007 ~ 2021 年间相关论文发表数（筛选自 Web of science）

Figure 1　Number of related papers published from 2007 to 2021 (Selected from the Web of science)

1　新型水缸潜水实验起源

动物的本性是暴露于新环境后出现显著躲避与逃跑行为,进而被认定为焦虑反应[12]。新水缸潜水实验利用了斑马鱼在新环境中寻求保护的本能行为,进入新的鱼缸后,斑马鱼的行为反应是潜入并在缸底呆滞,直到它们感觉安全才会试图四处探索[5]。新型水缸潜水实验是一种直接的、可重复的测量斑马鱼焦虑行为的方法[13]。Peitsaro 等[14]的研究中只对斑马鱼水平维度的运动进行了探索。Levin 等[5]在此基础上引入了新型水缸潜水实验,是首次在垂直维度上评估斑马鱼焦虑程度,以及药物刺激后对新环境适应的能力[5]。斑马鱼被放入一个容积为 1.5 L 的塑料梯形水缸(长 22.5 cm、宽 7.1 cm、高 18.2 cm),加入过滤后配制的养殖水[15]。该实验将水缸分为上、中、下 3 个部分,斑马鱼对水缸位置的选择及停留时间(底部、中层与上层)被认为是反映焦虑程度的重要指标。在后来的实验中,新型水缸被分成两个相等的虚拟水平部分(图 2)。中线以上的区域代表新型水缸的“上半部(顶部)”,而下方区域代表新型水缸的“下半部(底部)”[16]。所有设备都放置在水平、稳定的平台上(图 2)。研究人员能够收集和比较行为参数(如呆滞、不稳定运动、顶底比)来评估焦虑。首次进入上半部分的迟疑时间更长,在上半部分花费的时间减少,以及不稳定或呆滞行为的增加表明焦虑加剧[17]。

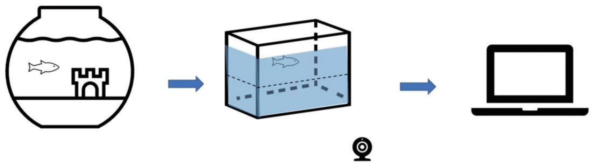

图 2　新型水缸潜水实验流程图

Figure 2　Novel tank diving test experimental flow chart

2　行为参数

2.1　呆滞时间

在斑马鱼新型水缸潜水实验中,呆滞(freezing duration)(见表 1)被定义为受试鱼移动速度低于 0.5 cm/s[18]。也有实验把呆滞定义为鱼除鳃和眼睛,其他部位完全不运动且持续时间> 2 s[19]。导出的行为视频轨迹由两名训练有素的观察者根据统一的标准进行独立评判,在软件中手动标记斑马鱼呆滞持续时间及频次。最后采用自动视频跟踪进一步补充对斑马鱼行为的手动观察[3]。

当斑马鱼进入新环境中会立即表现出呆滞反应,然而随着它们习惯于新的环境,便开始表现出探索行为[18]。在正常快速移动的开放水域中,斑马鱼呆滞行为反应非常罕见,而且通常只持续很短的时间[20]。因此,使用呆滞作为衡量焦虑程度的一个常见问题是:需要较大的样本量来减少个体间的差异。值得注意的是,还可以通过提高最小速度的阈值(例如,认定低于 0.75 cm/s 为呆滞),从而增加呆滞数据点的数量来减少方差[20]。

2.2　不稳定运动

不稳定运动(erratic movement)(表 1)被定义为斑马鱼在水中运动方向或速度的急剧变化并且重

复快速游窜的行为[21],是较好地衡量焦虑的指标。通常采用绝对转角和绝对角速度量化不稳定运动。当斑马鱼被放置在一个新的环境中时,绝对转角随斑马鱼进入新水缸时间的增长而减小,这表明随着动物适应环境,类似焦虑的行为减少[22]。与绝对转角相似,绝对角速度也呈下降趋势,被认为是斑马鱼适应了新环境的表现。导出的轨迹也由两名训练有素的观察者根据统一标准进行独立评判[23],手动记录斑马鱼不规则运动时长,用自动视频跟踪进一步补充。

2.3　顶底比

顶底比(top: bottom ratio)(见表 1)是指受试斑马鱼在顶部区域与底部区域花费时间的比例[23]。在顶部与底部区域花费的时间可由 Ethovision XT7 软件自动获取。通过计算得出在顶部与底部区域花费时间的比例[17,23]。顶底比越高,斑马鱼焦虑程度越低。在隔离实验中表明,雌性与雄性的压力相关的行为存在内在的差异,雌性斑马鱼的顶底比大于雄性[24]。

2.4　在顶部区域花费的时间与到顶部的次数

到顶部区域的次数(number of transitions and time spent in the upper portion of the tank)(见表 1)是指斑马鱼从底部区域进入顶部区域的次数。在顶部区域花费的时间是指斑马鱼在新型水缸顶部区域花费的总时间。进入顶部区域的次数和顶部区域花费的时间可由 Ethovision XT7 软件自动获取。水底栖息行为被认为是一种保护性或反捕食行为,可能是为了抵御空中和/或其他水生捕食者[25]。随着斑马鱼适应新水缸的环境,斑马鱼会逐渐探索新水缸的顶部区域。斑马鱼在顶部和底部穿梭次数越多,在水缸顶部区域时间越长,表明焦虑程度越低[17]。

2.5　第一次潜伏期

第一次潜伏期(latency to enter the top)(表 1)为斑马鱼第一次从新型水缸的底部区域穿越中线到顶部区域之前所经历的时长[26]。第一次潜伏期可由 Ethovision XT7 软件自动捕获。斑马鱼在一个新的环境中,会习惯性地潜伏在水缸底部。第一次潜伏期越长,表明斑马鱼在此环境中焦虑程度越高[20,27]。

2.6　平均速度和总移动距离

斑马鱼在新型水缸中移动速度的平均值和总的移动距离(average velocity and total traveled distance)(见表 1)可由 Ethovision XT7 软件自动采集[20]。视频跟踪不仅增加了数据量,而且保证了其他行为参数的准确性。例如,一种药物减少了到顶部区域的探索,但是平均速度和总的移动距离并不改变,可以解释为该鱼没有明显的呆滞行为[17]。

表 1　斑马鱼新水缸潜水实验常用行为指标定义及含义

Table 1　Definition and meaning of behavior index in novel tank diving test of zebrafish

行为参数 Behavioral parameter	定义 Definition	焦虑相关性 Correlation with anxiety
呆滞时间(s) Freezing duration(s)	受试斑马鱼移动速度低于 0.5 cm/s 的持续时间。 The duration when the moving speed of the tested zebrafish is lower than 0.5 cm/s.	正相关 Positive
不稳定运动(%) Erratic movement(%)	受试斑马鱼在水中运动方向或速度的急剧变化,并且重复快速游窜的行为。 The zebrafish's movement direction or speed changes sharply in the water, and it repeats the rapid swimming behavior.	正相关 Positive
顶底比 Top: bottom ratio	受试斑马鱼在顶部与底部花费时间的比率。 The ratio of time spent by zebrafish at the top to the bottom.	负相关 Negative
到顶部区域的次数(freq) Number of transitions and spent in the upper portion of the tank(freq)	受试斑马鱼从底部区域到达顶部区域的累计次数。 Cumulative times of zebrafish from the bottom area to the top area.	负相关 Negative
在顶部区域花费的时间(s) Number of time spent in the upper portion of the tank(s)	受试斑马鱼在新型水缸顶部区域花费的总时间。 Total time spent by zebrafish in the top area of the new water tank.	负相关 Negative
第一次潜伏期(s) Latency to enter the top(s)	受试斑马鱼第一次从新型水缸的底部区域穿越中线到顶部区域。 The zebrafish crossed the midline from the bottom area to the top area of the new tank for the first time.	正相关 Positive
平均速度(m/s)和总移动距离(m) Average velocity(m/s) and total distance traveled(m)	受试斑马鱼在新型水缸中移动速度的平均值和总的移动距离。 Mean value of moving speed and total moving distance of zebrafish in a new type of water tank.	极度活跃 Extremely active

3 新型水缸潜水实验的应用

斑马鱼被广泛应用于焦虑和恐惧行为研究[28-30]，已成为多个生物学科分支中最有潜力的脊椎动物模式生物[31]，其在胚胎发育及神经行为毒理学研究中发挥了极大作用。斑马鱼新水缸行为评价范式推动了抗焦虑药物在某些疾病和神经系统方面的研究，进而使研究人员能够探索与人类发病和临床治疗相关的机制和途径；新水缸行为评价范式应用于报警物质的研究有利于加深理解鱼的捕食-反捕食机制；通过斑马鱼行为的改变能够直接反映水环境的变化，便于对水体污染物的研究。

3.1 抗焦虑药物

新型水缸潜水实验可用于研究抗焦虑药物的作用机制，如苯二氮卓类药物和 5-羟色胺能系统。研究表明 GABA-A 受体有效激动剂氯氮卓，在大剂量范围内没有显示出任何抗焦虑作用，尽管这种作用通常见于大鼠；而非苯二氮卓类抗焦虑性丁螺环酮，其作用通过 5-羟色胺能系统（5HT1A 受体激动剂）介导，以及可能的多巴胺 D2 发挥作用，在新水缸潜水实验中显示出显著的抗焦虑作用[32]。在氟西汀慢性刺激实验中，受试鱼进入上半区域的迟疑时间、不规则运动等焦虑指标都呈现明显下降趋势。苯丙胺衍生物 2,5-二甲氧基-4-溴苯丙胺氢溴酸盐（DOB）和对甲氧基苯丙胺（PMA）与 3,4-亚甲基二氧基甲基苯丙胺（MDMA）及其受体抑制剂暴露后斑马鱼表现出抗焦虑作用[33-34]。新型水缸潜水实验也被发展成为评价东莨菪碱抗焦虑活性的实验范式[35-36]。根据对 γ-氨基丁酸、单胺能、胆碱能、谷氨酸能和阿片苷能等多种调控系统的研究结果得出[37]，斑马鱼新型水缸潜水实验可以成功检测药物的抗焦虑作用，尤其是 5-羟色胺能和烟碱药物，同时也提示该模型的缺陷性及适用性。

3.2 成瘾性/致幻性药物与食品

新型水缸潜水实验可用于研究成瘾性物质，如尼古丁、咖啡因。首次进行的斑马鱼新水缸潜水实验是针对尼古丁抗焦虑作用的研究[5]，而慢性尼古丁作用却产生焦虑行为[38]。斑马鱼大脑中存在对咖啡因做出焦虑反应的神经中枢，同时加入抗焦虑药物时，斑马鱼焦虑行为减轻[39-40]。斑马鱼新水缸模型有助于筛选尼古丁相关受体阻断剂[41]，以补充现有的啮齿动物实验范例，对成瘾行为及烟草戒除研究有深远意义。

新型水缸潜水实验广泛应用于药物与食品的研究中。帽柱木碱（mitragynine）是一种被阿片成瘾者广泛用于减轻药物戒断痛苦的植物成分，研究结果表明其具有显著降低暴露于吗啡后斑马鱼体内的皮质醇水平的功效，可用于减轻阿片类药物戒断期间焦虑反应[42]。斑马鱼暴露于浓度111 mmol/L 的葡萄糖溶液，表现为进入上层次数和在上部的时间都减少，由此推测葡萄糖诱发斑马鱼焦虑行为[43]。牛磺酸可以消除新水缸实验中斑马鱼在同种报警物质诱导下产生的不稳定运动等焦虑行为，产生对大脑有益的影响，有必要对其进行进一步的研究，阐明其对脊椎动物神经保护作用的潜在机制[44]。草本植物天麻已经显示出对神经系统疾病的有益效果，如帕金森病[45]、痴呆症[46]和癫痫[47]。使用新型水缸潜水实验来评估天麻提取物对斑马鱼利血平诱导的抑郁症保护作用[48]。与利血平组相比，利血平加入天麻提取物 25 mg/L、50 mg/L 和 100 mg/L 组斑马鱼在水缸顶部区域停留的时间显著增加，结果表明天麻提取物可以抑制斑马鱼的抑郁行为[49]。

3.3 报警物质

自然界中报警信息素是广泛存在的。当个体遭到捕食者攻击时，会释放报警信号，以提醒附近同种个体躲避潜在的风险，提升种群的适应性[50-52]。从皮肤粘液中分离得到的一些寡糖片段能够引起斑马鱼的恐惧反应，并且硫酸软骨素可能是主要的活性成分[53-54]。此外，研究发现一些二元胺类物质、尼古丁等也能诱发斑马鱼的一些焦虑反应[55-57]。因此，在研究鱼类报警物质时，应引起足够的重视，排除这些干扰[58-59]。斑马鱼的恐惧反应有以下特征：呆滞频率和时间增加、不稳定运动加快、第一次潜伏期变长、到底部距离方差减少[17,26,40,60]。对于报警物质的研究有利于加深理解鱼类捕食-反捕食机制[40]，完善斑马鱼作为人类焦虑模型的研究。

3.4 环境污染物

环境内分泌激素是广泛存在于环境中的污染物，对水生生物存在严重影响，值得对环境毒理效应进行深入研究。斑马鱼作为经典脊椎动物模型，适用于游泳水生动物行为表型的研究。研究表明乙炔雌二醇（25 ng/L）慢性暴露 2 周对雄性斑马鱼游泳行为有一定影响，表现为更多的探索行为，表明焦虑减小，该研究增加了对雌激素物质污染水生

环境的生态后果的理解[61]。溴代阻燃剂斑马鱼胚胎暴露以后,幼鱼也表现出行为变化[62]。环境中小剂量的替布康唑对斑马鱼成虫也表现出焦虑相关效应,诱导基因毒性和致突变性,以及改变与成鱼行为变化相关的神经功能[63]。环境中的精神活性药物曲马多对斑马鱼的行为也有一定影响,但是由于剂量关系,没有得出可靠结论[64]。环境中对羟基苯甲酸酯对斑马鱼胚胎的低剂量暴露引起焦虑行为[65]。

斑马鱼对水环境的变化非常敏感[66],尤其是金属离子[67]。斑马鱼在铜离子暴露下,表现为改变运动规律和自然倾向,避免明亮的区域[68-70]。斑马鱼在含汞无机化合物($HgCl_2$)暴露下未表现出行为变化[71],对甲基汞的作用也有所评价[72]。Liu 等[73]、Tu 等[74]也采用该模型评价环境中三丁基锡增加雄鱼攻击性行为的作用,为评价水生环境中微量污染物的影响提供了实例。斑马鱼的行为改变程度与污染物的毒性大小和浓度密切相关[75],量化分析污染物暴露下斑马鱼的游动速度、游动加速度和转弯次数[76],通过监测鱼类的行为变化可以实现水质污染事件的在线报警。

4 新水缸潜水实验中存在的问题

4.1 基因型

目前常用的斑马鱼有多种品系,其中野生型和 Albino 品系的斑马鱼多被用于行为实验。对 4 种行为指标进行检测,结果表明 4 种品系的斑马鱼在总游动距离和平均速度未表现出显著性差异。相比较于野生型斑马鱼,Leopard 品系在进入上半部迟疑时间显著增加,在上半部总停留时间显著减少,表明 Leopard 品系斑马鱼可能具有更易受惊吓的特性。Albino 品系的斑马鱼在上半部总停留时间上与野生型相比表现出显著降低。不同的基因型斑马鱼可用于不同实验意图,如对焦虑具有高敏感性 Leopard 品系适用于缓解焦虑的药物的效果评价,而非焦虑敏感型的野生型品系更适用于致焦虑药物的评价。品系间对焦虑的敏感性差异也为人类焦虑敏感差异提供了研究思路[77]。Deng 等[78]对常见的两种实验室斑马鱼品系 Tubingen 品系和 AB 品系进行了基因编码区的结构变异比较,揭示两个品系斑马鱼之间遗传差异,有助于更深入地探讨不同品系斑马鱼表型差异背后的遗传基础。Vossen 等[79]证明野生斑马鱼更适用于药物筛选。对于品系、突变体等问题的研究引起了学者的广泛重视[80-81],在实验中需要谨慎选择。

4.2 行为评价

斑马鱼的测试水缸宽度多为 5 cm,可以容纳受试鱼自由转身,但是缺乏对趋边性的实验数据。这样设计的目的是为了将三维空间运动模拟成为二维平面,从正面拍摄机位可以记录受试鱼体的垂直运动及其选择性。这种测试水缸往往与平时饲养的水缸大小一致,这对新水缸潜水实验中的运动参数也存在一定的影响[82]。例如,较为宽大的饲养水缸的受试鱼体在运动速度与底部停留时间这两个重要参数上表现出更大时间差异性[32]。由此,建议使用与平时饲养过程中一致的水缸进行实验以确保一致性。同时也考虑使用较宽的立方体水缸,并架设第二摄影位,即在水缸正上方向下拍摄,这样可以更好地记录趋向性运动[83]。

5 其他模型

García-González 等[81]提出了斑马鱼的明/暗水缸模型的设想,后来被 Maximino 等[4,84]进一步发展并验证。明/暗水缸模型针对成年斑马鱼对黑暗环境的自然偏好(趋暗性)和对明亮区域的躲避进行研究[85-86]。实验设备是定制的矩形玻璃水缸(长 × 宽 × 高,30 cm × 16 cm × 15 cm),被均匀地纵向分成两部分,即亮部分和暗部分[87](图 3)。水缸一侧的侧壁和底座用黑色塑料包裹,顶部用不透明的黑色盖子遮挡光线[88],形成暗区;而另一侧是不透明的白色塑料壁且顶部无遮挡,形成光区。水箱里装一定量的水,具体水量可根据矩形水缸的大小进行调节。根据 Shi 等[89]的方法,采用摄像设备记录运动轨迹,使用 Image J 软件分析获得的视频数据,用进入光区的潜伏期(LEL)、在光区停留时间(DL)、在光区的持续时间与整个测试的持续时间的比率(LTR)等参数估计斑马鱼的焦虑程度。进入光区的潜伏期越长、在光区停留时间越短、在光区的持续时间与整个测试的持续时间的比率越低则表明焦虑程度越高[81,90-93]。

相比较于新型水缸潜水模型,明/暗水缸被认为是评估斑马鱼焦虑行为的更具体的方法[94]。药理学证据表明,明/暗水缸对抗焦虑药物敏感,但对抗恐惧药物不敏感[95]。尽管两者都对焦虑药物/抗焦虑药物敏感,但药理学特征并无一定相关性。例如,新型水缸潜水实验对地西泮敏感,但对苯二氮

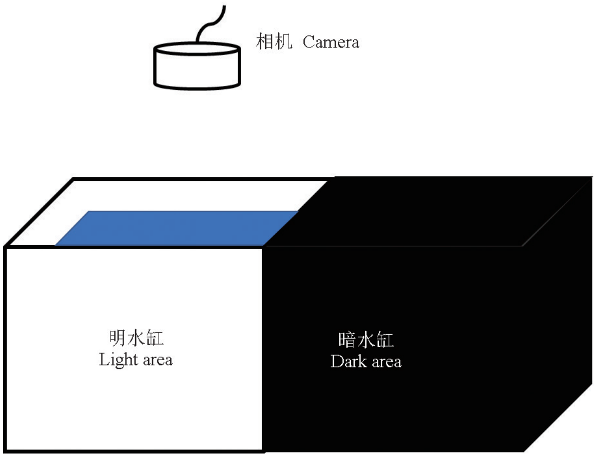

图 3　明暗水缸实验示意图

Figure 3　Schematic illustration of the light and dark preference test

卓不敏感,而明/暗水缸对两者都敏感[32,95]。当动物暴露于氟西汀时,两种行为实验也显示出不同的结果[17,96],明/暗水缸显示对吗氯贝胺(一种 MAO-A 抑制剂)缺乏敏感性[95]。开发一个包含多种参数的评估焦虑的模型有助于提高实验的可靠性和检测效率[86]。在明/暗水缸实验中,光照影响是导致实验结果差异的重要因素。研究表明在较低和较高的光照水平下,动物在暗区停留时间更长,在中等光照水平下则对动物行为没有影响[96]。因此,在进行明/暗水缸实验时,需要对光照等多因素进行考虑。新型水缸潜水模型和明/暗水缸模型结合,将有利于提高评估斑马鱼的焦虑行为实验结果的准确度[97]。

6　展望

新水缸潜水实验已经被应用于多个研究场景,包括对内源性报警物质的评价[17]、药物评价[32,34,37,42]、食品评价[67]、环境残留物评价[98-99]等。近年来,应用范畴扩宽到各种天然药用植物提取物[100-103]、辐射刺激[104]、锻炼效果[105]、鱼类“个性”[106-107]、纳米胶囊缓评价[108]、合成产品的生物兼容性评价[109]、突变体运动评价[110]、营养评价[111]、高脂肪饮食评价[93]、生殖评价[112]等方面。

研究焦虑行为的众多检测方法中,新型水缸检测方法易于执行、可重复性强,数据结果可靠。相较于基于鱼类的视觉趋向性的明暗测试[106],新型水缸实验专注于鱼类对潜在威胁的行为反应。同时,斑马鱼体内皮质醇水平是表征压力和焦虑的重要生理指标[17]。新型水缸实验诱导斑马鱼体内皮质醇升高,表明新型水缸实验比明暗测试更能激发生理响应[113],可以完善斑马鱼作为人类焦虑模型的研究,将为许多疾病的发病机理、新药研发提供科学依据。

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